



# COMBINED COMPENDIUM OF FOOD ADDITIVE SPECIFICATIONS

**Joint FAO/WHO Expert Committee on Food Additives**

All specifications monographs from the  
1st to the 65th meeting (1956–2005)

**Volume 4**

**Analytical methods, test procedures and  
laboratory solutions used by and referenced  
in the food additive specifications**



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## ACKNOWLEDGEMENTS

FAO Food and Nutrition Paper 52, “Compendium of Food Additive Specifications” (FNP 52) was first published in 1992. It combined all of the food additive specifications prepared by JECFA from its first meeting in 1956 to its thirty-seventh meeting in 1990. A companion publication, FAO Food and Nutrition Paper 5, “Guide to Specifications” (FNP 5) contained the analytical methods, test procedures and laboratory solutions used in and referenced by JECFA food additive specifications. It was first published in 1978 and revised in 1991.

The decision was made to prepare an updated second edition of FNP 52 to include all food additive specifications prepared by JECFA through its sixty-fifth meeting in 2005. It was further decided that an updated and revised version of FNP 5 would not be published separately as before, but would be incorporated into the FNP 52 Compendium second edition. The new Compendium of Food Additive Specifications is therefore now comprised of four volumes. The first three volumes are the food additive specifications themselves in alphabetical order. This fourth volume includes the revised and updated analytical methods, test procedures and laboratory solutions which first appeared in FNP 5, plus newer procedures and an expanded section on laboratory instrumentation.

The review, revision and updating of the material in FNP 5, to prepare Volume 4, was a long and arduous process. The Food and Agriculture Organization of the United Nations (FAO) wishes to acknowledge and thank the following experts who were instrumental in the preparation and finalization of this document: (Listed in alphabetical order)

- Dr. Julie Barrows, US Food and Drug Administration, USA
- Dr. Richard Cantrill, American Oil Chemists Society, USA.
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- Mrs. Harriet Wallin, National Food Agency, Finland
- Dr. Brian Whitehouse, Consultant, United Kingdom

FAO also wishes to thank Consultant John Weatherwax, USA, who coordinated this effort and who prepared the first three volumes of food additive specifications for publication. Although several JECFA experts were involved in the revision and editing of those first three volumes, FAO wishes to identify and thank two experts in particular, who both provided significant contribution. They are Dr. Chris Fisher and Dr. Brian Whitehouse, both Consultants residing in the United Kingdom.

### **SPECIAL NOTE**

The methods and analytical procedures described in this Compendium are designed to be carried out by properly trained personnel in a suitably equipped laboratory. In common with many laboratory procedures, the methods quoted frequently involve hazardous materials.

For the correct and safe execution of these methods it is essential that laboratory personnel follow standard safety procedures for the handling of hazardous materials.

While the greatest care has been exercised in the preparation of this information, FAO expressly disclaims any liability to users of these procedures for consequential damages of any kind arising out of, or connected with, their use.

# COMBINED COMPENDIUM OF FOOD ADDITIVE SPECIFICATIONS

## INTRODUCTION

### Introduction Contents

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## FOREWORD

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) was established following the recommendation of the first Joint FAO/WHO Conference on Food Additives that the two organizations collect and disseminate information on food additives (Joint FAO/WHO Conference on Food Additives, Report. FAO Nutrition Meetings Report Series, No. 11; WHO Technical Report Series, No. 107, 1956). JECFA was first convened in 1956, and has met annually, with a few exceptions, since that time.

As part of its work, JECFA establishes specifications of identity and purity for food additives. These were originally published in FAO Nutrition Meetings Reports Series (NMRS), the WHO Technical Report Series (TRS) or as FAO Food and Nutrition Papers (FNP). However many of these are now out of print, and the first edition of this Compendium was published in 1992 in order to consolidate all of the then current JECFA specifications into a single publication. Since 1992, separate Addenda to this Compendium have been published which contain both newly established specifications and revisions to earlier specifications. The original Compendium and the succeeding Addenda were all published in the FNP series as number 52. This new Combined Compendium replaces the earlier edition and incorporates all the additions and revisions made since 1992, up to and including those contained in FNP 52 Addendum 13. It is being published as the first document under a new publication series, the FAO JECFA Monographs.

Many additive specifications have been revised two or more times as new information or circumstances require. The specifications contained in this Combined Compendium are the most recent version or revision for each additive. This new Combined Compendium is in four volumes as follows:

- Volume 1 – Specifications for food additives A through D.
- Volume 2 – Specifications for food additives E through O.
- Volume 3 – Specifications for food additives P through Z.

- Volume 4 – Analytical methods, test procedures and laboratory solutions used by and referenced in the food additive specifications.

This Introduction is intended to replace and update the sections in the IPCS document *Principles for the Safety Assessment of Food Additives and Contaminants in Food* (Environmental Health Criteria 70 (EHC 70), WHO, 1987) that describe the purpose and function of specifications of identity and purity of food additives. It also incorporates the General Notices section of the *Guide to Specifications* originally published in Food and Nutrition Paper 5 (FNP 5), revision 2. The remainder of FNP 5 has been extensively revised and updated and is now included in Volume 4.

The term ‘specifications’ refers to the full set of individual specifications criteria for an additive. Specifications are regarded as one of the outputs of JECFA’s risk assessment of additives. The term ‘safety evaluation’ is used to describe the output of JECFA’s assessment of the toxicology and other safety data relating to a food additive.

As previously noted, the specifications published in this Combined Compendium represent the specifications current at the time of publication. These are, however, subject to future review and revision as part of JECFA’s continuing work. New and revised specifications are published as soon as practicable after the JECFA meetings when these were considered, and JECFA specifications are also available online on the FAO website.

Specifications for flavouring agents are not included in this Compendium, excepting those few which have an additional technological function as a food additive (e.g. carrier solvent). Flavouring agent specifications will, however, still be available online in a searchable database at the FAO website.

## **PURPOSE AND FUNCTION OF SPECIFICATIONS OF IDENTITY AND PURITY OF FOOD ADDITIVES**

### **General**

The specifications of identity and purity established by JECFA are intended to ensure that the Committee's safety evaluations apply, with a high degree of confidence, to all food additives manufactured according to those specifications. In setting specifications, the Committee also takes into account the need to encourage good manufacturing practice and to maintain the quality of additives on the market, noting that these considerations may add to the assurance of safety that the Committee is seeking as part of its risk assessment function.

JECFA's specifications are also intended to set manufacturing standards for food additives traded in international commerce either as such, or incorporated in food products. In some cases, JECFA specifications will differ from specifications developed by other national and international organizations. JECFA's safety evaluations can, however, be taken to apply to additives complying with these other specifications, provided that the additives are of equal or higher purity than those required by the JECFA specifications, and provided that the test criteria in the JECFA specifications are met.

The setting of specifications of identity and purity is an essential part of the risk assessment of food additives, and current thinking emphasizes the need for the safety evaluation and the specifications to be seen as joint outputs of the risk assessment process. The safety evaluation of an additive should therefore always be read in conjunction with the specifications of identity and purity that describe the additive. The link between the two parts of the risk assessment process is also emphasized by the fact that the latest safety evaluation is quoted in each of the specifications, together with a reference to the JECFA meetings(s) at which the specifications were elaborated and the safety evaluation carried out.

### **Formulation of Specifications and Information Requirements**

The formulation of satisfactory specifications requires detailed information to be made available to the Committee on the method of manufacture of the additive, including information on raw materials and on its chemical characterization. The Committee requires such information to be provided as part of the total data package whenever an additive is submitted for risk assessment, and all such information will be regarded as suitable for making publicly available unless requested otherwise and agreed by the Secretariat. Those submitting data for a JECFA evaluation are advised to consult existing specifications for further guidance, taking note also of the format of specifications described later in this introduction. They should also seek further advice as needed from the JECFA Secretariat.

Specifications may be revised where there is new information available on methods of manufacture or on the characteristics of the substance, or where changes or revisions in analytical methods are needed. Such changes may also prompt a review of the safety evaluation. Similarly, a review of the specifications may be needed if the safety evaluation is reconsidered.

Additives are mainly defined by a combination of (i) a description of their manufacture, (ii) a minimum requirement for the content of the principal functional component(s) of the additive, and (iii) maximum limits for undesirable impurities. The relative importance of these criteria, however, depends on the nature of the additive. Thus, for example, additives composed largely of single components are mainly defined in terms of their chemical purity,



whereas the definition of more complex materials, e.g. natural gums, relies more on a description of the raw materials and the method of manufacture.

In some cases, there may be insufficient information for the Committee to elaborate what it regards as fully acceptable specifications. The Committee may then decide to publish the incomplete specifications, but with a designation of 'Tentative'. In such cases the Committee will also state what additional information is required, and set a date by which this must be provided. The Committee will reconsider the specifications once the necessary information has been received, and if it considers that the information is sufficient, it will remove the 'Tentative' designation. If, however, the information is still deficient, or if no information has been provided by the due date, the specifications will be withdrawn.

JECFA specifications incorporate the methods of analysis that are to be used in testing the individual criteria. Information provided in submissions to JECFA should, therefore, always include details of the analytical methods to be used.

### **Stability and Fate of Additives in Food**

Specifications are intended to apply to the additive as marketed. However, some food additives are designed to perform their function by interacting with components of food as in the case of flour improvers, or, for example by removing undesirable constituents, as in the case of antioxidants. Others may be subject to chemical degradation in food or may interact with food components, sometimes with the production of undesirable reaction products.

In order to ensure that test data are relevant to the way the additive is used in food, the Committee requires information on potential reactivity to be provided as part of submissions for the safety evaluation of additives. This information should include data on (a) the general chemical reactivity of the additive; (b) its stability during storage and reactions in model systems; and (c) the reactions of the additive in food systems. The Committee may also set specific limits on potential degradation products in those cases where the additive may be subject to degradation during storage.

## JECFA SPECIFICATIONS AND THE CODEX SYSTEM

### JECFA, CCFAC and the Risk Analysis Framework

The respective roles of JECFA and the Codex Committee on Food Additives and Contaminants (CCFAC) have been extensively discussed in recent years in the context of the Codex risk analysis framework. In this context, JECFA is regarded as the expert risk assessment body on additives, contaminants and natural toxicants in food, with CCFAC fulfilling the corresponding risk management role. Thus, CCFAC endorses maximum use levels only for those additives for which JECFA (i) has established specifications of identity and purity and (ii) has completed a safety assessment or has performed a quantitative risk assessment

CCFAC also makes recommendations to the Codex Alimentarius Commission on the possible adoption of JECFA specifications as Codex Advisory Specifications. National food control authorities use Codex Advisory Specifications for enforcement purposes, and for ensuring that additives in international commerce meet agreed standards. National governments may also draw on Codex Advisory Specifications when developing their own regulatory standards.

### Referrals to JECFA and JECFA/CCFAC Interaction

Additives can be referred to JECFA by CCFAC, Codex member nations, FAO or WHO. However, CCFAC provides, as part of its risk management function, the primary forum for handling these referrals, and the JECFA Secretariat works closely with CCFAC in setting the agendas for JECFA meetings. CCFAC also has an important role in ensuring that those requesting JECFA evaluations understand that they bear responsibility for providing the supporting information, and for submitting this by the date set by JECFA in its pre-meeting Call for Data.

Requests to JECFA may be for a full risk assessment of an additive, in which case the information provided will need to include both toxicology and other safety data, as well as the data required to develop specifications. Alternatively, requests may be limited to a reconsideration of information relating to specifications. In such cases the Committee will, however, bear in mind the need to ensure that the specifications still provide the requisite assurance of safety, and will, therefore, only make revisions that it considers will not affect the validity of the safety evaluation.

The current mechanism for considering JECFA's specifications at CCFAC meetings involves the convening of an *ad hoc* Working Group that meets prior to the plenary CCFAC session. The Working Group, which includes delegates from Codex member states and non-Governmental observers, provides a dedicated forum for the discussion of specifications and other issues relating to the work of JECFA, and the presence of both the JECFA Secretariat and past members of JECFA, attending as part of CCFAC member countries' delegations, provides an opportunity for direct interchange between the risk assessors and risk managers. In this way the Working Group can be seen as fulfilling an important risk communication function in the work of JECFA and CCFAC. The Working Group makes recommendations for consideration at the plenary session of CCFAC, including recommendations on future items to be referred to JECFA and recommendations on which JECFA specifications CCFAC should consider referring to the Codex Alimentarius Commission for possible adoption as Codex Advisory Specifications.

## **SPECIFICATIONS AND METHODS OF ANALYSIS**

Information submitted to JECFA on the identity and purity of food additives should always include details of the relevant analytical methods. Information on the potential compositional variability of the substance should also be given, together with details of any sampling protocols used to assess this. Insufficient information on analytical methodology is one reason why JECFA may be unable to elaborate suitable specifications, or why it may decide that it is only able to assign a 'Tentative' designation, pending receipt of the further information required.

JECFA specifications incorporate guidance on the analytical techniques that should be used to verify the information. Wherever possible, this is done by reference to Volume 4. Otherwise details of the test procedures are set out in the individual specifications monographs.

As JECFA specifications are elaborated for worldwide use, the Committee prefers to quote methods that require the use of apparatus and equipment that is available in most laboratories, provided that such methods are capable of providing results meeting the requirements of the specifications. Methods involving more recently developed techniques or equipment will not normally be quoted until such techniques are accepted internationally and are generally available at reasonable cost. However, reference to specific methods of analysis should not be taken as precluding the use of other methods, provided that these give results of equivalent accuracy and specificity to those quoted.

Changes to analytical methods are reviewed from time to time as part of JECFA's ongoing work. Changes may also be considered when substances are evaluated for the first time, or when new information becomes available on substances that have been previously considered. Changes in analytical methodology may also prompt further consideration of specifications, for example where these changes reveal the possible presence of previously unsuspected contaminants.

## FORMAT OF SPECIFICATIONS

### **Additives other than enzyme preparations and flavouring agents**

JECFA specifications for food additives other than enzymes and flavouring agents normally include the headings listed below, which are given in the order in which they appear in the specifications. Note also that aluminium lakes of colouring matters must comply with the *Aluminium Lakes of Colouring Matters - General Specifications* included in Volume 1 of the Compendium.

#### ***TITLE***

The ***TITLE*** includes the name selected for the individual additive. This is the name that, in the view of JECFA, most appropriately identifies the substance or substances specified. It will normally correspond with the name given to the substance that was subject to safety evaluation, although in some cases there may be discrepancies, as when the substance is a member of a group that has been evaluated collectively, e.g. the phosphates.

In proposing names for additives, those submitting information should consult the guidelines for designating names set out in WHO Technical Report Series 1989, No 776, part 2.3.4. These allow the use of non-proprietary names established by international bodies, or used in national legislation, and indicate that in the absence of these, the name may be chosen from existing common or trivial names of the substance. The selected name must, however, be non-proprietary, and should be distinctive enough to enable the substance to be clearly distinguished from other food additives.

The ***TITLE*** also indicates those cases where the specifications have been designated as 'Tentative'. This designation is used when the Committee regards the information on the additive as insufficient to elaborate fully acceptable specifications, and in such cases the specifications will include an indication of the further information required.

The ***TITLE*** further includes a statement referring to the JECFA meeting at which the specifications were prepared. In cases where the specifications supersede earlier specifications, reference will also be made to these earlier specifications. The statement also includes a reference to the Committee's latest safety evaluation of the substance.

#### ***SYNONYMS***

Listed in this section are names, acronyms, and abbreviations under which the substance is widely known, other than those used in the ***TITLE*** or in the *Chemical name(s)* (see below). The Codex Alimentarius International Numbering System (INS) number and the USA FD&C number (for colours) are also included here where applicable. Common or trivial names may also be included as synonyms, but registered trade names are not used.

#### ***DEFINITION***

This section normally includes information on the raw materials used in the manufacture of the additive, together with a brief description of the salient points of the manufacturing method. Proprietary manufacturing information may be

excluded provided that this has been agreed with the JECFA Secretariat. For some substances, for example those of natural origin or those containing a number of different components, more detailed information, including manufacturing and purification methods, is given as necessary. The possible presence of other substances included in commercial additive preparations - for example anti-caking agents and antioxidants - may also be noted.

*Chemical name(s)*

Where an IUPAC or IUBMB name exists for an additive, this is generally included under this heading and listed first among the chemical names, whether or not it is the systematic name or the recommended common name.

*CAS number(s)*

The Chemical Abstract Service registry number(s) (CAS number(s)) for the major component(s) of the substance is normally given here. CAS numbers for substances that are encompassed by the specifications, e.g. specific isomeric forms of the main component(s), may also be included along with suitable descriptions.

*Chemical formula(e)*

The chemical formula(e) of the major component(s) of the additive is given here.

*Structural formula(e)*

The structural formula(e) corresponding to the major component(s) given under the previous heading is given here, as appropriate.

*Formula weight*

Formula weights are quoted in JECFA specifications in preference to molecular weights in order to avoid the improper use of the latter form. Formula weights are calculated from values quoted by IUPAC in its Table of Standard Atomic Weights.

*Assay*

A quantitative assay requirement is provided here, where applicable, to indicate the minimum acceptable content, or maximum acceptable content range, of the principal functional component(s) of the additive.

**DESCRIPTION**

Information on physical appearance and other significant properties, e.g. stability and odour, is provided in this section, and will normally include any special conditions required for the storage and use of the additive. Such information is by its nature descriptive and should not be interpreted as rigidly as, for example, the requirements under the **DEFINITION** section. For safety reasons, descriptions of the taste of a substance are not included.

**FUNCTIONAL USES**

Functional uses are included in specifications to indicate the technological functions of the additive as used in foods or in food processing. The stated functional uses are not necessarily an exhaustive list, however, and an additive may have uses other than those listed. The functional uses are intended to

conform as far as possible to the harmonized Codex Alimentarius International Numbering System (INS) list of functional uses. Where this is not possible, an unlisted term may be used, and in these cases the new term will be referred for further discussion by the Codex Committee on Food Additives and Contaminants (CCFAC) as part of its consideration of the possible adoption of the specifications as Codex Advisory Specifications.

## **CHARACTERISTICS**

### *IDENTIFICATION*

Identification criteria are generally qualitative and provide part of the means for defining the specified additive. Such criteria typically include solubility in water, solubility in organic solvents, colour reactions, absorption spectra, and pH values.

Where possible, test methods refer to procedures detailed in Volume 4. If the test procedure is not included in the published tests given in Volume 4, it will be included here provided it can be described briefly. Otherwise the details will be set out in the **TESTS** section under the heading *IDENTIFICATION TESTS*.

### *PURITY*

Items relating to the purity of the additive, such as limits on impurities and, where appropriate, criteria for microbiological purity, are included under this heading. Limits for trace impurities and for other parameters relating to purity, for example physical properties, are based on the information available on the manufacturing process at the time the specifications were prepared. Limits are set to be consistent with good manufacturing practice and to help provide an assurance of safety, taking into account the use of the additive.

Where possible, test methods refer to procedures detailed in Volume 4. If the test procedure is not included in the published tests given in Volume 4, it will be included here provided it can be described briefly. Otherwise the details of the test will be set out in the **TESTS** section under the heading *PURITY TESTS*.

## **TESTS**

### *IDENTIFICATION TESTS*

This section describes in full those test procedures referenced in the **CHARACTERISTICS** section under the heading *IDENTIFICATION*. The basic principle behind the analytical method is normally included in the narrative, in addition to details of the apparatus and reagents, the analytical procedure, and the method for calculating results.

### *PURITY TESTS*

This section describes in full those test procedures referenced in the **CHARACTERISTICS** section under the heading *PURITY*. The basic principle behind the analytical method is normally included in the narrative, in addition to details of the apparatus and reagents, the analytical procedure, and the method for calculating results. Suppliers of reference standards are named in the text.

### **METHOD OF ASSAY**

The Method of Assay includes a description of the principle of the method, a list of the apparatus and reagents required, details of the analytical procedure, and the

method for calculating results. Where possible, these are described by reference to procedures listed in Volume 4. Suppliers of reference standards are named in the text.

### **Enzyme preparations**

Enzyme preparations used in food processing, whether from animal, vegetable or microbial sources, must comply with the *General Specifications and Considerations for Enzyme Preparations used in Food Processing* found in the next section of this Introduction. In addition, enzyme preparations must meet the specifications criteria contained in the individual specifications monographs. These normally include the headings listed below, which are given in the order in which they appear in the specifications.

#### ***TITLE***

The ***TITLE*** of the specifications monograph includes the name given to the enzyme preparation. This will normally correspond to the name of the enzyme activity or activities that most accurately characterize the preparation. Where appropriate, the source material from which the preparation is derived is also included, so that the name will normally take the form [*Principal enzyme activity/activities*] from [*Systematic IUBMB name of source organism*] [*Description of genetic modification process where applicable*]. The name may or may not be the same as the systematic name recommended by the IUBMB Enzyme Commission Nomenclature Committee.

The ***TITLE*** also indicates those cases where the specifications have been designated as 'Tentative'. This designation is used when the Committee regards the information on the enzyme preparation as insufficient to elaborate fully acceptable specifications, and in such cases the specifications will include an indication of the further information required and the date by which this must be submitted.

The ***TITLE*** further includes a statement referring to the JECFA meeting at which the specifications were prepared. In cases where the specifications supersede earlier specifications, reference will also be made to these earlier specifications. The statement also includes a reference to the Committee's latest safety evaluation of the substance defined by the specifications.

#### ***SYNONYMS***

This section includes names and abbreviations under which the preparation is widely known, other than those used in the ***TITLE***. The INS number is also listed where applicable.

#### ***SOURCES***

This section identifies the animal, plant or microbial sources used to derive the enzyme preparation. The species, strains or variants, strain numbers and plasmid numbers, if from recognized culture collections/depositories (e.g. ATCC), are also given where appropriate. In cases where the source organism has been derived using recombinant DNA technology, a description of this process, including the identity of the host organism, is included.

*Active principles*

Listed in this section are the principal enzyme activities exhibited by the preparation. IUBMB-recommended names are preferred and will generally be listed first, but other names may also be included.

*Systematic names and numbers*

Where IUBMB systematic names and Enzyme Commission (EC) enzyme numbers exist, these are listed for each active principle. CAS numbers are also given here where these exist.

*Reactions catalysed*

This section includes a description of the substrates acted on by the enzyme preparation, the reactions catalysed, and the resultant products.

*Secondary enzyme activities*

Listed here, if appropriate, are minor enzyme activities that may be present in the enzyme preparation.

**DESCRIPTION**

Information on physical appearance is provided in this section, together with other information such as solubility in water, solubility in organic solvents, and relevant details of the manufacturing process. Information on the diluents, carriers, stabilisers, preservatives and immobilization agents that may be present in commercial products is also included in this section.

**FUNCTIONAL USES**

This section gives the principal and secondary technological function(s) of the enzyme preparation as used in foods or in food processing.

**GENERAL SPECIFICATIONS**

This gives a statement to the effect that all preparations have to conform to the *General Specifications and Considerations for Enzyme Preparations used in Food Processing* (see the next section of this Introduction).

**CHARACTERISTICS***IDENTIFICATION*

The enzyme activities of the active principles are listed here. Also listed here are criteria for trace impurities resulting from, for example, the use of carriers and immobilization agents, other than those noted in the *General Specifications and Considerations for Enzyme Preparations used in Food Processing* (see the next section of this Introduction).

Where possible, the assay methods refer to procedures detailed in Volume 4. Otherwise the details of the test are set out in the section on **TESTS**.

**TESTS**

Assay methods for the enzyme activities of the active principles listed in the **CHARACTERISTICS** section under the heading *IDENTIFICATION* are included here in cases where this cannot be done by reference to Volume 4. These describe



the principle of the method, apparatus and reagents required, and give details of the analytical procedure and the method of calculating results. Suppliers of standards for test materials are named in the text.

### Flavouring agents

Specifications for flavouring agents are set out in tabular format. They are not included in the Combined Compendium and, as noted above, may be found in the searchable Flavouring Agents database at the FAO website. The specific items included in flavouring agent specifications are set out below.

Specification Heading	Comment
No.	The number allocated by JECFA
Name	The name by which the flavouring agent is known in the trade
Chemical name	The IUPAC name or a similar, more familiar, name
Synonyms	Usually restricted to not more than four names
FEMA	The number allocated by the Flavour and Extract Manufacturers Association of the United States
COE	The number allocated by the Council of Europe. These will be superseded in due course by the European Commission number
CAS	Chemical Abstracts Service registry number
Mol Wt	Molecular weight
Formula	Empirical formula
Physical form/odour	Descriptions of the physical form and odour
Solubility	Solubility in water and in solvents other than ethanol.
Solubility in ethanol	For interpretation see Volume 4
Boiling point (°C)	At 760 mm Hg, unless specified otherwise
ID Test	Identification test method(s) (IR, NMR, MS)
Assay min	Minimum assay value. Where this is <95%, other components are also specified. See also <i>Other requirements</i>
Acid value max	Upper limit for acid value
Refractive index	At 20°C unless otherwise stated
Specific gravity	At 25°C unless otherwise stated
Other requirements	May include melting point (mp), content of significant other components, and other significant descriptors
JECFA	Contains a reference to the JECFA meeting at which the substance was considered. The abbreviations used are: N = New specifications; R = Specifications revised; T = 'Tentative' specifications (additional information required); S = Existing specifications maintained; N,T = New, 'tentative' specifications ; S,T = Existing, 'tentative' specifications maintained.
Data required	Listed in the case of 'Tentative' specifications

## GENERAL SPECIFICATIONS AND CONSIDERATIONS FOR ENZYME PREPARATIONS USED IN FOOD PROCESSING

*The following general specifications were prepared by the Committee at its sixty-seventh meeting (2006) for publication in FAO JECFA Monographs 3 (2006), superseding the general specifications prepared at the fifty-seventh meeting (1) and published in FAO JECFA Monographs 1 (2). These specifications were originally prepared by the Committee at its twenty-fifth meeting (3) and published in FAO Food and Nutrition Papers No. 19 and No. 31/2 (4,5). Subsequent revisions were made by the Committee at its thirty-fifth meeting and published in FAO Food and Nutrition Paper No. 52 (6). Additional amendments were made at the fifty-first meeting and published in FAO Food and Nutrition Paper No. 52 Add. 6 (7), and at the fifty-third meeting (8) and partially published in FAO Food and Nutrition Paper No. 52 Add. 7 (9).*

### Classification and Nomenclature of Enzymes

Enzymes are proteins that catalyse chemical reactions. The Enzyme Commission of the International Union of Biochemistry and Molecular Biology (formerly the International Union of Biochemistry) classified enzymes into six main classes: oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases (10). Based on the type of reaction catalysed, enzymes are assigned to one of these classes and given an Enzyme Commission (EC) number, a systematic name, and a common name. Other names are also provided, if available. Enzymes used in food processing are often referred to by their common or traditional names such as protease, amylase, malt, or rennet. For enzymes derived from microorganisms, the name of the source microorganism is usually specified, for example, “ $\alpha$ -amylase from *Bacillus subtilis*.” For enzymes derived from microorganisms modified by using recombinant DNA techniques (referred to as recombinant-DNA microorganisms or genetically modified microorganisms), the names of both the enzyme source (donor organism) and the production microorganism are provided, for example, “ $\alpha$ -amylase from *Bacillus licheniformis* expressed in *Bacillus subtilis*.”

### Enzyme Preparations

Enzymes are used in food processing as enzyme preparations. An enzyme preparation contains an active enzyme (in some instances a blend of two or more enzymes) and intentionally added formulation ingredients such as diluents, stabilizing agents, and preserving agents. The formulation ingredients may include water, salt, sucrose, sorbitol, dextrin, cellulose, or other suitable compounds. Enzyme preparations may also contain constituents of the source organism (i.e. an animal, plant, or microbial material from which an enzyme was isolated) and compounds derived from the manufacturing process, for example, the residues of the fermentation broth. Depending on the application, an enzyme preparation may be formulated as a liquid, semi-liquid or dried product. The colour of an enzyme preparation may vary from colourless to dark brown. Some enzymes are immobilized on solid support materials.

### Active Components

Enzyme preparations usually contain one principal enzyme that catalyses one specific reaction during food processing. For example,  $\alpha$ -amylase catalyses the hydrolysis of 1,4- $\alpha$ -D-glucosidic linkages in starch and related polysaccharides. However, some enzyme preparations contain a mixture of enzymes that catalyse two or more different reactions in food. Each principal enzyme present in an enzyme preparation is characterized by its systematic name, common name, and EC number. The activity of each enzyme is measured using an appropriate assay and expressed in defined activity units per weight (or volume) of the preparation.

### Source Materials

Enzymes used in food processing are derived from animal, plant, and microbial sources. Animal tissues used for the preparation of enzymes should comply with meat inspection requirements and be handled in accordance with good hygienic practice.

Plant material and microorganisms used in the production of enzyme preparations should not leave any residues harmful to health in the processed finished food under normal conditions of use.

Microbial strains used in the production of enzyme preparations may be native strains or mutant strains derived from native strains by the processes of serial culture and selection or mutagenesis and selection or by the application of recombinant DNA technology. Although nonpathogenic and nontoxigenic microorganisms are normally used in the production of enzymes used in food processing, several fungal species traditionally used as sources of enzymes are known to include strains capable of producing low levels of certain mycotoxins under fermentation conditions conducive to mycotoxin synthesis (11–15). Enzyme preparations derived from such fungal species should not contain toxicologically significant levels of mycotoxins that could be produced by these species.

Microbial production strains should be taxonomically and genetically characterized and identified by a strain number or other designation. The strain identity may be included in individual specifications, if appropriate. The strains should be maintained under conditions that ensure the absence of genetic drift and, when used in the production of enzyme preparations, should be subjected to methods and culture conditions that are applied consistently and reproducibly from batch to batch. Such conditions should prevent the introduction of microorganisms that could be the source of toxic and other undesirable substances. Culture media used for the growth of microbial sources should consist of components that leave no residues harmful to health in the processed finished food under normal conditions of use.

Enzyme preparations should be produced in accordance with good food manufacturing practice and cause no increase in the total microbial count in the treated food over the level considered to be acceptable for the respective food.

### Substances used in Processing and Formulation

Substances used in processing and formulation of enzyme preparations should be suitable for their intended uses.

In the case of immobilized enzyme preparations, leakage of active enzymes, support materials, crosslinking agents and/or other substances used in immobilization should be kept within acceptable limits established in the individual specifications.

To distinguish the proportion of the enzyme preparation derived from the source material and manufacturing process from that contributed by intentionally added formulation ingredients, the content of total organic solids (TOS) is calculated as follows:

$$\% \text{ TOS} = 100 - (A + W + D)$$

where:

A = % ash, W = % water and D = % diluents and/or other formulation ingredients.

### Purity

Lead: Not more than 5 mg/kg

Determine using an atomic absorption spectroscopy/inductively coupled atomic-emission spectroscopy (AAS/ICP-AES) technique appropriate to the specified level. The selection of the sample size and the method of sample preparation may be based on the principles described in the *Compendium of Food Additive Specifications*, Volume 4.

Microbiological criteria:

*Salmonella* species: absent in 25 g of sample

Total coliforms: not more than 30 per gram

*Escherichia coli*: absent in 25 g of sample (Determine using procedures described in the section on Microbiological Analyses, Volume 4)

Antimicrobial activity:

Absent in preparations from microbial sources.

### Other Considerations

Safety assessment of food enzyme preparations has been addressed in a number of publications and documents. Pariza & Foster (11) proposed a decision tree for determining the safety of microbial enzyme preparations. Pariza & Johnson (16) subsequently updated this decision tree and included information on enzyme preparations derived from recombinant-DNA microorganisms. The Scientific Committee on Food (17) issued guidelines for the presentation of data on food enzymes. The document includes a discussion on enzymes from genetically modified organisms including microorganisms, plants, and animals. Several international organizations, government agencies, and expert groups have also published discussion papers or guidelines that address safety assessment of food and food ingredients derived from recombinant-DNA plants and microorganisms (18–28). Certain information in these documents may be applicable to enzyme preparations derived from recombinant sources.

An overall safety assessment of each enzyme preparation intended for use in food processing should be performed. This assessment should include an evaluation of the safety of the production organism, the enzyme component, side activities, the manufacturing process, and the consideration of dietary exposure. Evaluation of the enzyme component should include considerations of its potential to cause an allergic reaction. For enzyme preparations from recombinant-DNA microorganisms, the following should also be considered:

- The genetic material introduced into and remaining in the production microorganism should be characterized and evaluated for function and safety, including evidence that it does not contain genes encoding known virulence factors, protein toxins, and enzymes involved in the synthesis of mycotoxins or other toxic or undesirable substances.
- Recombinant-DNA production microorganisms might contain genes encoding proteins that inactivate clinically useful antibiotics. Enzyme preparations derived from such microorganisms should contain neither antibiotic inactivating proteins at concentrations that would interfere with antibiotic treatment nor transformable DNA that could potentially contribute to the spread of antibiotic resistance.

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## TESTS AND ASSAYS

Descriptions and terms applying to the tests and assays in JECFA specifications include the following:

### *Analytical Samples*

The quantity of the analytical sample to be used is usually indicated in tests and assays. Unless otherwise specified, the quantity used may deviate by 10% from that stated. All quantitative determinations should be conducted on duplicate test portions and in these cases, the amount actually taken should be accurately weighed or measured and the result of the analysis calculated on this exact quantity. When substances are to be "accurately weighed" in a test or assay, the weighing is to be performed in such manner as to limit the error to  $\pm 0.1\%$  or less. Quantities smaller than 100 mg should be weighed to the nearest 0.1 mg.

### *Analytical Standards*

Certain procedures (e.g. chromatographic and spectrophotometric instrumental analyses, and antibiotic and enzyme assays) require the use of analytical reference standards. Where suitable standards are available from recognized international bodies, these are specified. In the absence of international standards, it has been necessary in some cases to specify the use of reference standards available from such organizations as the British Pharmacopoeia (BP), Food Chemicals Codex (FCC), National Formulary (NF) of the United States, or the United States Pharmacopoeia (USP). The addresses of these organizations may be found in the individual monographs.

### *Apparatus*

With the exception of volumetric flasks and other exact measuring or weighing devices, directions to use a certain size or type of container or other laboratory apparatus are intended only as recommendations, unless otherwise specified. In certain unavoidable cases, the Committee has found it necessary, for accurate description, to use a proprietary name to indicate a certain type of instrument (e.g. spectrophotometer or chromatograph) that is known to give satisfactory results in a particular analytical procedure. However, such a listing in specifications does not necessarily constitute endorsement of the specified instrument by the Committee, nor does it imply that similar instruments from other sources cannot be used with equal or better satisfaction, or that they are of lesser quality or utility than the instrument named.

### *Blank Tests*

The instruction to "perform a blank determination", or similar instructions, indicates that a reagent blank determination should be conducted, in which the same quantities of the same reagents are used, and the same procedure is repeated in every detail, except that the substance being tested is omitted.

### *Constant Weight*

A direction that a substance is to be "dried to constant weight" means that the drying should be continued until two consecutive weighings differ by not more

than 0.5 mg per g of sample taken, the second weighing to follow an additional hour of drying time at the temperature specified. The direction to "ignite to constant weight" means that the ignition should be continued at a temperature of 450°-550°, unless otherwise specified, until two consecutive weighings do not differ by more than 0.5 mg per g of sample taken, the second weighing to follow an additional 30 min ignition period, depending upon the nature of the sample tested.

### ***Desiccants and Desiccators***

The expression "in a desiccator" means the use of a tightly closed container of appropriate size and design in which a low moisture content can be maintained by means of a suitable desiccant. Preferred desiccants include, but are not limited to, anhydrous calcium chloride, magnesium perchlorate, phosphorus pentoxide, and silica gel.

### ***Indicators***

The quantity of an indicator solution used should be 0.2 ml (approximately 3 drops), unless another quantity is specified.

### ***Odourless***

The term "odourless" applies to the examination, after exposure to air for 15 min, of between 1 and 25 g of the substance that has been transferred from the original container to an open evaporating dish of about 100 ml capacity.

### ***Reagents***

Reagents used in tests and assays should be of appropriate analytical grade and should contain no interfering impurities.

### ***Significant Figures***

Where tolerance limits are expressed numerically, the values are considered to be significant to the number of digits shown. Thus, "not less than 99.0%" means 99.0% but not 99.00%. Values obtained in tests and assays should be rounded off to the nearest indicated digit according to the commonly used practice of rejecting or increasing numbers less than or greater than 5. For example, a requirement of not less than 96.0% would be met by a result of 95.96% but not by a result of 95.94%. When the digit to be dropped is exactly 5, the value should be rounded off to the closest even digit. Thus, both 1.4755 and 1.4765 would be rounded off to 1.476. When a range is stated, the upper and lower limits are inclusive, so that the range consists of the two values themselves, properly rounded off, and all intermediate values between them.

### ***Solutions***

All solutions, unless otherwise specified, are to be prepared with distilled or deionized water. Directions for the preparations of "TS" (test solutions), "TSC" (colorimetric solutions) and "PbT" (lead free solutions) are provided in Volume 4. Where volumetric solutions of definite concentration are directed to be used in quantitative determinations, standardized solutions of other concentrations may be employed, unless otherwise specified, if allowance is made for the calculation



factor and if the error of measurement is known not to be increased significantly thereby. Unless otherwise specified, it should be understood that concentrations of solutions prepared from liquids only are expressed in terms of volume in volume (v/v), and solutions of solids in liquids are expressed in terms of weight in volume (w/v). Thus, expressions such as "1 in 10" or "10%" mean that 1 part by volume of a liquid, or 1 part by weight of a solid, is to be dissolved in a volume of the diluent or solvent sufficient to make the finished solutions 10 parts by volume. For other types of solutions (e.g. gases in liquids), and where the above guidelines do not apply, the directions will specify the basis on which the concentration is determined (e.g. w/w, v/w).

### ***Temperatures***

Unless otherwise specified, temperatures are expressed in degrees Celsius, and all measurements are to be made at 20°. Ordinary procedures not involving precise instrumental measurements may be conducted at ambient temperature (approximately 15°-30°) unless a particular temperature is specified in a test or assay.

### ***Turbidity***

The terms "clear", "almost clear", "very slightly turbid", and "turbid", as specified in Purity Tests for "Clarity and colour of solution", are defined in the individual monographs. The term "no turbidity is produced" means that the clarity of the solution does not change.

### ***Vacuum***

The unqualified use of the term "in vacuum" or "in vacuo" means a pressure at least as low as that obtainable by an efficient aspirating water pump (i.e. not higher than about 20 mm of mercury).

### ***Water-bath***

The term "water-bath" means a bath of boiling water, unless water at some other temperature is indicated. An alternative form of heating may be employed, provided that the required temperature is approximately maintained and not exceeded.

## WEIGHTS AND MEASURES

JECFA generally uses SI units and units accepted for use with SI. The units and abbreviations commonly used in specifications are as follows:

m = metre  
 cm = centimetre ( $10^{-2}$  m)  
 mm = millimetre ( $10^{-3}$  m)  
 $\mu$ m = micrometre ( $10^{-6}$  m)  
 nm = nanometre ( $10^{-9}$  m)  
 g = gram  
 kg = kilogram ( $10^3$  g)  
 mg = milligram ( $10^{-3}$  g)  
 $\mu$ g = microgram ( $10^{-6}$  g)  
 ng = nanogram ( $10^{-9}$  g)  
 l = litre  
 ml = millilitre ( $10^{-3}$  l)  
 $\mu$ l = microlitre ( $10^{-6}$  l)  
 h = hour(s)\*  
 min = minute(s)\*  
 sec = second(s)  
 $^{\circ}$  = degrees Celsius  
 N = normality (gram equivalents per litre)  
 M = molarity (moles per litre)  
 $\text{cm}^{-1}$  = wave number  
 bar = unit of pressure ( $\text{kgm}^{-1}\text{sec}^{-2}$ )\*  
 mm Hg = mm of mercury, unit of pressure \*\*  
 $R_f$  = ratio of (distance of spot from point of application): (distance moved by solvent)  
 rpm = revolutions per minute

\*Time in minutes or hours, and volume in litres or decimals of litres, are outside the SI, but are accepted for use within it. The term 'bar' is also outside the SI, but is subject to review.

\*\* 'mm Hg' is outside the SI system, but is in common use and is retained: 760 mm Hg is equal to 1.013 bar.

## ABBREVIATIONS

JECFA specifications include various abbreviations and acronyms, as set out below.

ADI = Acceptable Daily Intake (expressed in mg/kg bw)  
AOAC = AOAC INTERNATIONAL, formerly the Association of Official Analytical Chemists  
ASTM = ASTM INTERNATIONAL, formerly the American Society for Testing and Materials  
ATCC = American Type Culture Collection  
CAC = Codex Alimentarius Commission  
CAS = Chemical Abstracts System  
C.I. = Colour Index  
EC = Enzyme Commission of IUBMB (for systematic nomenclature and numbering system of enzymes)  
FD&C = Food, Drug and Cosmetic  
FCC = Food Chemicals Codex (USA)  
FNP = FAO Food and Nutrition Paper  
FNS = FAO Food and Nutrition Series  
FW = Formula weight  
G(L)C = Gas (Liquid) chromatography  
(HP)LC = (High Performance) Liquid Chromatography  
INS = Codex Alimentarius International Numbering System (for food additives)  
ISO = International Organization for Standardization  
IUBMB = International Union of Biochemistry and Molecular Biology  
IUPAC = International Union of Pure and Applied Chemistry  
JECFA = Joint FAO/WHO Expert Committee on Food Additives  
i.d. = internal diameter  
o.d. = outer diameter  
IR = Infrared  
meq = milliequivalent  
MW = Molecular weight  
Mol. Wt = Molecular weight  
MTDI = Maximum Tolerable Daily Intake  
soln = solution  
NMRS = FAO Nutrition Meeting Report Series  
TLC = Thin Layer Chromatography  
TRS = WHO Technical Report Series  
UV = Ultraviolet  
VIS = Visible wavelength

**VOLUME 4**  
**Combined Compendium of Food Additive Specifications**

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## GENERAL INFORMATION

### Revision of Food and Nutrition Paper No. 5

In 2005, the decision was made by the Food and Agriculture Organization (FAO) to revise and update Food and Nutrition Paper (FNP) No. 5, "Guide to Specifications", containing general notices, general analytical techniques, identification tests, test solutions and other reference materials used in JECFA food additive specifications.

These revisions would be published in Volume 4 of the new Combined Compendium of Food Additive Specifications (FAO JECFA Monographs No. 1).

A number of JECFA and other national experts accepted to draft revisions and updates for selected chapters and other sections of FNP 5. The experts and the chapters or sections that they were requested to revise were as follows:

Dr. Madduri V. Rao (UAE University, United Arab Emirates)  
General Analytical Techniques  
Inorganic Components  
Phosphates  
Sweeteners

Mrs. Inge Meyland (Danish Institute of Food and Veterinary Research, Denmark)  
Appearance and Physical Properties  
Organic Components  
Polyols

Drs. Paul Kuznesof and Julie Barrows (US Food and Drug Administration, USA)  
Food Colours

Dr. Brian Whitehouse (Consultant, United Kingdom)  
Enzyme Preparations  
Flavouring Agents  
Flavour Enhancers

Dr. Richard Cantrill (American Oil Chemists Society, USA)  
Fats and Related Substances  
Hydrocarbons, Waxes and Oils

Dr. Keith Lampel (US Food and Drug Administration, USA)  
Microbiological Methods

In addition, Mr. John Howlett, Consultant, United Kingdom, was asked to prepare proposals for the overall reorganization of the revisions for incorporation into Volume 4.

A Technical Meeting was subsequently held at the Food and Agriculture Organization (FAO) headquarters in Rome, Italy, from 5 to 7 December 2005 to discuss and finalize the proposed revisions and updates prepared by the experts. That meeting was chaired by FAO Consultant John Weatherwax (USA) with FAO Consultant (and JECFA expert) Harriet Wallin (National Food Agency, Finland) as the Rapporteur. All experts involved in preparing revisions participated in the meeting, excepting Dr. Julie Barrows. The FAO JECFA Secretary, Dr. Annika Wennberg also participated.

## **Criteria for Replacement of Older Methods**

The meeting agreed that the following criteria would prompt replacement (if possible) of analytical methods being considered for revision:

- The method includes the use of packed gas chromatography columns. (It was noted that gas chromatographs designed for packed columns are no longer being manufactured).
- The method uses out-dated analytical techniques (e.g. polarography).
- The method includes use of hazardous reagents or solvents (see below).

Older analytical methods that are still in use and are functioning well were not deleted, but references were made to modern methods along with recommendations to use modern techniques.

Those methods which were not referred to by at least one food additive specification were deleted.

## **Food Chemicals Codex**

The Food Chemicals Codex (FCC), published by the National Academies, Washington, D.C., USA, has been an international reference for accepted standards of quality and purity of food chemicals since 1966. The current fifth edition of the FCC has updated many standards and analytical methods, based on changes in science and manufacturing since the previous edition. JECFA has used a number of FCC analytical methods in preparing food additive specifications. FCC methods used in this volume have been taken from the fifth edition.

## **Hazardous Reagents and Solvents**

In some cases, older methods specify the use of solvents or other reagents which are now known to be hazardous to human health. All such materials should be substituted by safer alternatives wherever possible and permitted by the analysis. Where safer substitutes are not available, and no alternative method can be used, the hazardous solvents or reagents should be used with appropriate caution.

## **Method Validation**

All analytical methods referenced in this volume have been shown to provide satisfactory analytical results in normal use. It is incumbent on the analyst, however, to appropriately validate any analytical procedure being used for the first time in their laboratories.

# ANALYTICAL TECHNIQUES

## CHROMATOGRAPHY

*(Note: The following is not intended to represent an exhaustive treatise on chromatographic methods, nor does it take into account numerous variations in procedures which may be necessary, depending upon the nature of analytes, particular reagents or instruments used. Though the fundamental principles on which the instruments work remain the same, the handling and operation of instruments vary from one manufacturer to the other. It is therefore recommended that, for more detailed instructions, the analyst follow the operation and maintenance manuals provided by the instrument manufacturers and directions provided by the supplier of the reagents for the particular additive under analysis.)*

### Introduction

Chromatography is defined as an analytical technique whereby a mixture of chemicals may be separated by virtue of their differential affinities for two immiscible phases. One of these, the stationary phase, consists of a fixed bed of small particles with a large surface area, while the other, the mobile phase or "eluant", is a fluid that moves constantly through, or over the surface of, the fixed phase. Chromatographic systems achieve their ability to separate mixtures of chemicals by selectively retaining some compounds in the stationary phase for varying times, while permitting others to move more freely. Therefore, the chromatogram may be evaluated qualitatively by determining the  $R_f$ , or retention factor, for each of the eluted substances. The  $R_f$  is a measure of that fraction of its total elution time that any compound spends in the mobile phase. Since this fraction is directly related to the fraction of the total amount of the solute that is in the mobile phase, the  $R_f$  can be expressed as

$$R_f = V_m C_m / (V_m C_m + V_s C_s),$$

where  $V_m$  and  $V_s$  are the volumes of the mobile and stationary phase, respectively, and  $C_m$  and  $C_s$  are the concentrations of the solute in either phase at any time. This can be simplified to

$$R_f = V_m / (V_m + K V_s),$$

where  $K = C_s / C_m$  and is an equilibrium constant that indicates this differential affinity of the solute for the two phases. Alternatively, a new constant  $k'$ , the capacity factor, may be introduced, giving another form of the expression:

$$R_f = 1 / (1 + k'),$$

where  $k' = K V_s / V_m$ . The capacity factor,  $k'$ , which is normally constant for small samples, is a parameter that expresses the ability of a particular chromatographic system to interact with a solute. The larger the  $k'$  value, the more the sample is retained.

Both the retention factor and the capacity factor may be used for qualitative identification of a solute or for developing strategies for improving separation. In terms of parameters easily obtainable from the chromatogram, the  $R_f$  is defined as the ratio of the distance travelled by the solute band to the distance travelled by the mobile solvent in a particular time. The capacity factor,  $k'$ , can be evaluated by the expression

$$k' = (t_r - t_0)/t_0,$$

where  $t_r$ , the retention time, is the elapsed time from the start of the chromatogram to the elution maximum of the solute, and  $t_0$  is the retention time of a solute that is not retained by the chromatographic system.

## Chromatography Principles

Retention of the solutes by the stationary phase may be achieved by one or a combination of mechanisms. Certain substances, such as alumina or silica gel, interact with the solutes primarily by adsorption, either physical adsorption, in which the binding forces are weak and easily reversible, or chemisorption, where strong bonding to the surface can occur. Another important mechanism of retention is partition, which occurs when the solute dissolves in the stationary phase, usually a liquid coated as a thin layer on the surface of an inert material or chemically bonded to it.

If the liquid phase is a polar substance (e.g. polyethylene glycol) and the mobile phase is nonpolar, the process is termed normal-phase chromatography. When the stationary phase is nonpolar (e.g. octadecylsilane) and the mobile phase is polar, the process is reversed-phase chromatography. For the separation of mixtures of ionic species, insoluble polymers called ion exchangers are used as the stationary phase. Solute ions in the mobile phase selectively displace an electrically equivalent amount of less strongly bound ions of the ion exchanger in order to maintain the electro neutrality of both phases.

The chromatographic separation of mixtures of large molecules such as proteins may be accomplished by a mechanism called size exclusion chromatography or gel permeation chromatography. The stationary phases used are highly cross-linked polymers that have incorporated a sufficient amount of solvent to form a gel. The separation is based on the physical size of the solutes; those that are too large to fit within the interstices of the gel are eluted rapidly, while the smaller molecules permeate into the pores of the gel and are eluted later.

Separation of certain molecules is accomplished by a mechanism called *affinity chromatography* in which specific binding between an antibody (stationary phase) and antigen (analyte) occur. In any chromatographic separation, more than one of the above mechanisms may be occurring simultaneously.

## Chromatography Techniques

Chromatographic separations may also be characterized according to the type of instrumentation or apparatus used. The types of chromatography that may be used are column, paper, thin-layer, gas and high-performance liquid chromatography. Each will be discussed below.

### Column Chromatography

#### *Apparatus*

The equipment needed for column chromatography is not elaborate, consisting only of cylindrical glass or Teflon tube that has a restricted outflow orifice. The dimensions of the tube are not critical and may vary from 10 to 40 mm in inner diameter and from 100 to 600 mm in length. For a given separation, greater efficiency may be obtained with a long narrow column, but the resultant flow rate will be lower. A fritted-glass disk may be seated in the end

of the tube to act as a support for the packing material. The column is fitted at the end with a stopcock or other flow-restriction device in order to control the rate of delivery of the eluant.

### *Procedure*

The stationary phase is introduced into the column either as a dry powder or as slurry in the mobile phase. Since a homogeneous bed free of void spaces is necessary to achieve maximum separation efficiency, the packing material is introduced in small portions and allowed to settle before further additions are made. Settling may be accomplished by allowing the mobile phase to flow through the bed, by tapping or vibrating the column if a dry powder is used, or by compressing each added portion using a tamping rod. The rod can be a solid glass, plastic, or metal cylinder whose diameter is slightly smaller than the column, or it can be a thinner rod onto the end of which has been attached a disk of suitable diameter. Ion-exchange resins and exclusion polymers are never packed as dry powders since after introduction of the mobile phase they will swell and create sufficient pressure to shatter the column. When the packing has been completed, the sample is introduced onto the top of the column. If the sample is soluble, it is dissolved in a minimum amount of the mobile phase, pipetted onto the column and allowed to percolate into the top of the bed. If it is not soluble or if the volume of solution is too large, it may be mixed with a small amount of the column packing. This material is then transferred to the chromatographic tube to form the top of the bed.

The chromatogram is then developed by adding the mobile phase to the column in small portions and allowing it to percolate through the packed bed either by gravity or under the influence of pressure or vacuum. Development of the chromatogram takes place by selective retardation of the components of the mixture as a result of their interaction with the stationary phase. In column chromatography, the stationary phase may act by adsorption, partition, ion exchange, exclusion of the solutes, or a combination of these effects.

When the development is complete, the components of the sample mixture may be detected and isolated by either of two procedures. The entire column may be extruded carefully from the tube, and if the compounds are coloured or fluorescent under ultraviolet light, the appropriate segments may be cut from the column using a razor blade. If the components are colourless, they may be visualized by painting or spraying a thin longitudinal section of the surface of the chromatogram with colour-developing reagents. The chemical may then be separated from the stationary phase by extraction with a strong solvent such as methanol and subsequently quantitated by suitable methods.

In the second procedure, the mobile phase may be allowed to flow through the column until the components of the mixture successively appear in the effluent. This eluate may be collected in fractions and the mobile phase evaporated if desired. The chemicals present in each fraction may then be determined by suitable analytical techniques.

### Paper Chromatography

In this type of chromatography, the stationary phase ordinarily consists of a sheet of paper of suitable texture and thickness. The paper used is made from highly purified cellulose, which has a great affinity for water and other polar solvents since it has many hydroxyl functional groups. The tightly bound water acts as the stationary phase, and therefore the mechanism that predominates is liquid-liquid or partition chromatography. Adsorption of solutes to the cellulose surface may also occur, but this is of lesser importance. Papers especially impregnated to permit ion-exchange or reverse-phase chromatography are also available.

### *Apparatus*

The essential equipment for paper chromatography consists of the following:

- *Vapour-tight chamber.* The chamber is constructed preferably of glass, stainless steel, or porcelain. It is provided with inlets for the addition of solvent or for releasing internal pressure, and it is designed to permit observation of the progress of the chromatographic run without being opened. Tall glass cylinders are convenient if they are made vapour-tight with suitable covers and a sealing compound.
- *Supporting rack.* The rack serves as a support for the solvent troughs and antisiphoning rods. It is constructed of a corrosion-resistant material about 5 cm shorter than the inside height of the chamber.
- *Solvent troughs.* The troughs, made of glass, are designed to be longer than the width of the chromatographic sheets and to contain a volume of solvent greater than that required for one chromatographic run.
- *Antisiphoning rods.* Constructed of heavy glass, the rods are placed on the rack and arranged to run outside of, parallel to, and slightly above the edge of the glass trough.
- *Chromatographic sheets.* Special chromatographic filter paper is cut to length approximately equal to the height of the chamber. The sheet is at least 2.5 cm wide but not wider than the length of the trough. A fine pencil line is drawn horizontally across the filter paper at a distance from one end such that when the sheet is suspended from the antisiphoning rods with the upper end of the paper resting in the trough and the lower portion hanging free into the chamber, the line is located a few cm below the rods. Care is necessary to avoid contaminating the paper by excessive handling or by contact with dirty surfaces.

### *Procedure for Descending Paper Chromatography*

Separation of substances by descending chromatography is accomplished by allowing the mobile phase to flow downward on the chromatographic sheet.

The substance or substances to be analyzed are dissolved in a suitable solvent. Convenient volumes of the resulting solution, normally containing 1 to 20  $\mu\text{g}$  of the compound, are placed in 6 to 10 mm spots along the pencil line not less than 3 cm apart. If the total volume to be applied would produce spots of a diameter greater than 6 to 10 mm, it is applied in separate portions to the same spot, each portion being allowed to dry before the next is added.

The spotted chromatographic sheet is suspended in the chamber by use of the antisiphoning rod and an additional heavy glass rod that holds the upper end of the sheet in the solvent trough. The bottom of the chamber is covered with a mixture containing both phases of the prescribed solvent system. It is important to ensure that the portion of the sheet hanging below the rods is freely suspended in the chamber without touching the rack or the chamber walls. The chamber is sealed to allow equilibration (saturation) of the chamber atmosphere and the paper with solvent vapour. Any excess pressure is released as necessary. For large chambers equilibration overnight may be necessary.

A volume of the mobile phase in excess of the volume required for complete development of the chromatogram is saturated with the immobile phase. After equilibration of the chamber, the prepared mobile solvent is introduced into the trough through the inlet. The inlet is closed, and the mobile phase is allowed to travel down the paper the desired distance. Precautions must be taken against allowing the solvent to run down the sheet when opening

the chamber and removing the chromatogram. The location of the solvent front is quickly marked, and the sheets are dried.

The chromatogram is observed and measured directly or after suitable development to reveal the location of the spots of the isolated components of the mixture.

#### *Procedure for Ascending Paper Chromatography*

In ascending chromatography, the lower edge of the sheet (or strip) is dipped into the mobile phase to permit the mobile phase to rise on the chromatographic sheet by capillary action.

The test materials are applied to the chromatographic sheet as directed under *Procedure for Descending Paper Chromatography*. Enough of both phases of the solvent mixture to cover the bottom of the chamber are added. Empty solvent troughs are placed on the bottom of the chamber, and the chromatographic sheet is suspended so that the end near which the spots have been added hangs free inside the empty trough.

The chamber is sealed, and equilibration is allowed to proceed as described under *Procedure for Descending Paper Chromatography*. Then the solvent is added through the inlet to the trough in excess of the quantity of solvent required for complete moistening of the chromatographic sheet. The chamber is resealed. When the solvent front has reached the desired height, the chamber is opened and the sheet is removed, the location of the solvent front is quickly marked, and the sheet is dried.

Small cylinders may be used without troughs so that only the mobile phase is placed on the bottom. The chromatographic sheet is suspended during equilibration with the lower end just above the solvent, and chromatography is started by lowering the sheet so that it touches the solvent.

#### *Detection of Chromatographic Bands*

After the chromatogram has been fully developed, the bands corresponding to the various solutes may be detected by means similar to those described in Column Chromatography. If the compounds are coloured or fluorescent under ultraviolet light, they may be visualized directly. Colourless compounds may be detected by spraying the paper with colour-developing reagents. The bands corresponding to the individual components can be cut from the paper, and the chemical substances eluted from the cellulose by the use of a strong solvent such as methanol.

#### *Identification of Solutes*

Since the chromatographic mobilities of the solutes may change from run to run due to varying experimental conditions, presumptive identification of a substance should be based on comparison with a reference standard. The  $R_f$  values of the unknown substance and the standard on the same chromatogram must be identical. Alternatively, the ratio between the distances travelled by a given compound and a reference substance, the  $R_r$  value, must be 1.0. Identification may also be made by mixing a small amount of the reference substance with the unknown and chromatographing. The resulting chromatograph should contain only one spot. Definitive identification of solutes may be achieved by eluting them from the paper and subjecting them to UV, IR, NMR, or mass spectrometry.

#### Thin-layer Chromatography

In thin-layer chromatography (TLC), the stationary phase is a uniform layer of a finely divided powder that has been coated on the surface of a glass or plastic sheet and that is held

in place by a binder. The capacity of the system is dependent on the thickness of the layer, which may range from 0.1 to 2.0 mm. The thinner layers are used primarily for analytical separations, while the thicker layers, because of their greater sample-handling ability, are useful for preparative work.

Substances that are used as coatings in TLC include silica gel, alumina, cellulose and reversed phase packings. Separations occur due to adsorption of the solutes from the mobile phase onto the surface of the thin layer. However, adsorption of water from the air or solvent components from the mobile phase can give rise to partition or liquid-liquid chromatography. Specially coated plates are available that permit ion-exchange or reverse-phase separations.

### *Apparatus*

Acceptable apparatus and materials for thin-layer chromatography consist of the following:

- *Glass plates*: Flat glass plates of uniform thickness throughout their areas. The most common sizes are 20 x 20 cm and 5 x 20 cm. (Aluminum plates also are commonly used).
- *Aligning tray*: An aligning tray or other suitable flat surface is used to align and hold plates during application of the adsorbent.
- *Adsorbent*: The adsorbent may consist of finely divided adsorbent materials for chromatography. It can be applied directly to the glass plate, or it can be bonded to the plate by means of plaster of Paris or with starch paste. Pretreated chromatographic plates are available commercially.
- *Spreader*: A suitable spreading device that when moved over the glass plate applies a uniform layer of adsorbent of desired thickness over the entire surface of the plate.
- *Storage rack*: A rack of convenient size to hold the prepared plates during drying and transportation.
- *Developing chamber*: A glass chamber that can accommodate one or more plates and can be properly closed and sealed as described under Paper Chromatography. It is fitted with a plate-support rack that can support the plates when the lid of the chamber is in place.
- *UV Chamber*: A UV viewing chamber with eye protection fitted with ultraviolet light source of short (254 nm) and long (360 nm) ultraviolet wavelengths suitable for observations may be required.

### *Procedure*

Clean the plates scrupulously, as by immersion in a chromic acid cleansing mixture, and rinse them with copious quantities of water until the water runs off the plates without leaving any visible water or oily spots, and then dry.

Arrange the plate or plates on the aligning tray, and secure them so that they will not slip during the application of the adsorbent. Mix an appropriate quantity of adsorbent and liquid, usually water, which when shaken for 30 sec gives a smooth slurry that will spread evenly with the aid of a spreader. Transfer the slurry to the spreader, and apply the coating at once before the binder begins to harden. Move the spreader smoothly over the plates from one end of the tray to the other. Remove the spreader, and wipe away excess slurry. Allow the plates to set for 10 min, and then place them in the storage rack and dry at 105° for 30 min or as directed in the monograph. Store the finished plates in a desiccator.



Equilibrate the atmosphere in the developing chamber as described under the *Procedure for Descending Paper Chromatography* in the section on Paper Chromatography.

Apply the Sample Solution and the Standard Solution at points about 1.5 cm apart and about 2 cm from the lower edge of the plate (the lower edge is the first part over which the spreader moves in the application of the adsorbent layer), and allow to dry. A template will aid in determining the spot points and the 10 to 15 cm distance through which the solvent front should move.

Arrange the plate on the supporting rack (sample spots on the bottom), and introduce the rack into the developing chamber. The solvent in the chamber must be deep enough to reach the lower edge of the adsorbent, but must not touch the spot points. Seal the cover in place, and maintain the system until the solvent ascends to a point 10 to 15 cm above the initial spots, this usually requiring from 15 min to 1 h. Remove the plates, and dry them in air. Measure and record the distance of each spot from the point of origin. If so directed, spray the spots with the reagent specified, observe, and compare the sample with the standard chromatogram.

#### *Detection and Identification*

Detection and identification of solute bands is done by methods essentially the same as those described in Paper Chromatography and Column Chromatography. However, in TLC an additional method called fluorescence quenching is also used. In this procedure, inorganic phosphorus is mixed with the adsorbent before it is coated on the plate. When the developed chromatogram is irradiated with ultraviolet light, the surface of the plate fluoresces with a characteristic colour except in those places where ultraviolet-adsorbing solutes are situated. These quench the fluorescence and are detectable as dark spots.

#### *Quantitative Analysis*

Two methods are available if quantitation of the solute is necessary. In the first, the bands are detected and their position marked. Those areas of adsorbent containing the compounds of interest are scraped from the surface of the plate into a centrifuge tube. The chemicals are extracted from the adsorbent with the aid of a suitable strong solvent, the suspension is centrifuged, and the supernatant layer is subjected to appropriate methods of quantitative analysis.

The second method involves the use of a scanning densitometer. This is a spectrophotometric device that directs a beam of monochromatic radiation across the surface of the plate. After interaction with the solutes in the adsorbent layer, the radiation is detected as transmitted or reflected light and a recording of light intensity versus distance travelled is produced. The concentration of a particular species is proportional to the area under its peak and can be determined accurately by comparison with standards.

## **Gas Chromatography**

This type of chromatography differs from the others in that the mobile phase is a gas and therefore the solutes must be vaporized in order to allow their movement through the chromatographic column. Gas chromatography is further divided into:

- Gas-solid chromatography, GSC (where stationary phases that are used are solids) or;
- Gas-liquid chromatography, GLC (where stationary phases that are used are liquids).

In gas-solid chromatography, the stationary phase is an active adsorbent, such as alumina, silica, carbon or a polyaromatic porous resin, packed into a column. The passage of solute

through the column will be retarded by adsorption or exclusion mechanisms. In gas-liquid chromatography, the stationary phase is a high boiling point liquid which is finely coated on an inert solid support, such as diatomaceous earth or a porous polymer which is packed into a column (packed column) or is coated as a thin film on the inside of a column (capillary column).

When a volatile compound is introduced into the carrier gas and carried into the column, it is partitioned between the gas and stationary phases by a dynamic countercurrent distribution process. The compound is carried down the column by the carrier gas, retained to a greater or lesser extent by sorption and desorption in the stationary phase. The elution of the compound is characterized by the partition ratio,  $k$ , a dimensionless quantity also called the capacity factor. It is equivalent to the ratio of the time required for the compound to flow through the column (the retention time) to the retention time of an unretained compound. The value of the capacity factor depends on the chemical nature of the compound; the nature, amount, and surface area of the liquid phase; and the column temperature. Under a specified set of experimental conditions, a characteristic capacity factor exists for every compound. Separation by gas chromatography occurs only if the compounds concerned have different capacity factors

#### *Apparatus*

*(Note: Most gas chromatographic methods presented in this manual are based on capillary columns as they provide better separation and have replaced the traditional packed columns. As a result, manufacturers of gas chromatographs (GC) have discontinued the manufacture of packed column GCs. However, certain methods using packed columns are listed under individual monographs either due to non-availability of a suitable capillary column or an equivalent method using capillary column has not been developed as yet. In the absence of availability of a packed column GC, analyst may choose an equivalent capillary column, develop and validate proper method for use. The JECFA Secretariat highly appreciates receiving updates on such developments to include in future publications).*

The essential components of a basic gas chromatograph are a carrier gas supply, an injection port, column oven, column, detector, and a suitable data-recording device. The injection port, column and detector are carefully temperature controlled.

Carrier gas supply: Typical carrier gas is helium or nitrogen, depending on the column and detector in use. The gas is supplied from a high-pressure cylinder, suitable pressure or flow controllers are used to regulate the pressure or flow of the carrier gas. Carrier gas shall be highly pure (minimum of 99.999%, water < 1ppm, oxygen <0.1 ppm) and free from any particulate matter. Additionally, gas purifiers such as activated charcoal to remove hydrocarbons, molecular sieve to remove traces of water and oxygen trap may be used to further purify the carrier gas. On line filters (2  $\mu$ m) may also be used to remove any particulate matter.

Sample injection device: Sample injectors depend on the type of column connected to the injector. They can be classified into packed column injectors (for use with packed columns) and capillary injectors (for use with capillary columns). Sample injection devices range from simple syringes to fully programmable automatic injectors. The amount of sample that can be injected into a capillary column without overloading is small compared with the amount that can be injected into a packed column, and may be less than the smallest amount that can be manipulated satisfactorily by syringe. The injected sample is required to be split into two fractions prior to reaching the column.

Capillary injectors have the capability to split sample into two fractions, a small one that enters the column and a large one that goes to waste (split injector). Injector can be operated either in split mode or splitless mode depending on the quantity of the sample injected. Temperature programmable injectors are also available where the sample is injected into an injector at low temperature (about 50°) and the injector temperature is quickly raised (250°/sec) to the required temperature. This helps in preventing thermal degradation of solutes in the injector.

Compounds to be chromatographed, either in solution or as gases, are injected into the gas stream at the injection port. Depending on the configuration of the instrument, the test mixture may be injected directly into the column or be vaporized in the injection port and mixed into the flowing carrier gas before entering the column. Purge and trap injectors are equipped with a sparging device by which volatile compounds in solution are carried into a low-temperature trap. When sparging is complete, trapped compounds are thermally desorbed into the carrier gas by rapid heating of the temperature-programmable trap.

Headspace injectors are equipped with a thermostatically controlled sample-heating chamber. Solid or liquid samples in tightly closed containers are heated in the chamber for a fixed period of time, allowing the volatile components in the sample to reach equilibrium between the nongaseous phase and the gaseous or headspace phase. After this equilibrium has been established, the injector automatically introduces a fixed amount of the headspace in the sample container into the gas chromatograph.

Column oven: Chromatographic columns are housed in an oven and its temperature is carefully controlled. Column oven may be operated either in isothermal or temperature programming modes. Compounds in the test mixture are separated by virtue of differences in their capacity factors, which in turn depend on their vapor pressure and degree of interaction with the stationary phase. The capacity factor, which governs resolution and retention times of components of the test mixture, is also temperature dependent. The use of temperature programmable column ovens takes advantage of this dependence to achieve efficient separation of compounds differing widely in vapor pressure. As resolved compounds emerge from the column, they pass through a detector, which responds to the amount of each compound present. The type of detector to be used depends on the nature of the compounds to be analyzed.

Columns: Two types of columns, packed or capillary **are** available for use in gas chromatography. Packed columns consist of tubes made up of glass, stainless steel or aluminium which are packed with the stationary phase. Columns of various dimensions may be used, but they usually range from 0.6 to 1.8 m in length and from 2 to 4 mm id.

Capillary columns with 0.25 mm inner diameter and lengths of 30 m or more, have replaced traditional packed columns due to their high efficiency. They are usually made of fused silica or aluminum clad. Fused silica columns are externally coated with polyamide to prevent breakage when they are coiled. Capillary columns are classified into three categories depending on their id. 0.15 – 0.25 id (narrow bore), 0.30-0.53 id (wide bore), .0.53 id (megabore). Wide bore and megabore columns withstand relatively high sample loading as compared to narrow bore columns. The liquid or stationary phase coated is 0.1 to 1.0 mm thick, although nonpolar stationary phases may be up to 5 mm thick. Three types of capillary columns are available:

- Wall coated open tubular columns (WCOT),
- Support coated open tubular columns (SCOT) and:

- Porous layer coated open tubular columns (PLOT).

WCOT columns are the most popular. Because of the absence of a solid support, capillary columns are much more inert than packed columns. Retention time and the peak efficiency depend on the carrier gas flow rate; retention time is also directly proportional to column length, while resolution is proportional to the square root of the column length.

Solid support materials must be as inert as possible in order to prevent interaction of the solutes with active surfaces, resulting in degradation, rearrangement, or loss of peak symmetry (tailing). The most commonly used supports are derived from silicates, usually diatomaceous earth. Before use they are acid-washed, calcined, and treated with a silanizing reagent to render surface hydroxyls inactive. They are available in various particle sizes from 30- to 120-mesh, with the 80- to 100-mesh and 100- to 120-mesh fractions most often used. Porous polymeric materials, which may be coated if desired or used as supplied, are available for the separation of low-molecular-weight compounds.

Liquid phases for partition chromatography may be chosen from a large variety of compounds, ranging from the very polar polyethylene glycols to the nonpolar methyl silicone gums. The choice of a liquid phase for a particular separation is mainly empirical, but usually polar phases are used for the analysis of mixtures of polar compounds. Chemically bonded and cross-lined phases can be used as they withstand higher temperatures (little 'bleeding' at about 300°) and can also be rinsed to restore column performance. They are useful for the analysis of high-boiling-point compounds. Capillary columns with stationary phases with varying polarities, lengths and id are commercially available.

For packed columns, the carrier gas flow rate is usually expressed in milliliters per minute at atmospheric pressure and room temperature. It is measured at the detector outlet with a soap film flow meter while the column is at operating temperature. Unless otherwise specified in the individual monographs, flow rates for packed columns are 60 to 75 ml/min for 4-mm id columns and ~30 ml/min for 2-mm id columns.

Before use, a packed column should be conditioned in the chromatograph to reduce the level of extraneous detector signals produced by the bleeding of volatile substances from the support and the liquid phase. This can be accomplished by increasing the column temperature gradually above its expected operating temperature, while maintaining a low flow of carrier gas through it and leave it overnight at the maximum temperature. During this process, the column should be disconnected from the detector. A suitable test for support inertness should be done.

Capillary columns can be protected by connecting them using a 'retention gap' (an empty or low polar capillary column of about 1.5 m in length depending on the polarity of the analytical column) through a quick seal connector. This will retain some unwanted matrix components and protect the column from contamination. For capillary columns, linear flow velocity is often used instead of flow rate. This is conveniently determined from the length of the column and the retention time of a dilute methane sample, provided a flame-ionization detector is in use. Typical linear velocities are 20 to 60 cm/s for helium. At high operating temperatures there is sufficient vapor pressure to result in a gradual loss of liquid phase, a process called "bleeding."

Capillary columns must be tested to ensure that they comply with the manufacturers' specifications before they are used. These tests consist of the following injections: a dilute methane sample to determine the linear flow velocity; a mixture of alkanes (e.g., C14, C15, and C16) to determine resolution; and a polarity test mixture to check for active sites on the column. The latter mixture may include a methyl ester, an unsaturated compound, a phenol,

an aromatic amine, a diol, a free carboxylic acid, and a polycyclic aromatic compound, depending on the samples to be analyzed.

*Detectors:* GC detectors can be classified into two groups, universal and selective detectors based on their general response or its response to specific elements or ions. Flame-ionization detector (FID) is the most commonly used detector in GC. The other detectors include electron-capture detector (ECD), thermal conductivity detector (TCD), nitrogen-phosphorus or thermionic specific detector (NPD or TSD), flame photometric detector (FPD) and mass spectrometric detectors (MSD). For quantitative analyses, detectors must have a wide linear dynamic range: the response must be directly proportional to the amount of compound present in the detector over a wide range of concentrations.

- FID has a wide linear range ( $\sim 10^6$ ) and is sensitive to organic compounds. Unless otherwise specified in individual monographs, FID with either helium or nitrogen carrier gas are to be used for packed columns, and helium is used for capillary columns.
- TCD detects changes in the thermal conductivity of the gas stream as solutes are eluted. Although its linear dynamic range is smaller than that of the FID, it is quite rugged and occasionally used with packed columns, especially for compounds that do not respond to FID.
- NPD contains a thermionic source, such as an alkali-metal salt or a glass element containing rubidium or other metal that results in the efficient ionization of organic nitrogen and phosphorus compounds. It is a selective detector that shows little response to hydrocarbons. This detector can be selectively operated either in the NP mode or P mode by altering the hydrogen flow rate and bead current to achieve better sensitivity of either nitrogen or phosphorus compounds.
- ECD contains a radioactive source (usually  $^{63}\text{Ni}$ ) of ionizing radiation. It exhibits an extremely high response to compounds containing halogens and nitro groups but little response to hydrocarbons. The sensitivity increases with the number and atomic weight of the halogen atoms.
- FPD can be operated either in phosphorus or sulphur mode by changing the emission filter and selecting proper wavelength for phosphorous or sulphur compounds.

(**Note:** Refer to the section on gas chromatography- mass spectrometry for details on mass specific detectors).

*Data Collection Devices:* Modern data stations receive the detector output, calculate peak areas, and print chromatograms, complete with run parameters and peak data. Chromatographic data may be stored and reprocessed, with integration and other calculation variables being changed as required. Data stations are used also to program the chromatograph, controlling most operational variables and providing for long periods of unattended operation. Data can also be collected using integrators whose capabilities range from those providing a printout of chromatogram and peak areas/ heights and data stored for possible reprocessing. Simple recorders are available for manual measurement.

### Qualitative Analysis

Since it is impracticable in gas chromatography to measure the  $R_f$ , presumptive identification of a solute should be done by comparing its position on the chromatogram with that of a reference standard. The position of a solute is characterized by its retention time, the time from injection to the peak maximum; its retention volume, the product of retention time and

carrier gas flow rate; or its retention distance, the distance from injection to the peak maximum. Since conditions may vary between determinations, it is more appropriate to identify a substance by its relative retention,

$$\alpha = (t_2 - t_0)/(t_1 - t_0),$$

Where  $t_2$  is the retention time, volume, or distance of the desired chemical,  $t_1$  is that of the reference compound, preferably determined on the same chromatogram, and  $t_0$  is the retention of an inert compound that is not retained by the column.

A method of definitive identification is to trap and condense the effluent for each peak and subject the condensate to analysis by IR, NMR, mass spectrometry, or other suitable methods.

A measure of the efficiency of a column is the number,  $N$ , of theoretical plates it contains for a given compound:

- $N = 16(t_r/w_b)^2$ , where  $t_r$  is the retention time of the peak, and  $w_b$  is its width in units of time at the baseline.

A measure of the efficiency of the separation of two adjacent peaks is given by the dimensionless constant  $R$ , the resolution factor, which can be calculated by the equation:

- $R = 2(t_2 - t_1)/(w_1 + w_2)$ , where  $t_2$  and  $t_1$  are the retention times of the two peaks, and  $w_1$  and  $w_2$  are the baseline widths determined by the intersection of the tangents of the inflection points of the peaks with the baseline. A resolution of 1.0 corresponds to a peak overlap of approximately 2% and is usually considered to be adequate for analytical purposes.

### Quantitative Analysis

In a gas chromatography, the parameter that is proportional to the concentration of any solute is the area under its peak or height. The following techniques can be used for the quantitation of solutes.

#### *Area normalization*

This method is based on the assumption that a peak is obtained on the chromatogram for each component of the mixture. The areas of all the peaks, each corrected by multiplying by its response factor, are added together to obtain the total area. Then the percentage of any component is equal to its corrected area divided by the total area and multiplied by 100. This method is reliable only if all components of the sample give a peak and if the various response factors are known.

#### *External standard*

A series of standards containing known amounts of the analyte are chromatographed under identical conditions. From the data obtained, a standard or working curve can be constructed by plotting area versus amount of standard that passes through the origin. If the compound to be analyzed is adsorbed within the system, the calibration curve will intersect the abscissa at a nonzero value. This may result in error, particularly for compounds at low concentrations determined by a procedure based on a single reference point. At high concentrations, the liquid phase may be overloaded, leading to loss of peak height and symmetry. A major source of error is irreproducibility in the amount of sample injected, notably when manual injections are made with a syringe. Auto samplers provide better precision.

*Internal standard*

In order to correct for errors that might occur when injection volumes vary or the chromatographic conditions change slightly from run to run, the internal standard method may be used. In this method, another standard, which is chemically similar to the unknown component and which elutes separately from all other peaks, is added in a constant amount to all standard and test solutions of the analyte. After chromatographing, a standard curve is constructed by plotting the area ratios of the standard solutions (area of analyte standard per area of internal standard) versus the weight or concentration ratios of each standard. The unknown is then chromatographed, its area ratio is determined, and the corresponding weight ratio is found by interpolation using the calibration curve. Since the amount of internal standard is constant and known, the concentration of the unknown component can be calculated.

**Headspace-Gas Chromatography**

Headspace-gas chromatography (headspace-GC) is an analytical technique in which the analyte in its gas phase and its GC analysis have been combined. The method can be applied to the analysis of compounds with a low boiling point, which therefore vapourize easily at low temperatures.

There are two different forms of headspace-GC: static and dynamic headspace-GC. In static headspace-GC the gaseous sample is taken from a sealed headspace vial into a loop and the analytes are transferred to the GC with the help of carrier gas. In dynamic headspace-GC the gaseous sample is forced out of the headspace vial with the help of an external source, usually the same gas as that used as the carrier gas. Headspace injectors are equipped with a thermostatically controlled sample-heating chamber. Solid or liquid samples in tightly closed headspace vials incubated in the chamber for a fixed period of time, allowing the volatile components in the sample to reach equilibrium between the nongaseous phase and the gaseous or headspace phase. After incubation at a specified temperature a gas sample from the head-space vial is sampled.

Purge and Trap Analysis

Purge and trap injectors are equipped with a sparging device by which volatile compounds in solution are carried into a low-temperature trap. When sparging is complete, trapped compounds are thermally desorbed into the carrier gas by rapid heating of the temperature-programmable trap.

**Analysis of Samples - General Method Instructions**

*(Note: The instructions detailed below provide guidance on performing gas chromatographic analysis. However, analyst should consult individual monographs for sample preparation, appropriate GC column, detector and other conditions)*

The following need careful consideration during the analysis:

Analyte characteristics: The selection of a proper gas chromatographic system depends on the analyte characteristics such as its molecular weight, polarity, boiling point, thermal stability and other properties (refractive index, functional groups, presence of nitrogen, phosphorus, halogens etc. which help in choosing a selective detector).

Column and detector selection: The selection of the right chromatographic column depends on the polarity of analytes and other matrix components. The general rule is that polar compounds can be separated on polar columns. Capillary columns with stationary

phases in different polarities are commercially available. Kovat's index, McReynolds constants and CP Index guide selection of an appropriate column. Choice of selective detectors is based on the analyte characteristics (analytes containing halogens- ECD, nitrogen and phosphorus – NPD/FPD, sulphur-FPD). FID is the most commonly used. The analyst may use either the columns or equivalent as specified in the monograph. Use the detector as specified in the monograph. Use of a different detector requires establishing the limit of detection and limit of determination to ensure that the sensitivity of the method meets the specification requirements.

*Optimizing separation:* The analyst may follow the GC operating conditions (injector, detector, column temperature - isothermal/temperature programming) detailed in the monographs. Temperature programming is suitable with most capillary columns to achieve optimum separation of analytes and other sample components. However, changes in column length, slight changes in polarity and flow rates demand slight adjustments to the temperatures. It is recommended that the analyst optimize the GC conditions to achieve better separation and peak symmetry by injecting analyte mixture.

*Sample preparation:* Sample preparation is considered as one of the critical steps in GC analysis and generally involves extraction, cleanup and concentration steps. Sample cleanup involves either single or a combination of steps which include: liquid-liquid extraction, column cleanup, GPC cleanup, SPE cleanup etc. It is recommended to follow the sample preparation method specified in the monograph.

*Derivatization:* Derivatization is carried out improve volatility, thermal stability, changes in separation properties or paves way for selective detection. General derivatization methods include:

- Methylamine: Trans esterification of carboxylic acids to corresponding methyl esters using sodium methoxide
- Silylation of functional groups [-OH, -COOH, -NH<sub>2</sub>, =NH, -SH], where the H is replaced by -O-Si(CH<sub>3</sub>)<sub>3</sub>. Common silylation agents are DMCS, TMS, BSTA, BTSFA
- Heptafluoro-derivatives for [-OH] groups - derivatization reagent HFBI.

Most analytes are volatile at the specified GC temperatures and do not require derivatization. It is recommended to follow the derivatization method specified in the monograph.

*Qualitative analysis:* Comparison of retention times of standard and the analyte is most commonly used for the identification of analytes. Wrong identification is possible in complex matrices where matrix components interfere. Techniques such as use of another column or mass spectral confirmation may be used in such cases. For such cases, specific instructions are detailed in the additive specifications. In the absence of any instructions, follow comparison of retention times for identification.

*Quantitative analysis:* The external standard method is most commonly used. Instructions are detailed in the additive specifications if alternate methods (normalization technique or internal standard method) are used. Follow the external standard method in the absence of any instructions.

*Method validation:* It is recommended to validate each test method to ensure accurate and reliable results. Method performance characteristics such as resolution, peak asymmetry, precision, accuracy, limit of detection, limit of determination, linearity and percent recovery provide data on method selectivity, sensitivity and applicability.



Reporting Results: Results need to be reported to three significant figures, unless otherwise specified.

## Gas Chromatography – Mass Spectrometry (GC-MS)

Gas chromatography-mass spectrometry is a hyphenated technique widely used for the confirmation and quantitation of analytes. In this technique, effluents from a GC column are passed into a mass selective detector or mass spectrometer and subjected to analysis. The gas chromatography part is detailed in the above section.

### Mass Spectrometry

Mass spectrometry is perhaps the most widely used analytical tool to provide information on molecular weight, identification and confirmation of compounds through their mass spectra as well as quantitation of compounds. In the mass spectrometry, the analyte molecule is bombarded with a stream of electrons that lead to the loss of an electron from the analyte molecule forming a charged molecular ion ( $M^+$ ). The collision between electrons and analyte molecules usually imparts enough energy to excite the molecules to the higher energy states. Relaxation then often occurs by fragmentation of part of the molecular ion to produce ions of lower masses. The positive ions produced on electron impact are attracted through the slit of a mass spectrometer where they are sorted according to their mass-to-charge ratios and displayed in the form of a mass spectrum.

The plot is in the form of a bar graph that relates the relative intensity (abundance) of mass peaks to their mass-to-charge ratio. The peak having highest intensity is termed as a base peak, and is arbitrarily assigned a value of 100. The heights of the remaining peaks are then computed as a percentage of the base-peak height. Mass spectrometers have the capability to identify base peak and normalize the remaining peaks to that peak. Figure 1 shows a typical mass spectrum of ethyl benzene.

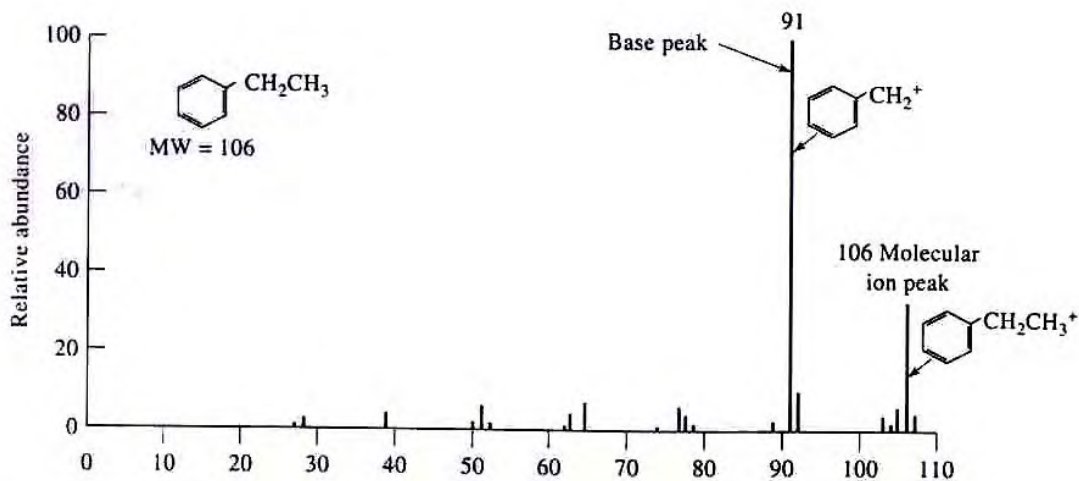


Figure 1

### Apparatus:

The essential components of a basic mass spectrometer are a sample inlet system, an ion source, a mass analyzer, a detector, a vacuum system, and a data processing device. Figure 2 shows the block diagram of a typical quadrupole GCMS.

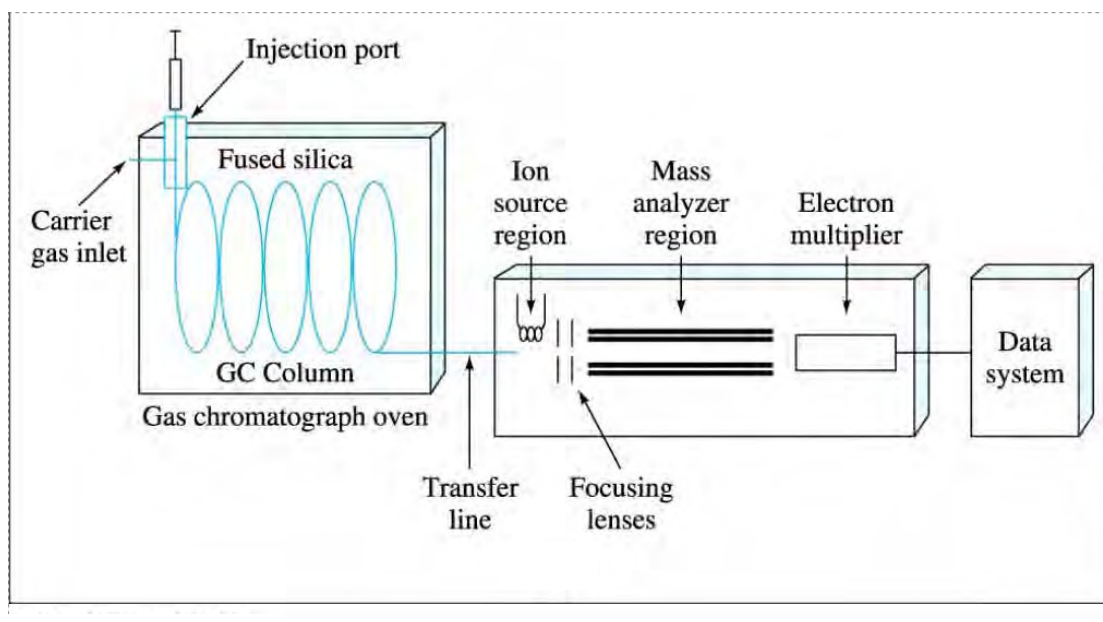


Figure 2

**Sample inlet system:** The sample inlet system permits introduction of a representative sample into the ion source with minimal loss of vacuum. These include batch inlets, direct probe inlets, chromatographic inlets and capillary electrophoretic inlets. In GCMS, chromatographic inlets are used where the tip of the capillary column is precisely inserted into the inlet.

**Ion Source:** The purpose of an ion source is to ionize the molecule to produce gaseous analyte ions. Electron impact ionization (EI), chemical ionization (CI) and field ionization (FI) are used to ionize the analytes.

EI is the most common ionization technique used, in which the sample is brought to a temperature high enough to produce molecular vapour, which is then ionized by bombarding with a beam of energetic electrons. Despite certain disadvantages (excessive fragmentation leading to disappearance of molecular ion peak at times and the need to volatilize the sample), this technique produces a reproducible mass spectra of a compound and is the basis on which many mass spectral libraries are built.

In CI, also termed as soft ionization, a gaseous sample is ionized by collision with ions produced by electron bombardment of a reagent gas such as methane or ammonia. Collision between the sample molecule (M) and highly reactive reaction products aroused from the reagent gas usually involves proton or hydride transfer leading to formation of either  $(M+1)^+$  or  $(M-1)^+$  ions. Relative to EI spectrum, CI spectrum is simple and provides molecular weight information.

**Mass Analyzer:** The mass analyzer separates the mass fragments produced by the ionization sources. The capability of a mass spectrometer to differentiate between masses is usually stated in terms of its resolution (R) which is defined as  $R = m/\Delta m$ , where  $\Delta m$  is the mass difference between two adjacent peaks that are just resolved and m is the nominal mass of the first peak (mean of the two peaks is some times used). The mass spectrometer with a resolution of 4000 would resolve peaks with m/z values of 400.0 and 400.1 or 40.00 and 40.01. Several low and high resolution mass analyzers are available which include single stage quadrupole, triple stage quadrupole, ion trap, magnetic sector and time-of-flight.

Single stage quadrupole and ion trap are most commonly used. In a quadrupole mass spectrometer, the mass analyzer consists of four parallel cylindrical rods that serve as electrodes, one pair being attached to positive side of a variable dc source and the other pair to the negative terminal. Variable radio-frequency ac potentials are applied to each pair of rods. Meanwhile, ac and dc voltages on the rods are increased simultaneously while maintaining their ratio constant. Fragment ions are accelerated into the space between the rods by a potential of 5 to 10 V. All the ions except those having the  $m/z$  value strike the rods and are converted into neutral molecules. Thus, the resonance ions will pass through the quadrupole and reach the transducer. Quadrupole resolves ions that differ in mass by one unit. Quadrupole mass analyzers are termed as mass filters.

A ion trap analyzer consists of a central doughnut-shaped ring electrode and a pair of end cap electrodes. A variable radio-frequency is applied to the ring electrode while the end cap electrodes are grounded. Fragment ions circulate in a stable orbit within the cavity surrounded by the ring. As the radio-frequency energy is increased, the orbits of heavier ions become stabilized, while those with lighter ions become destabilized, causing them to collide with the walls of the ring electrode. When radio-frequency is scanned, the destabilized ions leave the cavity of the ring electrode via openings in the lower end cap and pass into a transducer.

Detectors: Detectors used in the mass spectrometer include electron multiplier detectors, Faraday cup collector and photomultiplier detectors. Electron multiplier detectors are most commonly used.

Vacuum system: The ion source, mass analyzer and the detector must be kept under high vacuum ( $10^{-4}$  to  $10^{-8}$  torr) because charged particles interact with components of the atmosphere and are annihilated as a consequence. Several vacuum pumps are used in achieve the required vacuum. Pumps include rotary pumps, oil diffusion pumps and turbo molecular pumps. Differential pumping is recommended using rotary/oil diffusion pump at the first stage and turbo molecular pump at the second stage to crate a proper vacuum environment.

Data processing device: The data processing devices have the capability to control the instrument as well as process a large quantity of data and provide mass spectrum of compounds.

Identification of compounds: Independent identification of compounds is achieved by comparing the sample mass spectrum with several mass spectra available in the mass spectral library. Several EI mass spectral libraries are available. Most modern mass spectrometers have the facility of making custom mass libraries for use. Modern mass spectrometers have the facility to shift from EI to the CI mode which will provide molecular weight information. Tandem mass spectrometry (MS/MS) provides additional confirmation in identification of compounds. In MS/MS, a mass ion (usually the ion with high abundance) is selected and is subjected to further ionization followed by analyzing its mass spectrum. The ion selected for further ionization is termed as parent ion and the ions produced are termed as daughter ions. This process requires a triple quadrupole or ion trap-quadrupole system. In triple quadrupole, the first and third ones work as analyzers, where as the middle one works as an ionizer. The daughter ions so produced are very specific to the parent ions, providing a highly reliable confirmation of the presence of the analyte.

Quantitation: Quantitation is usually carried out using a single ion or a group of ions following selective ion monitoring. The area under the curve for standard as well as sample is used in the quantitation.

## High Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) is a separation technique based on a solid stationary phase and a liquid mobile phase. Separations are achieved by partition, adsorption, exclusion, or ion-exchange processes, depending on the type of stationary phase used. HPLC has distinct advantages over gas chromatography for the analysis of nonvolatile organic compounds. Compounds to be analyzed are dissolved in a liquid, and most separations take place at room temperature.

As in gas chromatography, the elution time of a compound can be described by the capacity factor,  $k$ , which depends on the chemical nature of the composition and flow rate of the mobile phase, and the composition and surface area of the stationary phase. Column length is an important determinant of resolution. Only compounds having different capacity factors can be separated by HPLC.

HPLC is divided into two types namely normal phase chromatography and reversed phase chromatography. In the normal phase chromatography, non-polar to polar stationary phases and non-polar to polar mobile phases separate compounds by their increasing polarity. In the reversed-phase chromatography, the stationary phase has been modified to be a non-polar substance (silica gel has been bonded with a long-chain non polar substance e.g. octadecylsilyl), and polar solvents are used as mobile phase. The order of elution is from polar to non polar compounds.

Reversed-phase chromatography has become a highly powerful technique because its selectivity over a wide range of solutes that can be adjusted by varying the polarity of the mobile phase. In most cases, mobile phases consist of water:methanol or water:acetonitrile. Adjustment of the pH of a mobile phase prevents ionization of weak acids and bases (ion suppression). Addition of an ionic reagent (e.g. heptane-sulfonate) to form a less polar ion pair with a charged solute (ion pairing) aids the retention of polar compounds (e.g. food colours).

### Apparatus

The basic components of a liquid chromatograph are a solvent delivery system, a sample injection device, a chromatographic column, a detector, and a suitable data-recording device.

*Solvent delivery systems:* consists of one or more pumps capable of delivering a pulse-less flow of mobile phase at pressures up to 6000 psig. Flow rates up to 10 ml/min with increments of 0.01 ml/min are typical. In the isocratic mode, where a mobile phase of constant composition is used throughout the run, a single pump and solvent reservoir are required. The mobile phase needs to be prepared externally by mixing the liquids in the required proportion and degassing it by sparging with helium.

For the separation of mixtures where the  $k'$  values vary over a wide range, gradient-elution analysis may be used. In this method, the composition of the mobile phase is constantly changed during the chromatographic run. Modern gradient HPLCs (binary, ternary and quaternary systems) have the advantage of internally mixing the liquids, in a mixing chamber, in the required proportions, and have the facility for continuous degassing using a vacuum degasser.

*Injectors:* After dissolution in a mobile phase or another suitable solution, compounds to be chromatographed are injected into the mobile phase, either manually by syringe or loop injectors, or automatically by auto samplers. The latter consist of a carousel or rack to hold sample vials with tops that have a pierce able septum and an injection device to transfer sample from the vials to a loop from which it is loaded into the chromatograph. Auto

samplers are programmed to control sample volume, the number of injections and loop rinse cycles, the interval between injections, and other operating variables. Some valve systems incorporate a calibrated sample loop that is filled with test solution for transfer to the column in the mobile phase. In other systems, a test solution is transferred to a cavity by syringe and then switched into the mobile phase.

Columns used for analytical separations usually have internal diameters of 2 to 4.6 mm and lengths from 5.0 – 30 cm and are usually made of stainless steel. Glass cartridge columns are also used with a cartridge column holder. Larger diameter columns are used for preparative chromatography.

Stationary phases for modern reversed-phase liquid chromatography typically consist of an organic phase chemically bound to silica or other materials. Particles are usually 3, 5, or 10  $\mu\text{m}$  in diameter. Column polarity depends on the polarity of the bound functional groups, which range from relatively nonpolar octadecyl silane to very polar nitrile groups. The percent carbon load (amount of bonded phase material loaded on to the silica support, measured as weight percentage of bulk silica) is important in determining the polarity of reversed phase columns. Increasing carbon load and chain length increases retentivity. End capping of columns (a chemical process that reduces the number of free silanol groups attached to the base silica support material) minimizes competing mechanisms.

Columns used for normal phase chromatography are polar in nature (silica). Use of guard columns in front of analytical column protects it and extends its life. Guard columns retain the non-polar substances and any particulate matter that may be in the sample. Guard columns in different sizes (0.5 – 5.0 cm in length) are available for both normal and reversed-phase columns.

Columns are housed in column housing with a thermostatic system to control the temperature. Columns may be heated to give more efficient separations, but only rarely are they used at temperatures above 60° because of potential stationary phase degradation or mobile phase volatility (Certain resin based columns e.g. carbohydrate columns are heated to 90° to achieve efficient separation of sugars). Unless otherwise specified, columns are used at an ambient temperature.

Ion-exchange chromatography is used to separate water-soluble, ionizable compounds of molecular weights that are less than 2000. The stationary phases are usually synthetic organic resins. Cation-exchange resins contain negatively charged active sites and are used to separate basic substances such as amines, while anion-exchange resins have positively charged active sites for separation of compounds with negatively charged groups such as phosphate, sulfonate, or carboxylate groups. Water-soluble ionic or ionizable compounds are attracted to the resins, and differences in affinity bring about the chromatographic separation. The pH of the mobile phase, temperature, ion type, ionic concentration, and organic modifiers, all affect the equilibrium. These variables can be adjusted to obtain the desired degree of separation.

In size-exclusion chromatography, columns are packed with a porous stationary phase. Molecules of the compounds being chromatographed are filtered according to size. Those too large to enter the pores pass unretained through the column (total exclusion). Smaller molecules enter the pores and are increasingly retained as molecular size decreases. These columns are typically used to remove high molecular weight matrices or to characterize the molecular weight distribution of a polymer or separation of a mixture of proteins with varying molecular weights.

*Detectors:* The types of detectors most frequently used in HPLC are spectrophotometric, fluorometric, refractometric, potentiometric, voltammetric, or polarographic, electrochemical and mass detectors. The detectors consist of a flow-through cell (8 - 16  $\mu$ l) to which the column outlet is connected.

In spectrometric detectors, a beam of ultraviolet radiation passes through the flow cell and into the detector. As compounds elute from the column, they pass through the cell and absorb the radiation, resulting in measurable energy level changes. Fixed, variable, and photodiode array (PDA) detectors are widely available. Fixed wavelength detectors operate at a single wavelength, typically 254 nm, emitted by a low-pressure mercury lamp. Variable wavelength detectors contain a continuous source, such as a deuterium or high-pressure xenon lamp, and a monochromator or an interference filter to generate monochromatic radiation at a wavelength selected by the operator. Modern variable wavelength detectors can be programmed to change wavelength while an analysis is in progress.

Multi-wavelength detectors measure absorbance at two or more wavelengths simultaneously. In diode array multi-wavelength detectors, continuous radiation is passed through the sample cell, and then resolved into its constituent wavelengths, which are individually detected by the photodiode array. These detectors acquire absorbance data over the entire UV-visible range, thus providing the analyst with chromatograms at multiple, selectable wavelengths and spectra of the eluting peaks. Since the absorption spectrum is continuously collected (from start to the end of the peak) PDA has the additional capability to overlay normalized absorption spectra at different points of the peak and assesses peak purity. Signals also can be extracted at different wavelengths depending on the absorption maximum of each analyte, which provides improved sensitivity. Diode array detectors usually have lower signal-to-noise ratios than fixed or variable wavelength detectors, and thus are less suitable for analysis of compounds present at low concentrations.

Differential refractometer detectors measure the difference between the refractive index of the mobile phase alone and that of the mobile phase containing chromatographed compounds as it emerges from the column. Refractive index detectors are used to detect non-UV absorbing compounds, but they are less sensitive than UV detectors. They are sensitive to small changes in solvent composition, flow rate, and temperature, so that a reference column may be required to obtain a satisfactory baseline.

Fluorometric detectors are sensitive to compounds that are inherently fluorescent or that can be converted to fluorescent derivatives either by chemical transformation of the compound or by coupling with fluorescent reagents at specific functional groups. If derivatization is required, it can be done before chromatographic separation or, alternatively, the reagent can be introduced into the mobile phase just before it enters the detector.

Potentiometric, voltammetric, or polarographic electrochemical detectors are useful for the quantitation of species that can be oxidized or reduced at a working electrode. These detectors are selective, sensitive, and reliable, but require conducting mobile phases free of dissolved oxygen and reducible metal ions. A pulse-less pump must be used, and care must be taken to ensure that the pH, ionic strength, and temperature of the mobile phase remain constant. Working electrodes are prone to contamination by reaction products with consequent variable responses.

Electrochemical detectors with carbon-paste electrodes may be used advantageously to measure nanogram quantities of easily oxidized compounds, notably phenols and catechols.

Mass spectrometric detectors provide additional advantage of independent identification of compounds. However, the mobile phase needs to be removed and compounds need to be

ionized. This is achieved by connecting the column outlet to an inlet using electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI). For further details, refer the section on mass spectrometry.

Data Collection Devices: Modern data stations receive and store detector output and print out chromatograms complete with peak heights, peak areas, sample identification, and method variables. They are also used to program the liquid chromatograph, controlling most variables and providing for long periods of unattended operation. Data also may be collected on simple recorders for manual measurement or on stand-alone integrators, which range in complexity from those providing only a printout of peak areas to those providing a printout of peak areas and calculated peak heights plus storing the data for possible use in subsequent reprocessing.

### System Suitability

System suitability tests are an integral part of both gas and liquid chromatographic methods. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. The tests are based on the concept that the equipment, electronics, analytical operations, and samples to be analyzed constitute an integral system that can be evaluated as such.

Figure 3 shows the chromatographic separation of two substances. The resolution,  $R$ , is a function of column efficiency,  $N$ , and is specified to ensure that closely eluting compounds are resolved from each other, to establish the general resolving power of the system, and to ensure that internal standards are resolved from the analyte. Column efficiency may be specified also as a system suitability requirement, especially if there is only one peak of interest in the chromatogram; however, it is a less reliable means to ensure resolution than direct measurement. Column efficiency is a measure of peak sharpness, which is important for the detection of trace components. Replicate injections of a standard preparation used in the assay or other standard solution are compared to ascertain whether requirements for precision are met. Data from five replicate injections of the analyte are used to calculate the relative standard deviation if the requirement is 2.0% or less; data from six replicate injections are used if the relative standard deviation requirement is more than 2.0%.

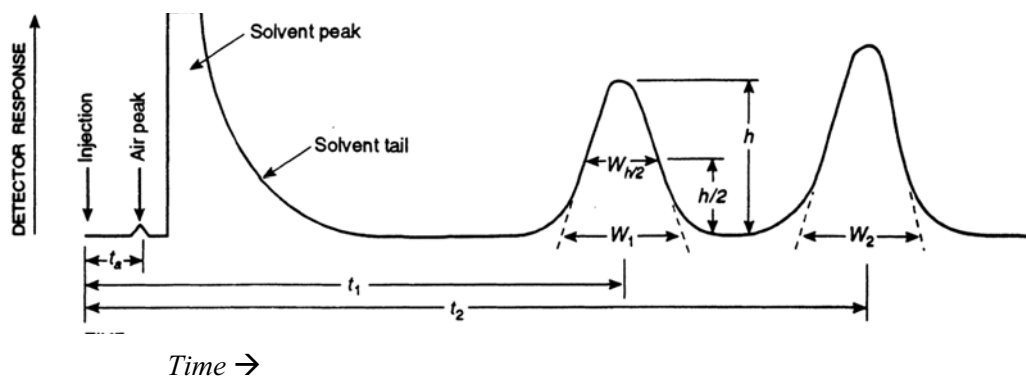


Figure 3: Chromatographic Separation of Two Substances.

Figure 4 shows an asymmetrical chromatographic peak with tailing. The tailing factor,  $T$ , a measure of peak symmetry, is unity for perfectly symmetrical peaks, and its value increases as tailing becomes more pronounced. In some cases, values less than unity may be observed. As peak asymmetry increases, integration, and hence precision, becomes less reliable. The calculation is expressed by the equation

$$\text{Tailing factor} = T = W_{0.05}/2f,$$

Where  $W_{0.05}$  is the width of the peak at 0.05 height and  $f$  is the width of the first half peak (see details in Figure 4).

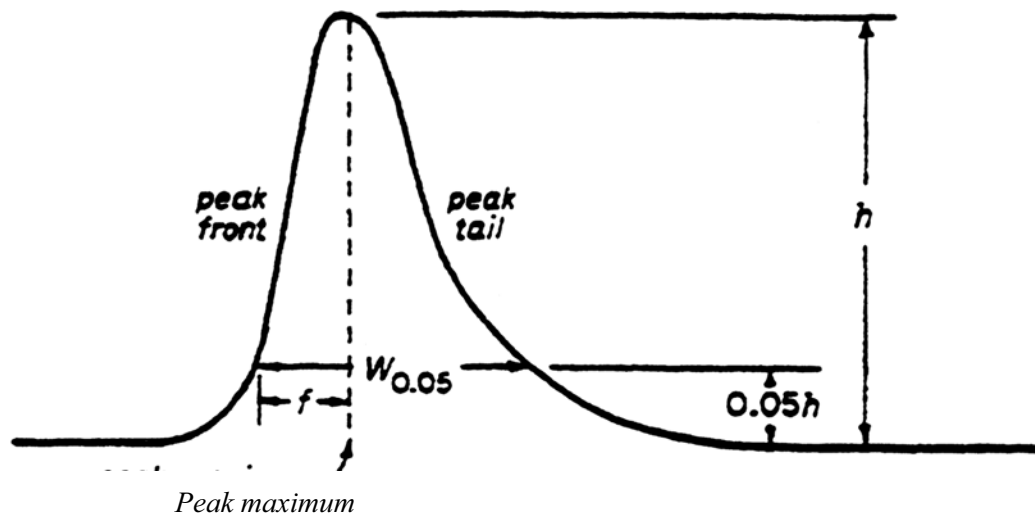


Figure 4: Asymmetrical Chromatographic Peak

These tests are performed by collecting data from replicate injections of standard or other suitable solutions. Adjustments of operating conditions to meet system suitability requirements may be necessary. It is common to determine system suitability parameters from the analyte peak.

To ascertain the effectiveness of the final operating system, it should be subjected to a suitability test before use and during testing whenever there is a significant change in equipment or in a critical reagent or when a malfunction is suspected.



## SPECTROPHOTOMETRY AND SPECTROSCOPY

### Definitions

#### Absorption Spectrophotometry

Is the measurement of the selective absorption by molecules or ions, of electromagnetic radiation having a definite and narrow wavelength range, approximating monochromatic light.

Absorption spectrophotometry encompasses the following wavelength and wave number regions: ultraviolet (185 nm to 380 nm), visible (380 nm to 780 nm), near-infrared (0.78 – 2.5  $\mu\text{m}$  or 12,800 - 4,000  $\text{cm}^{-1}$ ) and mid-infrared (2.5 – 50  $\mu\text{m}$  or 4000 - 200  $\text{cm}^{-1}$ ).

#### Colourimetry

Has been commonly accepted as the measurement of "filtered" light in the visible region; however, it is more prudent to restrict its use to those applications where human perception of colour is involved, i.e., the visible region.

#### Atomic Absorption Spectroscopy

Is the measurement of the radiation absorbed by the unexcited atoms in their gaseous phase, that have been aspirated into a flame or, in the absence of a flame, directly into the path of the radiation.

#### Flame Emission Spectroscopy (Flame Photometry)

Is the measurement of the intensity of radiation emitted from electronically excited atoms or molecular species. The excitation is brought about by aspirating a solution of the sample into a hot flame.

#### Fluorescence Spectrophotometry

Or "fluorometry", is the measurement of light emitted from a chemical substance while it is being exposed to electromagnetic radiation. The maximum intensity of the emitted fluorescence is usually at a wavelength longer (i.e., of lower energy) than the exciting radiation.

#### Turbidimetry and Nephelometry

Are two light-scattering techniques that involve the measurement of light scattered due to its passage through a transparent medium containing a suspended particulate phase. As a result of this scattering, an attenuation or decrease in intensity is suffered by the beam along its axis of travel. Turbidimetry involves the measurement of the degree of attenuation of the light beam by particles suspended in a medium, the measurement being made in the axis of the transmitted beam. Nephelometry involves the measurement of the light scattered by the suspended particles, the measurement being made at right angles to the incident beam.

### Terminology

#### Radiant power, P

Is the energy of radiation per sec that reaches certain areas of a detector. Incident radiant power is usually given the symbol  $P_0$ . Alternate terminology is radiation intensity with symbols  $I$  and  $I_0$ .

Absorbance,  $A = \text{Log}_{10}(P_0/P)$ 

Is the logarithm to the base 10 of the quotient of the incident radiant power upon a specimen divided by the radiant power transmitted by the specimen. Former terms were optical density "OD", absorbanacy, and extinction.

Specific absorbance,  $A^{1\%}_{1\text{ cm}} = A/bc \times 10$ 

Is the quotient of the absorbance, A, divided by the product of the adsorption path length, b, in cm, and the concentration, c, of the specimen, expressed in g per 100 ml. In general the specific absorbance of a substance is a constant and is independent of the intensity of the incident radiation, path length and concentration. Previously designated by the symbol

$$E^{1\%}_{1\text{ cm}}$$

Transmittance,  $T = (P/P_0)$ 

Is the quotient of the radiant power transmitted by a specimen divided by the incident power upon the specimen. Transmittance is often expressed as a percentage and is related to the absorbance by the equation  $\text{Log}_{10}T = -A$ , or  $A = 2.3 - \text{Log}_{10}\%T$ . Other terms are transmission and transmittancy.

Absorptivity,  $a = A/bc$ 

Is the quotient of the absorbance, A, divided by the product of the absorption path length, b, in cm, and the concentration, c, of the specimen, expressed in g per 1,000 ml. In general, the absorptivity of a substance is a constant and is independent of the intensity of the incident radiation, path length, and concentration.

Molar absorptivity,

Is the quotient of the absorbance, A, divided by the product of the absorption path length, in cm, and the specimen concentration, expressed in moles per liter. Former terms were molar absorbanacy index, molar extinction coefficient and molar absorption coefficient.

Absorption spectrum

Is a graphic representation of the absorbance of a specimen or any of its functions, e.g., transmittance, as the ordinate and the wavelength of the incident radiation as the abscissa.

Fluorescence intensity, I

Is a descriptive term for the fluorescence activity of a substance and is commonly expressed in units related to the detector response. An alternate term is fluorescence power, with the symbol F.

Fluorescence excitation spectrum

Is a graphic representation of the incident (activating) radiation intensity as the ordinate and its wavelength as the abscissa.

Fluorescence emission spectrum

Is a graphic representation of the radiation intensity emitted by an activated species for a specific excitation wavelength as the ordinate and its wavelength as the abscissa.

Turbidance

Is the light-scattering effect of the suspended particles in a turbid medium.

### Turbidity

Is a measure of the attenuation in the incident beam power per unit length of a turbid medium. The former term is turbidity coefficient.

### **Theory and Formulas for Calculations**

When electromagnetic radiation travels through a medium containing atoms, molecules, or ions of a chemical substance, radiation at certain frequencies may be partially or totally removed in a process called "absorption". As a result of this absorption, these species are activated from their lowest energy state (ground state) to higher energy states (excited states). For absorption to occur, the energy of the exciting radiation must match the quantized energy difference between the ground state and one of the excited states of the specimen. In atomic absorption, excitation occurs only through electronic transition. Visible and ultraviolet radiation can excite only the outermost or bonding electrons to a higher energy level. Inner-shell electrons are excited only by X-ray radiation (less than 1 nm).

In the case of polyatomic molecules, vibrational and rotational transitions can occur in addition to electronic excitation, and as a result the molecular spectrum consists of closely spaced absorption bands instead of the sharp lines generally observed in the atomic absorption spectrum. Pure vibrational transitions can be achieved by infrared radiation in the range of 1 to 15  $\mu\text{m}$ , while changes in rotational levels are detectable in the region from 10 to 100  $\mu\text{m}$ .

The decrease in the radiant power of a monochromatic beam of light has been found to be proportional to both the distance the radiation travelled through the absorbing medium and the concentration of the absorbing species encountered in that medium. This decrease in energy can be described quantitatively by the Beer-Lambert law:

$$\text{Log}_{10}(P_0/P) = \text{Log}_{10}(1/T) = A = abc$$

Therefore, if the absorptivity and the cell thickness are kept constant during a specific determination, a plot of the absorbance as the ordinate versus concentration as the abscissa should yield a linear relationship. The practical application of the Beer-Lambert law, however, necessitates the use of a reference standard solution of known concentration in order to compare its absorbance with that of the sample solution of unknown concentration. If absorption measurements are conducted in two matching cells having the same path lengths, the absorptivity,  $a$ , and the cell thickness,  $b$ , will be the same. Therefore the following general formula can be used for the calculation of the unknown concentration of the sample solution,

$$C_u = C_s (A_u/A_s)$$

where

$C_u$  = the concentration of the sample solution

$C_s$  = the known concentration of the standard solution

$A_u$  = the absorbance of the sample solution

and  $A_s$  = the absorbance of the standard solution.

The Beer-Lambert law is usually satisfactory, provided a thorough understanding of its limitations is taken into consideration. Some of these are of such a fundamental nature that they constitute a real limitation of the law. They are due to the fact that the law does not take into consideration the effects of temperature, wavelength, or solute-solvent and solute-solute interactions, e.g., association, dissociation, chemical reaction, etc. Due to these limitations,

the law usually applies only to dilute solutions, where these interactions are insignificant. Another limitation to the Beer-Lambert law is due to the inability of most instruments to provide monochromatic radiation.

Fluorescence can be observed in a number of gaseous, liquid, or solid substances. However, it is only applied analytically to a relatively small number of organic compounds. Fluorescence occurs when a molecule absorbs sufficient radiation at a certain wavelength to promote it to an excited singlet state with higher levels of energy. The gained energy is released as radiation or "fluorescence" of wavelengths longer than the incident radiation. In most cases, in order for fluorescence to occur the electronic transition involved is a  $\pi \rightarrow \pi^*$  system. To a lesser extent,  $\pi \rightarrow \pi$  and  $\pi \rightarrow \sigma^*$  transitions occur. There is a delay between the absorption and emission of radiation of about  $10^{-9}$  sec. This short delay period distinguishes fluorescence from phosphorescence, which has a delay period of about  $10^{-3}$  sec and is due to release of weaker radiations from an excited triplet state and not a singlet state as is true of fluorescence. The effect of concentration on the fluorescence intensity can be described by a slightly modified version of the Beer-Lambert law. A linear relationship exists between the fluorescence intensity,  $I$ , of the solution and the concentration of the emitting species:

$$I = 2.3K \times bcP_0$$

where  $K$  is a constant dependent upon the quantum efficiency of the fluorescence process and instrumental parameters. At constant  $P_0$ , a simple relationship as in the Beer-Lambert law can be obtained:  $I = Kc$ . Thus a plot of the fluorescence intensity of a solution as the ordinate versus concentration of the emitting species as the abscissa should be linear at low concentrations (see Figure 5).

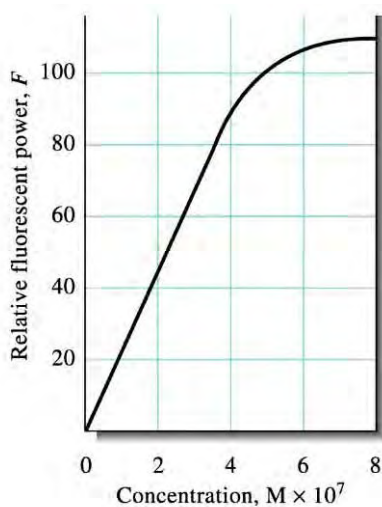


Figure 5

When light passes through a transparent medium containing a suspended particulate phase, scattering occurs in all directions, and as a result the beam loses power along its axis of travel. For dilute suspensions and under fixed conditions (particles, shape, size, refractive index, wavelength of radiation), the loss in radiation intensity can be related to the number of particles (or concentration,  $c$ ) by an equation similar to the Beer-Lambert law.

$$\text{Log}_{10}(P_0/P) = kbc,$$

where  $\tau = kc/2.303$ . Therefore, in turbidimetric analysis a plot is constructed with standard solutions with  $\text{Log}_{10}(P_0/P)$  as the ordinate and  $c$  as the abscissa ( $P_0$  is determined by using the solvent as reference). In nephelometric analysis, the radiation intensity scattered at right angles to the incident beam is plotted as the ordinate versus concentration as the abscissa.

## **Apparatus**

The fundamental principles of optics and electronics that are used in manufacturing spectrophotometers are common to all regions of the spectrum from the vacuum ultraviolet to the far-infrared. However, due to important differences in detail, spectrophotometers are commercially available for use in the visible; in the visible and ultraviolet; in the visible, ultraviolet, and near-infrared; and in the infrared regions of the spectrum. In selecting the type of spectrophotometer to be employed, several factors have to be considered, including the nature of the specimen to be analyzed, the degree of accuracy required, sensitivity, and selectivity.

The essential parts of all spectrophotometers include a stable source of radiant energy; a device that permits the selection of a defined wavelength region such as a prism or grating monochromator; a slit for limiting the suitable bandwidth; a sample compartment; a radiation detector; and an indicator that may be a meter, a recorder, a digital counter, a printer, or a computer.

Radiation sources commonly employed are hydrogen or deuterium lamps for the ultraviolet region, tungsten lamps for the visible, and a Nernst glower, a global, or an incandescent wire for the infrared. Quartz or fused-silica cells or cuvettes can be used in the ultraviolet, visible, or near-infrared regions. For infrared spectrophotometry, cells or plates made of sodium chloride are usually used. The radiation detector of ultraviolet and visible radiation is usually a photomultiplier tube with associated amplifiers.

### UV/Visible Spectrophotometers:

Two types of spectrophotometers are available: a single-beam spectrophotometer, which adapts well to quantitative analysis that involves single-wavelength measurements, and a double-beam spectrophotometer, which is particularly useful for qualitative analysis and where continuous monitoring of absorbance is required. Some spectrophotometers are manually operated, while others are equipped for automatic and continuous recordings. Spectrophotometers employing the latest technology can be interfaced to a digital computer through an analog-digital converter for the direct determination of different spectra of analytes as well as for the storage of reference spectra. Fourier transformed infrared spectrophotometry is different from the regular dispersion type in that it employs an interferometric technique, whereby polychromatic radiations pass through the specimen to a detector on an intensity and frequency basis. Interfacing with a digital computer is required in order to process such complicated spectral data.

### Atomic Absorption Spectrophotometers (AAS):

Instruments for atomic absorption measurements have the same basic components as other spectrophotometers except for the radiation source and the sample container. The most common radiation source is the hollow-cathode lamp, the cathode of which is usually made of the element to be analyzed. The sample is aspirated as a fine mist into a flame that is produced by an optimized mixture of air and acetylene or other suitable gases. The flame vaporizes the solvent molecules and brings the solutes into a gaseous phase. Monochromatic light emitted from the hollow-cathode lamp is selectively absorbed by the analytes.

Photomultiplier tubes are used as detectors, with the electronics designed to accept the modulated radiation source output, thereby negating the continuous signal from the flame. Therefore only changes in the signal from the hollow-cathode lamp are monitored by the detector. These changes are proportional to the number of atoms in the analyte.

Both single- and double-beam atomic absorption spectrophotometers are available. One of the major limitations of Flame AAS is its low sensitivity for certain elements. For determination of elements in low concentrations, electro-thermal atomization technique (graphite furnace atomization) is useful. In the electro-thermal atomization technique, the laminar flow burner is replaced by a graphite furnace. A known volume of analyte solution is placed at the centre of a graphite tube placed in the furnace, the solution is dried and ashed using a controlled heating from electrodes attached to the furnace. Solutes in the dry matter are atomized to a vapour state in a short period to absorb light from the hollow-cathode lamp. Certain elements can be selectively brought to gaseous atomic state (e.g. mercury vapour) without using the flame. Such techniques use vapour generation accessory, in which mercury ions in the solution are reduced to metallic mercury and the mercury vapour is flushed with an inert gas such as nitrogen into an optical cell.

#### Inductively Coupled Plasma – Atomic Emission Spectrophotometers (ICP-AES):

Instruments for inductively coupled plasma - atomic emission measurements consist of a sample solution aspiration system, a high temperature ( $\sim 6000^{\circ}$ ) plasma source (a torch in which argon gas is subjected to collisions using a radio frequency source to produce high temperature plasma). The plasma vaporizes the solvent molecule, brings solutes to an atomic state and excites them to higher energy levels. The emitted light from the atoms is measured using a specific emission wavelength. The torch can be operated either in the axial or radial mode depending on the type and nature of elements to be quantitated and type of sample matrix.

Modern ICP instruments use a charge coupled detector (CCD) for the fast measurement of the intensity of emitted light consisting of different wavelengths. Two types of ICP systems are currently available: (a) The sequential system scans the emission spectrum from lower to higher wave lengths during a sample run, making the determination of elements sequentially and; (b) the simultaneous system that collects emission data from all wave lengths simultaneously. ICP instruments have the added advantage of having high linearity of standard curves as compared to AAS and more sensitivity than flame AAS for several elements.

#### Spectrofluorimeters:

The apparatus for fluorescence intensity measurement is either a fluorometer, which employs filters to restrict the bandwidth of both the excitation and emission beams, or a spectrofluorometer, where prism or grating monochromators are used to limit the excitation beam, the emission beam, or both.

Since a spectrofluorimeter requires a more intense radiation source than a spectrophotometer, either a mercury lamp with its strong discrete lines or a xenon lamp with its energy continuum from the ultraviolet to the infrared is used. Cells for fluorometric measurement are constructed of silica, and the cell compartment is designed to allow a minimum of scattered light to reach the photomultiplier. To minimize scattering interferences, the detector is placed at right angles to the incident excitation beam.

For turbidimetric measurements, a conventional photometer with a tungsten source is usually employed. However, it is preferable to make the measurements in the blue region of a mercury arc. For nephelometric measurements, standard fluorometers are commonly used.

#### Infrared Spectrometers:

Infrared spectroscopy deals with vibrational and rotational frequencies in the molecules. Infrared measurements may be carried out in the region of near-infrared ( $0.78 - 2.5 \mu\text{m}$  or  $12,800 - 4,000 \text{ cm}^{-1}$ ) or mid-infrared ( $2.5 - 50 \mu\text{m}$  or  $4000 - 200 \text{ cm}^{-1}$ ). The most commonly used region, however, is  $2.5 - 15 \mu\text{m}$  or  $4000 - 670 \text{ cm}^{-1}$ .

Two types of instruments are available: (a) dispersive grating spectrophotometers mainly used for qualitative analysis and (b) multiplex instruments, employing Fourier transform that are used for both qualitative and quantitative measurements. The dispersive grating spectrophotometers are replaced by Fourier transform instruments due to their speed, reliability and convenience. Most commercial Fourier transform infrared spectrometers (FTIR) are based on the Michelson interferometer.

The essential components of FTIR include a radiation source, interferometer, sample holder and a detector. Several infrared sources such as Nernst glower, globar source, incandescent wire source, high pressure mercury arc source (used in classical dispersion instruments), and tunable diode laser (helium-neon) sources may be used in FTIR. Michelson interferometer consists of a moving mirror, a fixed mirror and a beam splitter. Radiation from an infrared source is collimated by a mirror and the resultant beam is divided at the beam splitter, half the beam passes to a fixed mirror and the other half is reflected to the moving mirror. After reflection, the two beams recombine at the beam splitter and, for any particular wavelength, constructively or destructively interfere, depending on the difference in optical paths between the two arms of the interferometer. With a constant mirror velocity, the intensity of the emerging radiation at any particular wavelength modulates in a regular sinusoidal manner. In case of a broad band source the emerging beam is a complex mixture of modulation frequencies that after passing through the sample compartment, is focused into the detector. Detectors such as thermal detector (deuterated triglycine sulphate, DTGS) and quantum detector (mercury cadmium telluride, MCT) are used. Detector signal is sampled at precise intervals during the mirror scan. Both the sampling rate and the mirror velocity are controlled by the reference signal incident upon a detector, which is produced by modulation of beam from the helium-neon laser. The resulting signal from the detector is known as an interferogram which contains all the information to reconstruct the spectrum via the mathematical process known as Fourier transformation.

#### Nuclear Magnetic Resonance Spectrometers (NMR):

Nuclear magnetic resonance spectrometers consist of a continuously spinning superconducting magnet capable of producing a magnetic field ( $\sim 11$  tesla) cooled with liquid nitrogen in a double jacketed closed system. Sample solutions using liquid inlets or solids directly using solid inlets are introduced into the core of the magnetic field. The magnetic field produced by the spinning nucleus of elements such as  $^1\text{H}$  or  $^{13}\text{C}$  depend on their environment (functional moieties) in which they exist. Resonance takes place when the magnetic field of the nucleus matches with that of external magnetic field, producing a signal.

The NMR spectrum of a molecule consists of signals produced by a specific nucleus existing in different functional groups (environments) which help in its identification. A study of NMR spectrums helps in the structural elucidation of an unknown molecule. The intensity of the signal produced by a nucleus at a particular resonating frequency is dependent on the

number of such resonating atoms, which makes it possible to quantify the number of atoms and provides a way for the quantitative determination. Instruments ranging from 60 MHz to 1000 MHz are currently available for NMR measurements.

## Procedures

Instruction manuals supplied by manufacturers should always be consulted for such matters as care, calibration, handling techniques, and operating procedures. Calibration of both the wavelength and the photometric scales should be conducted at fixed intervals. For wavelength calibration in the ultraviolet and visible regions, a quartz-mercury arc and a holmium oxide glass filter are the most common standards employed. For the near-infrared and infrared regions, a polystyrene film may be used. The photometric scale can be checked by a number of standard inorganic glass filters or by standard solutions of known transmittance.

In absorption spectrophotometry, comparisons of the sample and reference standard are best made at or within  $\pm 1$  nm of the wavelength at which maximum absorbance occurs. If matched cells are unavailable, both cells are filled with the selected solvent and any difference in absorbance should be corrected instrumentally or mathematically. The solvent should be transparent in the spectral range of interest. Water, lower alcohols, chloroform, aliphatic hydrocarbons, and many other organic solvents can be used as solvents for ultraviolet and visible measurements. For best results, the concentration of the sample solution should produce an absorbance in the range of about 0.2 to 0.7. For the infrared region, however, few solvents are suitable for sample preparation.

The solvent used in infrared spectrophotometry must not affect the material, usually sodium chloride, of which the cell is made. No solvent in appreciable thickness is completely transparent throughout the infrared spectrum. Infrared spectral grade solvents such as carbon tetrachloride R is practically transparent (up to 1 mm in thickness) from 4,000 to 1,700  $\text{cm}^{-1}$  (2.5 to 6  $\mu\text{m}$ ). Chloroform R, dichloromethane R, and dibromomethane R are other useful solvents. Carbon disulfide IR (up to 1 mm in thickness) is suitable as a solvent to 250  $\text{cm}^{-1}$  (40  $\mu\text{m}$ ), except in the 2,400-2,000  $\text{cm}^{-1}$  (4.2-5.0  $\mu\text{m}$ ) and the 1,800-1,300  $\text{cm}^{-1}$  (5.5-7.5  $\mu\text{m}$ ) regions, where it has strong absorption. Its weak absorption in the 875-845  $\text{cm}^{-1}$  (11.4-11.8  $\mu\text{m}$ ) region should also be noted. Other solvents have relatively narrow regions of transparency (carbon disulfide, chloroform, and carbon tetrachloride are the most frequently used). (**Note:** *These solvents are hazardous and appropriate precautions should be taken*).

In some cases, the sample can be dispersed in mineral oil to form a mull, which is transferred to the salt plates. In most cases, however, the sample is dispersed in dried potassium bromide and the mixture is compressed into a tablet or pellet. The proportion of substance to the halide should be about 1 to 200. The amount taken should be such that the weight of substance per area of the disc is about 5-15  $\mu\text{g}$  per  $\text{mm}^2$ , varying with the molecular weight and to some degree with the type of apparatus used. However, the concentration of the substance should be such that the strongest peak attributable to the substance reaches to between 5% and 25% transmittance. Although the infrared region extends from 2 to 40  $\mu\text{m}$ , for purposes of ascertaining compliance with a reference spectrum, the range from 2.5 to 15  $\mu\text{m}$  (3,800 to 650  $\text{cm}^{-1}$ ) is usually satisfactory.

For atomic absorption measurements, the solvent should not seriously interfere with the absorption or emission processes or with the production of neutral atoms. Also, both the analyte solution and the standard solution should be as much alike as possible, especially with respect to concentration, viscosity, and surface tension.



In fluorescence spectrophotometry, test solutions are usually very dilute ( $10^{-3}$  to  $10^{-7}$ M) in order to minimize the "inner filter" effect caused by significant absorption of incident radiation by the sample near the cell surface. Other undesirable effects of highly concentrated solutions in fluorometry are the "self-quenching" and "self-absorption" phenomena that cause significant deviation from linearity. Test solutions used in fluorometry should also be free from any dust and solid particles, as they cause interference in the measurement. In some cases, before any measurement it is advisable to remove dissolved oxygen from the test solutions, due to its quenching effect. Temperature control is usually needed for extremely sensitive determinations, and baseline correction may be critical.

In turbidimetric and nephelometric measurements, it is important to minimize the settling of the suspended particles. This is generally achieved through the addition of protective colloids.

When visual colour and turbidity comparisons are made, matched colour-comparison tubes that are of the same internal diameter must be used. The solutions to be compared should be at the same temperature (preferably room temperature). For colour comparisons, the tubes are usually held vertically and illuminated from below. Viewing is done from above along the axis of the tube, against a white background. If the colours to be compared are too dark to be viewed downward through the depth of the solutions, they may be viewed horizontally across the diameter of the tubes, with the aid of a light source directed from the back of the tubes. If two layers are present, the designated layer must be viewed horizontally across the diameter of the tube.

For visual turbidity comparisons, the tubes should be viewed horizontally across the diameter of the tubes, with the aid of a light source directed at a right angle against the sides of the tubes.

When conducting limit tests involving the comparison of colour or turbidities, suitable detection instruments may be used in place of the unaided eye.

## **Applications**

Ultraviolet and visible spectra provide only limited information about the chemical structure of a substance. However, because of the sensitivity of these techniques and the high degree of precision and accuracy in their measurements, they are employed extensively in assays and other quantitative determinations.

Near-infrared and infrared spectra, on the other hand, are unique for a given chemical compound, except for optical isomers, which have identical spectra in solution. Polymorphism and other factors, such as variations in crystal size and orientation, the grinding procedure, and the possible formation of hydrates may, however, be responsible for a difference in the infrared spectrum of a given compound in the solid state. The infrared spectrum is usually not greatly affected by the presence of small quantities of impurities (up to several percent) in the tested substance. For identification purposes the spectrum may be compared with that of a reference substance, concomitantly prepared or with a standard reference spectrum. Specificity makes the infrared spectrum one of the most valuable tools for structure elucidation and positive identification of complex organic molecules. Correlation charts and reference spectra of thousands of chemicals are readily available. The sensitivity of infrared analysis, however, is poor (about 1/100 to 1/1,000 of ultraviolet), and therefore it has only a very limited application in quantitative analysis.

Atomic absorption is the technique of choice for the quantitative determination of most of the common elements, even those in complex matrices. Although interferences may occur in the determination of some elements due to chemical interaction between different atoms in the

flame (e.g., cation-anion interference), they can usually be circumvented by preliminary treatment (e.g., addition of a complexing agent) or by the optimization of the instrumentation parameters (e.g., increasing the temperature of the flame to decrease anion-cation attraction). High background signals can be corrected using deuterium background correctors or use of Zeeman furnace techniques. Use of chemical modifiers also helps in reducing background in the furnace analysis.

Fluorescence spectrophotometry has the most inherent sensitivity of all the absorption and light-scattering techniques. Concentrations as low as  $10^{-7}$ M can be quantitatively determined with high precision and accuracy. Fluorescence, however, is not as widespread as the other techniques because of the limited number of organic compounds in which fluorescence can be induced.

Light-scattering techniques, including turbidimetry and nephelometry, are very useful in the determination of weight-average molecular weights in dispersed colloidal systems. Several common ions can be determined using these techniques after their precipitation with suitable reagents. Generally, turbidimetry is adequate for the analysis of heavy suspensions where excessive scattering occurs. Nephelometry, on the other hand, is more suitable for the analysis of cloudy liquids where the attenuation of the radiant power is minimal.

## GENERAL METHODS

### APPEARANCE AND PHYSICAL PROPERTIES

#### Boiling Point and Distillation Range

The following method employs 100 ml of sample. In cases where it is necessary or would be desirable to use a smaller sample, the method of McCullough et al. [J. Chem. Ed. 47, 57 (1970)], which employs only 50  $\mu$ l of sample, may be used.

#### Definitions

*Distillation range:* The difference between the temperature observed at the start of a distillation and that observed at which a specified volume has distilled, or at which the dry point is reached.

*Initial boiling point:* The temperature indicated by the distillation thermometer at the instant the first drop of condensate leaves the end of the condenser tube.

*Dry point:* The temperature indicated at the instant the last drop of liquid evaporates from the lowest point in the distillation flask, disregarding any liquid on the side of the flask.

#### Apparatus

*Distillation flask:* A 200-ml round-bottomed distillation flask of heat-resistant glass is preferred when sufficient sample (in excess of 100 ml) is available for the test. If a sample of less than 100 ml must be used, a smaller flask having the capacity of at least double the volume of the liquid taken may be employed. The 200-ml flask has a total length of 17.9 cm, and the inside diameter of the neck is 2.1 cm. Attached about midway in the neck, approximately 12 cm from the bottom of the flask, is a side arm 12.7 cm long and 5 mm in internal diameter, which is inclined downward at an angle of 75° from the vertical.

*Condenser:* Use a straight glass condenser of heat-resistant tubing, 56 to 60 cm long and equipped with a water jacket so that about 40 cm of the tubing is in contact with the cooling medium. The lower end of the condenser may be bent to provide a delivery tube or it may be connected to a bent adapter, which serves as the delivery tube.

**Note:** *All-glass apparatus with standard-taper ground joints may be used alternatively if the assembly employed provides results equal to those obtained with the flask and condenser described above.*

*Receiver:* The receiver is a 100-ml cylinder, which is graduated in 1-ml sub-divisions and calibrated "to contain". It is used for measuring the sample as well as for receiving the distillate.

*Thermometer:* A partial-immersion thermometer, calibrated for accuracy, having the smallest practical sub-divisions (not greater than 0.2°) is recommended in order to avoid the necessity for an emergent-stem correction.

*Source of heat:* A Bunsen burner is the preferred source of heat. An electric heater may be used, however, if it is shown to give results comparable to those obtained with the gas burner.

*Shield:* The entire burner and flask assembly should be protected from external air currents. Any efficient shield may be employed for this purpose.

*Flask support:* An asbestos board, 6.5 mm in thickness and having a 10 cm circular hole, is placed on a suitable ring or platform support and fitted loosely inside the shield to ensure that

hot gases from the source of heat do not come in contact with the sides of neck of the flask. A second 6.5 mm asbestos board, at least 225 square cm and provided with a 30 mm circular hole, is placed on top of the first board. This board is used to hold the 200 ml distillation flask, which should be fitted firmly on the board so that direct heat is applied to the flask only through the opening in the board.

#### Procedure

**Note:** *This procedure is to be used for liquids that distil above 50° in which case the sample can be measured and received, and the condenser water used, at room temperature (20-30°). For materials boiling below 50°, cool the liquid to below 10° before sampling, receive the distillate in a water bath cooled to below 10° and use water cooled to below 10° in the condenser.*

Measure  $100 \pm 0.5$  ml of the liquid in the 100-ml graduated cylinder and transfer the sample together with an efficient anti-bumping device to the distillation flask. Do not use a funnel in the transfer, or allow any of the sample to enter the side arm of the flask. Place the flask on the asbestos boards, which are supported on a ring or platform, and place in position the shield for the flask and burner. Connect the flask and condenser, place the graduated cylinder under the outlet of the condenser tube and insert the thermometer. The thermometer should be located in the centre of the neck end so that the top of the bulb (when present, auxiliary bulb) is just below the bottom of the outlet to the side arm. Regulate the heating so that the first drop of liquid is collected within 5 to 10 min. Read the thermometer at the instant the first drop of distillate falls from the end of the condenser tube and record as the initial boiling point. Continue the distillation at the rate of 4 or 5 ml of distillate per min, noting the temperature as soon as the last drop of liquid evaporates from the bottom of the flask (dry point) or when the specified percentage has distilled over.

Correct the observed temperature readings for any variation in the barometric pressure from the normal (760 mm Hg) by allowing  $0.1^\circ$  for each 2.7 mm of variation, adding the correction if the pressure is lower, or subtracting if higher than 760 mm Hg. When a total immersion thermometer is used correct for the temperature of the emergent-stem by the formula  $0.00015 \times N(T - t)$ , in which N represents the number of degrees of emergent-stem from the bottom of the stopper, T the observed temperature of distillation, and t the temperature registered by an auxiliary thermometer the bulb of which is placed midway of the emergent-stem, adding the correction to the observed readings of the main thermometer.

Alternatively, the following simplified correction formula may be applied:

$$t = t_0 - k(760 - b)$$

in which  $t_0$  is the boiling point at 760 mm, b is the observed pressure in mm Hg and k is the correction factor for each 1-mm difference with normal pressure.

The factor k depends on the substance under study; it is given in handbooks and varies between 0.033 and 0.057.

### **Determination of pH (Potentiometric Method)**

The pH of an aqueous solution may be determined accurately by potentiometry using a pH meter. The practical definition of pH in water may be given by the equation:

$$\text{pH} = \text{pH}_0 + [(E - E_0)/0.0591],$$

where pH is the value for the solution being measured,  $\text{pH}_0$  is the value for a standard buffer, E is the potential value for the solution being measured,  $E_0$  is the potential value for the

standard buffer, and 0.0591 is the value at 25° of the Nernstian constant. The equation does not apply to solvents other than water, or to mixed solvents that include water. However, the pH meter gives reproducible readings in other solvent systems, on the basis of calibration with aqueous buffers, and while the pH readings lack thermodynamic significance they are useful in setting specifications.

The measurement of pH using a pH meter is a matter of comparing the meter reading of an unknown solution with the meter readings of standard buffers whose pH values are accurately known. Standard buffer solutions are described in compendia, such as the Merck Index. Routine measurement uses only one buffer and an approximation of the electrode slope, usually made by a temperature compensator, pH measurement accurate to  $\pm 0.05$  pH unit or better requires the use of two buffers that bracket, if possible, the expected pH range. All samples and buffer should be at the same temperature.

The choice and care of glass and reference electrodes must be carefully considered. The ordinary glass electrode begins to be sensitive to alkali metal cations at pH values above about 9, leading to the so-called alkaline error. Electrodes with a greatly reduced alkaline error should be used for readings in the alkaline range. Store the electrodes in distilled water when not in use, in order to avoid dehydration. "Flow-type" electrodes may be used if evidence of validity of pH measurement with the electrode is demonstrated.

The measurement of the pH of "highly buffered solutions" (distilled water or solutions of non-ionic organic compounds in distilled water) is a particularly difficult measurement. The addition of 0.3 ml of a saturated solution of potassium chloride per 100 ml of distilled water helps by providing a small amount of electrolyte. However, it will usually be necessary to protect the solution being measured from the carbon dioxide in air by use of a blanket of nitrogen during the measurement. Measure the pH of successive portions of the distilled water or test solutions, with vigorous agitation, until the observed results for two successive portions agree within 0.1 pH unit.

#### Procedure

Use a suitable pH meter and follow the manufacturer's instructions. Each time the electrodes are used, rinse them with distilled or deionised water and carefully blot them dry with clean absorbent tissue. Form a fresh reference electrode liquid junction. Rinse the sample vessel three times with each new solution to be introduced.

Choose two standard buffers (standard buffer solutions are described in compendia, such as the Merck Index) to bracket, if possible, the anticipated pH of the unknown. Warm or cool these standards as necessary to match within 2° the temperature of the unknown, and initially set the temperature compensator to that temperature. Immerse the electrodes in a portion of the first standard buffer, and following the manufacturer's instructions adjust the appropriate standardization control (knob, switch, or button) until the pH reading is that of the buffer. Repeat this procedure with fresh portions of the first standard buffer until two successive readings are within  $\pm 0.02$  pH unit without an adjustment of the standardization control.

Rinse the electrodes, blot dry, and immerse them in a portion of the second standard buffer of lower pH. Do not change the setting of the standardization control. Following the manufacturer's instructions, adjust the slope control (thumbwheel switch, knob, or temperature compensator) until the exact buffer pH is displayed.

Repeat the sequence of standardization with both buffers until the pH readings are within  $\pm 0.02$  pH unit for both buffers without any adjustment of either control (the amount of sample to be used in sample preparation is given where applicable in the individual specification.). The pH of the unknown solution may then be measured (The difference between the results

of two pH determinations when carried out simultaneously on in rapid succession by the same analyst, under the same conditions, should not exceed 0.05 pH unit.).

Always re-standardize the instrument after even a short period during which the amplifier is turned off.

### **Melting Range/Melting point**

The melting point of a pure substance is the temperature at which the substance changes state from solid to liquid. A substance containing impurities will not melt at one specific temperature, but will melt over a range.

Before determining the melting range of a substance, the sample should be dried under the conditions specified for Loss on Drying in the individual monograph. If a temperature is not specified in the monograph, the sample should be dried for 24 h in a desiccator.

Transfer a quantity of the dried powder to a dry capillary-tube about 10 cm long and sealed at one end (thickness of the wall, 0.10-0.15 mm; i.d. 0.9-1.1 mm) and pack the powder by tapping the tube on a hard surface so as to form a tightly-packed column 2-4 mm in height.

Attach the capillary-tube and its contents to a standard thermometer so that the closed end is at the level of the middle of the bulb, and heat in a suitable apparatus containing an appropriate liquid (liquid paraffin or silicone oil) and fitted with a stirring device and an auxiliary thermometer. Regulate the rise in temperature during the first period to 3° per min. When the temperature has risen to 5° below the lowest figure of the range for the substance being tested, heat more slowly: if no other directions are given, the rate of rise in temperature should be 1-2° per min.

Unless otherwise directed, read the temperature at which the substance is observed to form droplets against the side of the tube and the temperature at which it is completely melted, as indicated by the formation of a definitive meniscus.

To the temperature readings, apply the emergent-stem correction determined as follows:

Before starting the determination of the melting range, adjust the auxiliary thermometer so that the bulb touches the standard thermometer at a point midway between the graduation for the expected melting range and the surface of the heating material. When the substance has melted, read the temperature on the auxiliary thermometer. Calculate the correction to be added to the temperature reading of the standard thermometer from the following formula:

$$0.00015 N(T - t)$$

in which T is the temperature reading of the standard thermometer, t is the temperature reading of the auxiliary thermometer and N is the number of degrees of the scale of the standard thermometer between the surface of the heating material and the level of the mercury.

The statement "melting range, a° - b°" means that the corrected temperature at which the material is observed to form droplets must be at least a°, and that the material must be completely melted at the corrected temperature b°.

### **Refractive Index**

The refractive index of a transparent substance is the ratio of the velocity of light in air to its velocity in that material under like conditions. It is equal to the ratio of the sine of the angle of incidence made by a ray in air to the sine of the angle of refraction made by the ray in the

material being tested. The refractive index values specified are for the D line of sodium (589 nm) unless otherwise specified.

The determination should be made at the temperature specified in the individual monograph or at 25° if no temperature is specified. This physical constant is used as a means for identification of, and detection of impurities in, volatile oils and other liquid substances. The Abbé refractometer, or other refractometers of equal or greater accuracy, may be employed at the discretion of the operator.

### **Solidification Point**

This method is designed to determine the solidification point of food grade chemicals having appreciable heats of fusion. It is applicable to chemicals having solidification points between -20° and +150°.

Solidification point is an empirical constant defined as the temperature at which the liquid phase of a substance is in approximate equilibrium with a relatively small portion of the solid phase. It is measured by noting the maximum temperature reached during a controlled cooling cycle after the appearance of a solid phase.

Solidification point is distinguished from freezing point in that the latter term applies to the temperature of equilibrium between the solid and liquid state of pure compounds. Some chemical compounds have two temperatures at which there may be a temperature equilibrium between solid and liquid state depending upon the crystal form of the solid that is present.

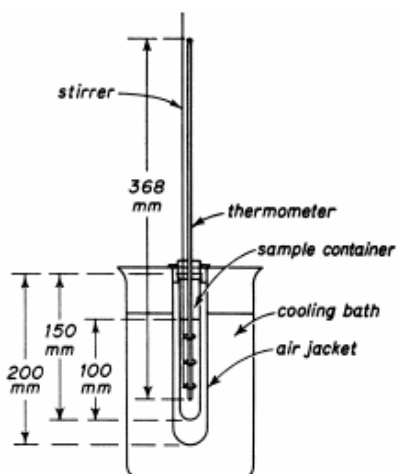
#### Apparatus

The apparatus is illustrated below and consists of the components described as follows:

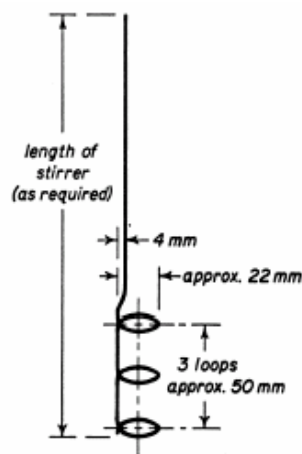
*Sample container:* Use a standard glass 25 x 150 mm test-tube with lip, fitted with a stopper bored to hold the thermometer in place and to allow stirring with stirrer.

*Thermometer:* A thermometer having a range not exceeding 30° graduated in 0.1° divisions, and calibrated for accuracy at 76 mm immersion, should be employed. A thermometer should be so chosen that the stopper of the sample container does not obscure the solidification point.

*Stirrer:* The stirrer consists of a 1 mm diameter (B & S gauge 18) corrosion-resistant wire bent in a series of 3 loops about 25 mm apart. It should be made so that it will move freely in the space between the thermometer and the inner wall of the sample container. The shaft of the stirrer should be of a convenient length designed to pass loosely through a hole in the stopper holding the thermometer. Stirring may be hand-operated or mechanically activated at 20 to 30 strokes per min.



Apparatus  
for determining Solidification Point



Stirrer  
for Solidification Point determination

*Air jacket:* Use a standard glass 38 x 200 mm test-tube with lip, fitted with a stopper bored with a hole into which the sample container can easily be inserted up to the lip.

*Cooling bath:* Use a 2-L beaker or similar suitable container as a cooling bath. Fill it with an appropriate cooling medium such as glycerine, mineral oil, water, water and ice or alcohol-dry ice.

*Assembly:* Assemble the apparatus in such a way that the cooling bath can be heated or cooled to control the desired temperature ranges. Clamp the air jacket so that it is held rigidly just below the lip and immerse it in the cooling bath to a depth of 160 mm.

*Preparation of sample:* The solidification point is usually determined on chemicals as they are received. Some may be hygroscopic, however, and require special drying. Where this is necessary it will be noted in the monograph. Products which are normally solid at room temperature must be carefully melted at a temperature about 10° above the expected solidification point. Care should be observed to avoid heating in such a way as to decompose or distil any portion of the sample.

### Procedure

Adjust the temperature of the cooling bath to about 5° below the expected solidification point. Fit the thermometer and stirrer with a stopper so that the thermometer is centred and the bulb is about 20 mm from the bottom of the sample container. Transfer a sufficient amount of the sample, previously melted if necessary, into the sample container to fill it to a depth of about 90 mm when in molten state. Place the thermometer and stirrer in the sample container and adjust the thermometer so that the immersion line will be at the surface of the liquid and the end of the bulb  $20 \pm 4$  mm from the bottom of the sample container. When the temperature of the sample is about 5° above the expected solidification point, place the assembled sample tube in the air jacket.

Allow the sample to cool while stirring at the rate of 20 to 30 strokes per min, in such a manner that the stirrer does not touch the thermometer. Stir the sample continuously during the remainder of the test.



The temperature at first will gradually fall, then become constant as crystallization starts and continues under equilibrium conditions, and finally will start to drop again. Some chemicals may super-cool slightly below ( $0.5^{\circ}$ ) the solidification point; as crystallization begins the temperature will rise and remain constant as equilibrium conditions are established. Other products may cool more than  $0.5^{\circ}$  and cause deviation from the normal pattern of temperature changes. If the temperature rise exceeds  $0.5^{\circ}$  after the initial crystallization begins, repeat the test and seed the melted compound with small crystals of the sample at  $0.5^{\circ}$  intervals as the temperature approaches the expected solidification point. Crystals for seeding may be obtained by freezing a small sample in a test-tube directly in the cooling bath. It is preferable that seeds of the stable phase be used from a previous determination.

Observe and record the temperature readings at regular intervals until the temperature rises from a minimum, due to super-cooling, to a maximum and then finally drops. The maximum temperature reading is the solidification point. Readings 10 sec apart should be taken in order to establish that the temperature is at the maximum level and continues until the drop in temperature is established.

### Solubility

Approximate solubilities, as specified in the Identification Tests, are to be interpreted according to the following descriptive terms:

Descriptive term	Parts of solvent required for 1 part of solute			
Very soluble	Less than 1			
Freely soluble	From	1	to	Less than 10
Soluble	From	10	to	Less than 30
Sparingly soluble	From	30	to	Less than 100
Slightly soluble	From	100	to	Less than 1,000
Very slightly soluble	From	1,000	to	Less than 10,000
Practically insoluble or in soluble	More than 10,000			

Procedure: Unless otherwise specified, transfer a known amount of the sample into a flask containing known amount of the specified solvent, shake for no less than 30 sec and no more than 5 min.

### Specific Gravity

Specific gravity is defined as the ratio of the mass of the sample to the mass of an equal volume of the standard material. The specific gravity ( $d_t^t$ ) means the ratio of the weight of the sample at  $t^{\circ}$  to that of an equal volume of water at  $t^{\circ}$ . Unless otherwise specified, specific gravity is noted as  $D_{20}^{20}$ . Specific gravity is determined by one of the following methods, unless otherwise specified.

#### Measurement by Pycnometer

A pycnometer is a vessel made of glass with a capacity of usually 10 to 100 ml. It has a ground-glass stopper fitted with a thermometer, and has a side tube with a mark and a ground-glass cap. Weigh a pycnometer previously cleaned and dried, and note the weight  $W$ . Remove the stopper and the cap, fill the pycnometer with a sample, keep at the temperature of about  $1^{\circ}$  to  $3^{\circ}$  lower than that specified, and stopper, taking care not to leave bubbles.

Raise the temperature gradually until the thermometer shows the specified temperature. Remove the sample above the mark from the side tube, replace the cap, and wipe the outside thoroughly. Weigh, and note the weight  $W_1$ . Using the same pycnometer, perform the similar determination with water. Weigh the pycnometer containing water at the specified temperature, and note the weight  $W_2$ . Calculate the specific gravity of the sample by the following formula.

$$d = (W_1 - W) / (W_2 - W)$$

#### Measurement by Mohr-Westphal Balance

Keep the balance horizontal; attach the glass tube in which a thermometer is enclosed by a wire onto the end of the arm. Immerse the glass tube in water in a cylinder, place the largest rider on the arm at the mark 10, and adjust the balance by moving the nut at the specified temperature.

After that, immerse the glass tube in the sample, adjust the balance by hanging riders on the arm, and read the specific gravity at the marks at which riders are placed. It is necessary to make the length of the part of wire that is immersed in a sample equal to that immersed in water by changing the height of the sample in the cylinder.

#### Measurement by Hydrometer

Use a hydrometer with a precision intended for use at the specified temperature. Clean the hydrometer with alcohol. Shake the sample well, and place in the hydrometer after bubbles have disappeared. At the specified temperature, when the hydrometer has settled, read the specific gravity at the upper rim of the meniscus. In case of any hydrometer, however, for which special directions are given, follow the directions.

#### Measurement by Sprengel-Ostwald Pycnometer

A Sprengel-Ostwald pycnometer (see figure) is a vessel made of glass with a capacity of usually 1 to 10 ml. As shown in the figure, both the ends are thick-walled fine tubes one of which has a mark on it. A platinum or an aluminium wire is attached to hang on the arm of a chemical balance.



Sprengel-Ostwald Pycnometer

Weigh the pycnometer, previously cleaned and dried ( $W$ ). Immerse the curved tube in the sample kept at a temperature  $3^\circ$  to  $5^\circ$  lower than the specified temperature, attach a rubber

tube at the end of the straight tube, and suck the sample gently until it comes up above the mark, taking care to prevent formation of bubbles. Immerse the pycnometer in a water bath kept at the specified temperature for about 15 min, and by attaching a piece of filter paper at the end of the curved tube, adjust the end of the sample to the mark. Remove the pycnometer from the water bath, and wipe the outside well. Weigh and note the weight W1. By using the same pycnometer, perform the same determination with water. Weigh the pycnometer containing water at the specified temperature, and note the weight W2. Calculate the specific gravity by the following formula:

$$d = (W1 - W) / (W2 - W)$$

### Specific Rotation

Optical rotation of chemicals is generally expressed in degrees, as either "angular rotation" (observed) or "specific rotation" (calculated with reference to the specific concentration of 1 g of solute in 1 ml of solution, measured under stated conditions).

Specific rotation usually is expressed by the term  $[\alpha]_{t_x}$ , in which t represents, in degrees centigrade, the temperature at which the rotation is determined, and x represents the characteristic spectral line or wavelength of the light used. Spectral lines most frequently employed are the D line of sodium (doublet at 589.0 and 589.6 nm and the yellow-green line of mercury at 546.1 nm). The specific gravity and the rotatory power vary appreciably with the temperature.

The accuracy and precision of optical rotation measurements will be increased if they are carried out with due regard for the following general considerations.

The source of illumination should be supplemented by a filtering system capable of transmitting light of a sufficiently monochromatic nature. Precision polarimeters generally are designed to accommodate interchangeable disks to isolate the D line from sodium light or the 546.1 nm line from the mercury spectrum. With polarimeters not thus designed, cells containing suitably coloured liquids may be employed as filters [see "Technique of Organic Chemistry", A. Weissberger. Vol. I, Part II, 3rd ed. (1960), Interscience Publishers, Inc., New York, N.Y.].

Special attention should be paid to temperature control of the solution and of the polarimeter. Observations should be accurate and reproducible to the extent that differences between replicates, or between observed and true values of rotation (the latter value having been established by calibration of the polarimeter scale with suitable standards), calculated in terms of either specific rotation or angular rotation, whichever is appropriate, shall not exceed one-fourth of the range given in the individual monograph for the rotation of the article being tested. Generally, a polarimeter accurate to  $0.05^\circ$  of angular rotation, and capable of being read with the same precision, suffices. In some cases, a polarimeter accurate to  $0.01^\circ$  or less, of angular rotation, and read with comparable precision, may be required.

Polarimeter tubes should be filled in such a way as to avoid creating or leaving air bubbles, which interfere with the passage of the beam of light. Interference from bubbles is minimized with tubes in which the bore is expanded at one end. However, with tubes of uniform bore, such as semimicro- or micro-tubes, care is required for proper filling. At the time of filling, the tubes and the liquid or solution should be at a temperature not higher than that specified for the determination, to guard against the formation of a bubble upon cooling and contraction of the contents.

In closing tubes having removable end-plates fitted with gaskets and caps, the latter should be tightened only enough to ensure a leak-proof seal between the end-plate and the body of the

tube. Excessive pressure on the end-plate may set up strains that result in interference with the measurements. In determining the specific rotation of a substance of low rotatory power, it is desirable to loosen the caps and tighten them again between successive readings in the measurement of both the rotation and the zero-point. Differences arising from end-plate strain thus generally will be revealed and appropriate adjustments to eliminate the cause may be made.

#### Procedure

In the case of a solid, dissolve the substance in a suitable solvent, reserving a separate portion of the latter for a blank determination. Make at least five readings of the rotation of the solution, or of the substance itself if liquid, at 25° or the temperature specified in the individual monograph. Replace the solution with the reserved portion of the solvent (or, in the case of a liquid, use the empty tube), make the same number of readings, and use the average as the blank value. Subtract the blank value from the average observed rotation if the two figures are of the same sign, or add if opposite in sign, to obtain the corrected angular rotation.

#### Calculation

Calculate the specific rotation of a liquid substance, or of a solid in solution, by application of one of the following formulas:

- For liquid substances,  $[\alpha]_x' = (a/ld)$ ;
- For solutions of solids,  $[\alpha]_x' = (100a/lpd) = (100a/lc)$ ;

in which a is the corrected angular rotation, in degrees, at temperature t; l is the length of the polarimeter tube in decimeters; d is the specific gravity of the liquid or solution at the temperature of observation; p is the concentration of the solution expressed as the number of g of substance in 100 g of solution; and c is the concentration of the solution expressed as the number of g of substance in 100 ml of solution.

The concentrations p and c should be calculated on the dried or anhydrous basis, unless otherwise specified.

## IDENTIFICATION TESTS

The following are chemical tests to identify specific inorganic ions or organic moieties. Test solutions (TS) used are defined under the section on Media, Reagents and Solutions.

### **Inorganic Ions**

#### Aluminium

Solutions of aluminum salts yield with ammonia TS a white, gelatinous precipitate which is insoluble in an excess of ammonia TS. A similar precipitate is produced by sodium hydroxide TS or sodium sulfide TS, but it dissolves in an excess of either reagent.

#### Ammonium

Sodium hydroxide TS decomposes ammonium salts with the evolution of ammonia, recognizable by its odour and its alkaline effect upon moistened red litmus paper. The decomposition is accelerated by warming.

#### Bicarbonate

See Carbonate.

#### Bisulfite

See Sulfite.

#### Bromate

Solutions of bromates acidified with nitric acid (1 in 20), yield a white, crystalline precipitate with the addition of 2 or 3 drops of silver nitrate TS, which dissolves by heating. A pale yellow precipitate is produced with the addition of 1 drop of sodium nitrite TS.

Solutions of bromates acidified with nitric acid (1 in 20), produce a yellow to reddish brown colour with the addition of 5 or 6 drops of sodium nitrite TS. With the addition of 1 ml of chloroform and stirring, the chloroform layer becomes a yellow to reddish brown colour.

#### Bromide

Free bromine is liberated from solutions of bromides upon the addition of chlorine TS, dropwise. When shaken with chloroform, the bromine dissolves, colouring the chloroform red to reddish brown. A yellowish white precipitate, which is insoluble in nitric acid and slightly soluble in ammonia TS, is produced when solutions of bromides are treated with silver nitrate TS.

#### Calcium

Insoluble oxalate salts are formed when solutions of calcium salts are treated in the following manner: using 2 drops of methyl red TS as indicator, neutralize a solution of a calcium salt (1 in 20) with ammonia TS. A white precipitate of calcium oxalate forms upon the addition of ammonium oxalate TS. This precipitate is insoluble in acetic acid but dissolves in hydrochloric acid.

Calcium salts moistened with hydrochloric acid impart a transient yellowish red colour to a non-luminous flame.

### Carbonate

Carbonates and bicarbonates effervesce with acids, yielding a colourless gas (carbon dioxide) which produces a white precipitate immediately when passed into calcium hydroxide TS. Cold solutions of soluble carbonates are coloured red by phenolphthalein TS, whereas solutions of bicarbonates remain unchanged or are slightly changed.

### Chloride

Solutions of chlorides yield with silver nitrate TS a white, curdy precipitate which is insoluble in nitric acid but soluble in a slight excess of ammonia TS. Chlorine, recognizable by its distinctive odour, is evolved when solutions of chloride are warmed with potassium permanganate and dilute sulfuric acid TS.

### Copper

When solutions of cupric compounds are acidified with hydrochloric acid, a red film of metallic copper is deposited upon a bright untarnished surface of metallic iron. An excess of ammonia TS, added to a solution of a cupric salt, produces first a bluish precipitate and then a deep blue-coloured solution. Solutions of cupric salts yield with potassium ferrocyanide TS a reddish brown precipitate, insoluble in dilute acids.

### Ferric salts

Potassium ferrocyanide TS produces a dark blue precipitate in acid solutions of ferric salts. With an excess of sodium hydroxide TS, a reddish brown precipitate is formed. Solutions of ferric salts produce with ammonium thiocyanate TS a deep red colour which is not destroyed by dilute mineral acids.

### Ferrocyanide

To 10 ml of a 1% solution of the sample add 1 ml of ferric chloride TS. A dark blue precipitate is formed.

### Ferrous salts

Potassium ferricyanide TS produces a dark blue precipitate in solutions of ferrous salts. This precipitate, which is insoluble in dilute hydrochloric acid, is decomposed by sodium hydroxide TS. Solutions of ferrous salts yield with sodium hydroxide TS a greenish white precipitate, the colour rapidly changing to green and then to brown when shaken.

### Iodide

Solutions of iodides, upon the addition of chlorine TS, dropwise, liberate iodine which colours the solution yellow to red. Chloroform is coloured violet when shaken with this solution. The iodine thus liberated gives a blue colour with starch TS. Silver nitrate TS produces in solutions of iodides a yellow, curdy precipitate which is insoluble in nitric acid and in ammonia TS.

### Iron

Solutions of ferrous and ferric compounds yield a black precipitate with ammonium sulfide TS. This precipitate is dissolved by cold dilute hydrochloric acid TS with evolution of hydrogen sulfide.

### Magnesium

Solutions of magnesium salts in the presence of ammonium chloride yield no precipitate with ammonium carbonate TS, but a white, crystalline precipitate, which is insoluble in ammonia TS, is formed upon the subsequent addition of sodium phosphate TS.

### Manganese

Solutions of manganese salts yield with ammonium sulfide TS a salmon-coloured precipitate which dissolves in acetic acid.

### Nitrate

When a solution of a nitrate is mixed with an equal volume of sulfuric acid, the mixture cooled, and a solution of ferrous sulfate superimposed, a brown colour is produced at the junction of the two liquids. Brownish red fumes are evolved when a nitrate is heated with sulfuric acid and metallic copper. Nitrates do not decolourize acidified potassium permanganate TS (distinction from nitrites).

### Nitrite

Nitrites yield brownish red fumes when treated with dilute mineral acids or acetic acid. A few drops of potassium iodide TS and a few drops of dilute sulfuric acid TS added to a solution of a nitrite liberate iodine which colours starch TS blue.

### Peroxide

Solutions of peroxides slightly acidified with sulfuric acid yield a deep blue colour upon the addition of potassium dichromate TS. On shaking the mixture with an equal volume of ether and allowing the liquids to separate, the blue colour is transferred to the ether layer.

### Phosphate

Neutral solutions of orthophosphates yield with silver nitrate TS a yellow precipitate, which is soluble in dilute nitric acid TS or in ammonia TS. With ammonium molybdate TS, a yellow precipitate, which is soluble in ammonia TS, is formed.

### Potassium

Potassium compounds impart a violet colour to a non-luminous flame if not masked by the presence of small quantities of sodium. In neutral, concentrated or moderately concentrated solutions of potassium salts, sodium bitartrate TS slowly produces a white, crystalline precipitate which is soluble in ammonia TS and in solutions of alkali hydroxides or carbonates. The precipitation may be accelerated by stirring or rubbing the inside of the test tube with a glass rod or by the addition of a small amount of glacial acetic acid or ethanol.

### Sodium

Sodium compounds, after conversion to chloride or nitrate, yield with cobalt-uranyl acetate TS a golden-yellow precipitate, which forms after several min agitation. Sodium compounds impart an intense yellow colour to a non-luminous flame.

### Sulfate

Solutions of sulfates yield with barium chloride TS a white precipitate which is insoluble in hydrochloric and nitric acids. Sulfates yield a white precipitate with lead acetate TS, which is

soluble in ammonium acetate solution. Hydrochloric acid produces no precipitate when added to solutions of sulfates (distinction from thiosulfates).

#### Sulfite

When treated with dilute hydrochloric acid TS, sulfites and bisulfites yield sulfur dioxide, recognizable by its characteristic odour. This gas blackens filter paper moistened with mercurous nitrate TS.

#### Thiosulfate

Solutions of thiosulfates yield with hydrochloric acid a white precipitate which soon turns yellow, liberating sulfur dioxide, recognizable by its odour. The addition of ferric chloride TS to solutions of thiosulfates produces a dark violet colour which quickly disappears.

#### Zinc

Zinc salts, in the presence of sodium acetate, yield a white precipitate with hydrogen sulfide. This precipitate, which is insoluble in acetic acid, is dissolved by dilute hydrochloric acid TS. A similar precipitate is produced by ammonium sulfide TS in neutral or alkaline solutions. Solutions of zinc salts yield with potassium ferrocyanide TS a white precipitate which is insoluble in dilute hydrochloric acid TS.

### **Organic Ions and Compounds**

#### Acetate

Acetic acid or acetates, when warmed with sulfuric acid and alcohol, form ethyl acetate, recognizable by its characteristic odour. With neutral solutions of acetates, ferric chloride TS produces a deep red colour which is destroyed by the addition of a mineral acid.

#### Alginate

Dissolve as completely as possible 0.01 g of the sample by shaking with 0.15 ml of 0.1 N sodium hydroxide and add 1 ml of acid ferric sulfate TS. Within 5 min, a cherry-red colour develops that finally becomes deep purple.

#### Ascorbate

To 2 ml of a 2% solution of the sample in water add 2 ml of water, 0.1 g of sodium bicarbonate and about 0.02 g of ferrous sulfate. Shake and allow to stand. A deep violet colour is produced, which disappears on the addition of 5 ml of dilute sulfuric acid TS.

#### Benzoate

Neutral solutions of benzoates yield a salmon-coloured precipitate with ferric chloride TS. From moderately concentrated solutions of benzoate, dilute sulfuric acid TS precipitates free benzoic acid, which is readily soluble in ether.

#### Citrate

When a few mg of a citrate are added to a mixture of 15 ml of pyridine and 5 ml of acetic anhydride, a carmine red colour is produced.



### Gluconates

Dissolve a quantity of the sample in water to obtain a solution containing 10 mg/ml, heating in a water bath at 60°, if necessary. Similarly, prepare a standard solution of potassium gluconate in water containing 10 mg/ml.

Apply separate 5- $\mu$ l portions of the test solution and the standard solution on a suitable thin-layer chromatographic plate coated with 0.25-mm layer of chromatographic silica gel, and allow to dry. Develop the chromatogram in a solvent system consisting of a mixture of ethanol, water, ammonium hydroxide, and ethyl acetate (50:30:10:10) until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the chamber, and dry at 110° for 20 min. Allow to cool, and spray with a reagent, prepared as follows: Dissolve 2.5 g of ammonium molybdate in about 50 ml of 2 N sulfuric acid in a 100-ml volumetric flask, add 1.0 g of ceric sulfate, swirl to dissolve, dilute with 2 N sulfuric acid to volume, and mix. Heat the plate at 110° for about 10 min. The principal spot obtained from the test solution corresponds in colour, size, and retention to that obtained from the standard solution.

### Glutamate

Proceed as directed under Thin Layer Chromatography (see Analytical Techniques) using the following conditions:

*Sample:* 1  $\mu$ l of 1 in 100 solution of the sample. Add a few drops of ammonium hydroxide TS if required to dissolve.

*Reference:* 1  $\mu$ l of a 1 in 100 solution of monosodium L-glutamate

*Solvent:* A mixture of 2 volumes of n-butanol, 1 volume of glacial acetic acid and 1 volume of water

*Adsorbent:* Silica gel

Stop the development when the solvent front has advanced about 10 cm from the point of application. Dry the plate at 80° for 30 min. Spray ninhydrin TS on the plate, heat at 80° for 10 min and observe the plate under natural light. The  $R_f$  value of the sample and that of the reference standard are identical.

### Glycerol

Heat a few drops of the sample in a test tube with about 0.5 g of potassium bisulfate; pungent vapours of acrolein are evolved.

### Lactate

When solutions of lactates are acidified with sulfuric acid, and potassium permanganate TS is added and the mixture heated, acetaldehyde, recognizable by its distinctive odour, is evolved.

### Malate

Transfer the solution described in the individual monograph into a porcelain dish and add 10 mg of sulfanilic acid. Heat the solution on a water bath for a few min, add 5 ml of a 1 in 5 solution of sodium nitrite and heat slightly. Make alkaline with sodium hydroxide TS. A red colour is produced.

### Organic Phosphate

To the solution given in the monograph add 2 ml of magnesia mixture TS. No precipitate is formed. Add 5 ml of nitric acid, boil for 10 min, neutralize with strong ammonia TS, add water to make to 100 ml, add ammonium molybdate TS, and warm. A yellow precipitate is formed, which dissolves in sodium hydroxide TS or ammonia TS.

### Ribose

To 3 ml of a 3 in 10,000 solution of the sample in water, add 0.2 ml of a 1 in 10 solution of orcinol in ethanol and subsequently 3 ml of a 1 in 1,000 hydrochloric acid solution of ferric ammonium sulfate. Heat in a water bath for 10 min. A green colour is produced.

### Tartrate

When a few mg of a tartrate are added to a mixture of 15 ml of pyridine and 5 ml of acetic anhydride, an emerald green colour is produced.

## INORGANIC COMPONENTS

### Acid-Insoluble Matter

Transfer 2 g of the sample, accurately weighed, into a 250-ml beaker containing 150 ml of water and 1.5 ml of sulfuric acid TS. Cover the beaker with a watch glass and heat the mixture on a steam bath for 6 h rubbing down the wall of the beaker frequently with a rubber-tipped stirring rod and replacing any water lost by evaporation. Weigh 500 mg of a suitable acid washed filter aid, pre-dried at 105° for 1 h, to the nearest 0.1 mg, add this to the sample solution and filter through a tared Gooch crucible provided with an asbestos pad. Wash the residue several times with hot water, dry the crucible and its contents at 105° for 3 h, cool in a desiccator and weigh. The difference between the total weight and the weight of the filter aid plus crucible and pad is the weight of the Acid-insoluble matter. Calculate as percentage.

### Arsenic Limit Test

**Note 1:** *Method I referenced in older specifications has been deleted. The colourimetric procedure described in Method II may be used. However, it is recommended that, whenever possible, that the determination of arsenic be carried out using AAS-hydride technique/ICP method.*

**Note 2:** *Metals or salts of metals such as chromium, cobalt, copper, mercury, molybdenum, nickel, palladium, and silver may interfere with the evolution of arsine (Method II). Antimony, which forms stibine, is the only metal likely to produce a positive interference. Stibine forms a red coloured complex with silver diethyldithiocarbamate reagent which has a maximum absorbance at 510 nm. But at 535 - 540 nm the absorbance of the antimony complex is so diminished that the results of arsenic would not be affected significantly.*

**Note 3:** *All reagents used in the limit test for arsenic should be very low in arsenic content.*

#### Method II (Colourimetric Procedure)

##### *Apparatus*

The general apparatus is shown in Figure 1. It consists of a 125-ml arsine generator flask with a 24/40 standard-taper joint fitted with a scrubber unit and an absorber tube connected by a capillary of inside diameter 2 mm and outside diameter 8 mm via a ball-and-socket joint, secured with a No. 12 clamp, connecting the units. Alternatively, an apparatus embodying the principle of the general assembly described and illustrated may be used.

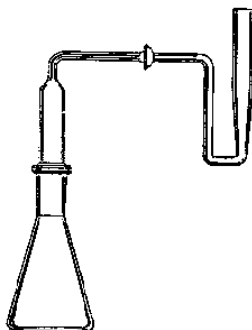


Figure 1. Apparatus for Arsenic Limit Test – Method II

## Reagents

### Silver Diethyldithiocarbamate Solution

Dissolve 1 g of recrystallized silver diethyldithiocarbamate,  $(C_2H_5)_2NCSSAg$ , in 200 ml of reagent grade pyridine in a fume hood. Store this solution in a light-resistant container and use within 1 month.

Silver diethyldithiocarbamate is available commercially or may be prepared as follows. Dissolve 1.7 g of reagent grade silver nitrate in 100 ml of water. In a separate container, dissolve 2.3 g of sodium diethyldithiocarbamate,  $(C_2H_5)_2NCSSNa \cdot 3H_2O$ , in 100 ml of water, and filter. Cool both solutions to about  $15^\circ$ , mix the two solutions, while stirring, collect the yellow precipitate in a medium-porosity sintered-glass crucible or funnel, and wash with about 200 ml of cold water.

Recrystallize the reagent, whether prepared as directed above or obtained commercially, as follows: Dissolve in freshly distilled pyridine, using about 100 ml of solvent for each g of reagent, and filter. Add an equal volume of cold water to the pyridine solution, while stirring. Filter off the precipitate, using suction, wash with cold water, and dry in vacuum at room temperature for 2 to 3 h. The dry salt is pure yellow in colour and should show no change in character after 1 month when stored in a light-resistant container. Discard any material that changes in colour or develops a strong odour.

### Standard Arsenic Solution

Weigh accurately 132.0 mg of arsenic trioxide that has been finely pulverized and dried for 24 h over a suitable desiccant, and dissolve it in 5 ml of sodium hydroxide solution (1 in 5). Neutralize the solution with diluted sulfuric acid TS, add 10 ml in excess, and dilute to 1,000.0 ml with recently boiled water, and mix. Transfer 10.0 ml of this solution into a 1,000-ml volumetric flask, add 10 ml of diluted sulfuric acid TS, dilute to volume with recently boiled water and mix.

Use this final solution, which contains 1  $\mu$ g of arsenic (As) in each ml, within 3 days.

### Stannous Chloride Solution

Dissolve 40 g of reagent grade stannous chloride dihydrate,  $SnCl_2 \cdot 2H_2O$ , in 100 ml of hydrochloric acid. Store the solution in a glass container and use within 3 months.

### Lead Acetate-Impregnated Cotton

Soak cotton in a saturated solution of reagent grade lead acetate, squeeze out the excess solution, and dry in a vacuum at room temperature.

**Note:** *When preparing and using the cotton, take great care to avoid lead contamination.*

### Preparation of the Sample Solution

The solution obtained by treating the sample as directed in an individual monograph is used directly as the Sample Solution in the Procedure. Sample solutions of organic compounds are prepared in the generator flask (Figure 3), unless otherwise directed, according to the following general procedure:

**Caution:** *Some substances may react unexpectedly with explosive violence when digested with hydrogen peroxide. Appropriate safety precautions must be employed at all times.*

**Note:** *If halogen-containing compounds are present, use a lower temperature while heating the sample with sulfuric acid, do not boil the mixture, and add the peroxide, with caution, before charring begins, to prevent loss of trivalent arsenic.*

Transfer 1.0 g of the sample into the generator flask, add 5 ml of sulfuric acid and a few glass beads, and digest at a temperature not exceeding 120° on a hot plate in a fume hood until charring begins. (Additional sulfuric acid may be necessary to completely wet some samples, but the total volume added should not exceed about 10 ml.) After the sample has been initially decomposed by the acid, add with caution, dropwise, 30% hydrogen peroxide, allowing the reaction to subside and reheating between drops. The first few drops must be added very slowly with sufficient mixing to prevent a rapid reaction, and heating should be discontinued if foaming becomes excessive. Swirl the solution in the flask to prevent unreacted substance from caking on the walls or bottom of the flask during digestion. Maintain oxidizing conditions at all times during the digestion by adding small quantities of the peroxide whenever the mixture turns brown or darkens. Continue the digestion until the organic matter is destroyed, gradually raising the temperature of the hot plate to 250° - 300° until fumes of sulfuric acid are copiously evolved, and the solution becomes colourless, or retains only a slight straw colour.

Cool, add cautiously 10 ml of water, again evaporate (fumes of sulfuric acid evolved), and cool. Add cautiously 10 ml of water, mix, wash the sides of the flask with a few ml of water, and dilute to 35 ml.

#### *Procedure*

If the sample solution was not prepared in the generator flask, transfer to the flask a volume of the solution, prepared as directed, equivalent to 1.0 g of the substance being tested and add water to make 35 ml.

Add 20 ml of dilute sulfuric acid (1 in 5), 2 ml of potassium iodide TS, and 0.5 ml of *Stannous Chloride Solution*, and mix. Allow the mixture to stand for 30 min at room temperature. Pack the scrubber tube with two plugs of Lead Acetate-Impregnated Cotton, leaving a small air space between the two plugs, lubricate the ground-glass joints with stopcock grease, if necessary, and connect the scrubber unit with the absorber tube. Transfer 3.0 ml of silver diethyldithiocarbamate solution to the absorber tube, add 3.0 g of granular zinc (20-mesh) to the mixture in the flask, and immediately insert the standard-taper joint in the flask. Allow the evolution of arsine and colour development to proceed at room temperature ( $25 \pm 3^\circ$ ) for 45 min, swirling the flask gently at 10-min intervals. (The addition of a small amount of isopropanol to the generator flask may improve the uniformity of the rate of gas evolution.) Disconnect the absorber tube from the generator and scrubber units, and transfer the Silver diethyldithiocarbamate solution to a 1-cm absorption cell. Determine the absorbance at the wavelength of maximum absorption between 535 nm and 540 nm with a suitable spectrophotometer or colorimeter, using Silver diethyldithiocarbamate solution as the blank. The absorbance due to any red colour from the solution of the sample does not exceed that produced by 3.0 ml of standard arsenic solution (3 µg As) when treated in the same manner and under the same conditions as the sample. The room temperature during the generation of arsine from the standard should be held to within  $\pm 2^\circ$  of that observed during the determination of the sample.

## **Ash**

### Ash (Total)

Accurately weigh a known quantity of sample (depending on the ash content such that about 20 mg of ash is obtained) in a tared crucible, ignite at a low temperature (about 550°), not to exceed a very dull redness, until free from carbon, cool in a desiccator, and weigh. If a carbon-free ash is not obtained, wet the charred mass with hot water, spread the residue using

a glass rod, dry it in an air oven and reignite. If a carbon-free ash is still not obtained, cool the crucible, add 15 ml of ethanol, break up the ash with a glass rod, then burn off the ethanol, again heat the whole to dull redness, cool in a desiccator, and weigh.

**Note:** *If difficulty with oxidizing organic material is found, the use of an ash aid such as ammonium nitrate may prove to be more satisfactory. Addition of a few drops of hydrogen peroxide facilitate oxidation of organic matter.*

#### Ash (Acid-insoluble)

Boil the ash obtained as directed under Ash (Total) above, with 25 ml of dilute hydrochloric acid TS for 5 min, collect the insoluble matter on a suitable ash-less filter, wash with hot water, ignite at  $800 \pm 25^\circ$ , cool, and weigh. Calculate the percentage of acid-insoluble ash from the weight of the sample taken.

#### Ash (Sulfated ash)

##### *Method I (for solids)*

Transfer the quantity of the sample directed in the individual monograph to a tared 50- to 100-ml platinum dish or other suitable container. Add sufficient diluted sulfuric acid TS to moisten the entire sample. Heat gently, using a hot plate, an Argand burner, or an infrared heat lamp, until the sample is dry and thoroughly charred, then continue heating until all of the sample has been volatilized or nearly all of the carbon has been oxidized, and cool. Moisten the residue with 0.5 ml of sulfuric acid TS, and heat in the same manner until the remainder of the sample and any excess sulfuric acid have been volatilized. Finally ignite in a muffle furnace at  $800 \pm 25^\circ$  for 15 min or longer, if necessary, to complete ignition, cool in a desiccator, and weigh.

**(Note:** *In order to promote volatilization of sulfuric acid, it is advisable to add a few pieces of ammonium carbonate just before completing ignition.*)

##### *Method II (for liquids)*

Unless otherwise directed, transfer the required weight of the sample to a suitable tared container, add 10 ml of diluted sulfuric acid TS, and mix thoroughly. Evaporate the sample completely by heating gently without boiling, and cool. Finally, ignite in a muffle furnace at  $800 \pm 25^\circ$  for 15 min or longer, cool in a desiccator, and weigh.

### **Chlorides Limit Test**

Unless otherwise specified, place the prescribed quantity of the sample in a Nessler tube, dissolve it in about 30 ml of water, and neutralize with dilute nitric acid TS if the solution is alkaline. Add 6 ml of dilute nitric acid TS and dilute to 50 ml with water. If the use of a sample solution is prescribed, transfer the sample solution into a Nessler tube and dilute to 50 ml with water. Transfer the prescribed volume of 0.01 N hydrochloric acid into another Nessler tube to serve as the standard, add 6 ml of dilute nitric acid TS, and dilute to 50 ml with water.

If the solution containing the sample is not clear, filter both solutions under the same conditions. Add 1 ml of silver nitrate TS to each solution, mix thoroughly, and allow to stand for 5 min protected from direct sunlight. Compare the turbidity of the two solutions by observing the Nessler tubes from the sides and the tops against a black background. The turbidity of the sample solution does not exceed that of the standard.

## Chromium Limit Test

**Note:** *The limit test described below is designed to show whether the sample contains more or less than 20 mg/kg of chromium. It is recommended to use an appropriate AAS/ICP method in the place of the limit test, if possible, for quantitative determination.*

### Procedure

Weigh 1.0 g of the sample into a quartz dish. Char the material, raising the temperature slowly. Allow to cool, add 10 ml of a 25% magnesium nitrate solution; evaporate, heating slowly until no more nitrous vapour evolves. Heat the material in an oven at 600° until all black particles have disappeared (1 h).

Dissolve the residue by adding 10 ml of 4 N sulfuric acid and 20 ml of water. Heat on a water bath for about 5 min.

Add 0.5 ml of 0.1 N potassium permanganate. Add more permanganate if the solution decolourizes. Cover with a watch glass and heat on a water bath for about 20 min. Add 5% sodium azide solution, one drop every 10 sec, until the excess potassium permanganate has been removed (avoid excess of sodium azide; 2 drops are usually sufficient). Cool the solution in running water, and filter if manganese dioxide (black precipitate) is evident. Transfer the solution to a 50-ml volumetric flask. Add 2.5 ml of 5 M sodium dihydrogenphosphate, add 2 ml of diphenyl carbazide TS and fill to the mark with water. Measure the absorbance at 540 nm 30 min after adding the diphenyl carbazide TS. A blank with the latter two reagents should show no colour or only a slight purple colour.

At the same time run a parallel test with 1.00 ml of standard chromate TS (1 ml = 20 µg Cr) and a few ml of saccharose placed into a second quartz dish. Treat the mixture exactly as the sample and measure the extinction at the same wavelength.

Calculate the chromium content of the sample from the two extinction values observed.

## Cyclic Phosphate Determination

**Note:** *The method uses perchloric acid in one of the reagents. Special care shall be taken while handling it and all operations shall be conducted in a perchloric acid fume cup board.*

### Principle

The method is based on two-dimensional paper chromatography, in which the development is first carried out in one direction using a basic solvent. The paper is then turned through 90° and chromatographed using an acidic solvent. Spots are revealed by spraying with a perchloric acid/molybdate reagent, and are identified and qualitatively assembled by reference to chromatograms of standard phosphates. Quantitative estimation is effected by cutting out the 'spots', washing the paper with ammonia, subsequent determination of the phosphorus content by colourimetry of the molybdenum blue complex and calculation of cyclic phosphate content as % NaPO<sub>3</sub>.

### Reagents

*Solvent A (basic):* Mix together: 400 ml isopropanol, 200 ml isobutanol, 300 ml deionised water and 10 ml 0.880 sp.gr. ammonia solution.

*Solvent B (acidic):* Mix together: 750 ml isopropanol and 250 ml deionised water. Add: 50 g trichloro-acetic acid and 2.5 ml 0.880 sp.gr. ammonia solution.

*Spray reagent:* To 50 ml deionised water, add: 5 ml 60% perchloric acid, 1 ml conc. HCl (1.18 sp.gr.) and 1 g ammonium molybdate. Make up to 100 ml with deionised water

*Standard Phosphate Solutions:* Prepare standard solutions of sodium tri, tetra, hexa, and octameta phosphates containing 2 µg/µl (0.2% w/v).

#### Procedure

Draw faint pencil lines 2.5 cm from the bottom edge and 2.5 cm from the right-hand side of a 23 x 23 cm square piece of the chromatography paper. Apply 1 µl of a 10% w/v solution of the sample at the intersection of the two pencil lines. Allow the paper to dry, curve it into a cylinder, and secure with plastic clips. Stand the cylinder in the tank containing the basic solvent (Solution A), the immersion depth being about 6 mm and allow the solvent front to rise to a height of 20 cm. Remove the paper from the tank and mark the position of the solvent front. Dry the paper in an air oven at 50° and cut off the excess paper above the solvent front.

Develop the paper in acid solvent (Solution B), with the previous right-hand edge to the bottom of the cylinder, until the solvent front has travelled 20 cm. Remove and dry the paper and spray with the acid ammonium molybdate solution. Develop the spots produced by placing the paper under the U.V. lamp at 250 nm for a few minutes.

Mark out a separate piece of chromatography paper as described above. At the intersection of the pencil lines apply 1 µl of each of the meta phosphate standard solutions in turn, drying the paper after each application. Treat this standard paper in a similar manner to that described for the sample. Both tests must be run concurrently using the same solvents, tanks and spray.

Compare the sample and standard chromatograms, and identify the 'spots' with the aid of the  $R_f$  values given in the Table below.

Phosphate	$R_f$ basic	$R_f$ acidic
Tri-meta	0.49	0.13
Tetra-meta	0.36	0.05
Hexa-meta	0.27	0.02
Octa-meta	0.21	0.01
Ortho	0.32	0.71
Pyro	0.26	0.40

$R_f$  values for ortho-, pyro- and cyclic phosphates  
(Values should be taken as a guide only).

If a spot of particular interest is too weak, the chromatogram should be repeated using 2 or 5 µl sample solution instead of 1 µl. About 2 µl of each of the various phosphates should be visible.

An approximation of the quantities of each component in the sample will be gained by a visual comparison of the two chromatograms. For a more accurate measurement, cut out each spot and analyze for total phosphorus by the following method.:

Soak each cut out area of chromatography paper in 25.00- ml of 0.1 N ammonium hydroxide solution for at least 1 h. Pipet a 20.00- ml aliquot of the resulting solution into a 50 -ml volumetric flask, add 5 ml of 10 N sulfuric acid and heat in a boiling water bath for 30 min



to hydrolyse the cyclic phosphates to orthophosphate. Cool to room temperature, add 1 ml of 12.5% ammonium molybdate solution, shake the flask and then add 1 ml of 0.6% hydrazine hydrochloride. Make up to volume with water and place the flask in a boiling water bath for exactly 10 min. Cool rapidly in a cold water bath and measure the absorbance of the solution in a spectrophotometer at 830 nm using distilled water as the reference solution. Perform a blank determination using an equal area of chromatography paper known not to include any phosphate spots and subtract the blank value from the test values. Determine the amount of phosphorus present by reference to a calibration curve of absorbance at 830 nm obtained using samples of standard amounts of potassium dihydrogen orthophosphate.

Where a spot is ill-defined, compare with the standard chromatogram and cut out the zone where the spot should appear. Cut out the area occupied by all the metaphosphates to obtain total cyclic content.

#### Calculation

If x = is  $\mu\text{l}$  of a 10% solution of sample put on paper and y is =  $\mu\text{g}$  P obtained by attached method, then:

$$\%P \text{ in sample} = y / x$$

$$\% \text{ cyclic phosphates expressed as } \text{NaPO}_3 = (102 / 31)(y / x)$$

### **Fluoride Limit Test**

#### Method I - Thorium Nitrate Colorimetric Method

This method should be used unless otherwise directed in the individual monograph.

**Caution:** *When applying this test to organic compounds, the temperature at which the distillation is conducted must be rigidly controlled at all times to the recommended range of 135° to 140° to avoid the possibility of explosion.*

**Note:** *To minimize the distillation blank resulting from fluoride leached from the glassware, the distillation apparatus should be treated as follows:*

Treat the glassware with hot 10% sodium hydroxide solution, followed by flushing with tap water and rinsing with distilled water. At least once daily, treat in addition by boiling down 15 to 20 ml of dilute sulfuric acid (1 in 2) until the still is filled with fumes; cool, pour off the acid, treat again with 10% sodium hydroxide solution, and rinse thoroughly. For further details, see sections 25.050 and 25.054 in Official Methods of Analysis of the AOAC, Thirteenth Edition, 1980.

Unless otherwise directed, place a 5.0 g sample and 30 ml of water in a 125 ml distillation flask having a side arm and trap. The flask is connected with a condenser and carries a thermometer and a capillary tube, both of which must extend into the liquid. Slowly add, with continuous stirring, 10 ml of perchloric acid, and then add 2 or 3 drops of silver nitrate solution (1 in 2) and a few glass beads. Connect a small dropping funnel or a steam generator to the capillary tube. Support the flask on an asbestos mat with a hole that exposes about one third of the flask to the flame. Distil until the temperature reaches 135°. Add water from the funnel or introduce steam through the capillary, maintaining the temperature between 135° and 140° at all times. Continue the distillation until 100 ml of distillate has been collected. After the 100 ml portion (Distillate A) is collected, collect an additional 50 ml portion of distillate (Distillate B) to ensure that all of the fluorine has been volatilized.

Place 50 ml of Distillate A in a 50 ml Nessler tube. In another similar Nessler tube place 50 ml of water distilled through the apparatus as a control. Add to each tube 0.1 ml of a filtered solution of sodium alizarinsulfonate (1 in 1,000) and 1 ml of freshly prepared hydroxylamine hydrochloride solution (1 in 4,000), and mix well. Add, dropwise and with stirring, either 1 N or 0.05 N sodium hydroxide, depending upon the expected volume of volatile acid distilling over, to the tube containing the distillate until its colour just matches that of the control, which is faintly pink. Then add to each tube 1.0 ml of 0.1 N hydrochloric acid, and mix well. From a burette, graduated in 0.05 ml, add slowly to the tube containing the distillate enough thorium nitrate solution (1 in 4,000) so that, after mixing, the colour of the liquid just changes to a faint pink. Note the volume of the solution added, then add exactly the same volume to the control, and mix. Now add to the control solution, sodium fluoride TS (10 µg F per ml) from a burette to make the colours of the two tubes match after dilution to the same volume. Mix well, and allow all air bubbles to escape before making the final colour comparison. Check the endpoint by adding 1 or 2 drops of sodium fluoride TS to the control. A distinct change in colour should take place. Note the volume of sodium fluoride TS added.

Dilute Distillate B to 100 ml, and mix well. Place 50 ml of this solution in a 50 ml Nessler tube, and follow the procedure used above for Distillate A. The total volume of sodium fluoride TS required for the solutions from both Distillate A and Distillate B should not exceed 2.5 ml.

#### Method II - Ion-Selective Electrode Method A

##### *Reagents*

*Buffer Solution:* Dissolve 36 g of cyclohexylenedinitrilotetraacetic acid (CDTA) in sufficient 1 M sodium hydroxide to make 200 ml. Transfer 20 ml of this solution (equivalent to 4 g of disodium CDTA) into a 1,000-ml beaker containing 500 ml of water, 57 ml of glacial acetic acid, and 58 g of sodium chloride, and stir to dissolve. Adjust the pH of the solution to between 5.0 and 5.5 by the addition of 5 M sodium hydroxide, then cool to room temperature, dilute to 1,000 ml with water, and mix.

##### *Procedure*

Unless otherwise directed in the individual monograph, transfer 8.0 g of sample and 20 ml of water to a 250-ml distilling flask, cautiously add 20 ml of perchloric acid, and then add 2 or 3 drops of silver nitrate solution (1 in 2) and a few glass beads. Following the directions, and observing the **Caution** and **Note**, as given under Method I, distil the solution until 200 ml of distillate has been collected.

Treat the glassware with hot 10% sodium hydroxide solution, followed by flushing with tap water and rinsing with distilled water. At least once daily, treat in addition by boiling down 15 to 20 ml of dilute sulfuric acid (1 in 2) until the still is filled with fumes; cool, pour off the acid, treat again with 10% sodium hydroxide solution, and rinse thoroughly. For further details, see sections 25.050 and 25.054 in Official Methods of Analysis of the AOAC, Thirteenth Edition, 1980.

Transfer a 25.0-ml aliquot of the distillate into a 250-ml plastic beaker, and dilute to 100 ml with the Buffer Solution. Place the fluoride ion and reference electrodes (or a combination fluoride electrode) of a suitable ion-selective electrode apparatus in the solution. Adjust the calibration control until the indicator needle points to the center of the logarithmic concentration scale, allowing sufficient time for equilibration (about 20 min) and stirring constantly during the equilibration period and throughout the remainder of the procedure. Pipet 1.0 ml of a solution containing 100 µg of fluoride (F) ion per ml (prepared by

dissolving 22.2 mg of sodium fluoride, previously dried at 200° for 4 h, in sufficient water to make 100.0 ml) into the beaker, allow the electrode to come to equilibrium, and record the final reading on the logarithmic concentration scale. (**Note:** *Follow the instrument manufacturer's instructions regarding precautions and interferences, electrode filling and check, temperature compensation, and calibration.*)

#### Calculation

Calculate the fluoride content, in mg/kg of the sample taken by the formula

$$[IA/(R - I)] \times 100 \times [200/25W]$$

in which I is the initial scale reading before the addition of the sodium fluoride solution; A is the concentration, in  $\mu\text{g}$  per ml, of fluoride in the sodium fluoride solution added to the sample solution; R is the final scale reading, after addition of the sodium fluoride solution; and W is the original weight of the sample in g.

### Method III - Ion-Selective Electrode Method B

#### Reagents

*Sodium Fluoride Solution (5  $\mu\text{g}$  F per ml):* Transfer 2.210 g of sodium fluoride, previously dried at 200° for 4 h and accurately weighed, into a 400-ml plastic beaker, add 200 ml of water, and stir until dissolved. Quantitatively transfer this solution into a 1,000-ml volumetric flask with the aid of water, dilute to volume with water, and mix. Store this stock solution in a plastic bottle. On the day of use, transfer 5.0 ml of the stock solution into a 1,000-ml volumetric flask, dilute to volume with water, and mix.

*Standard Curve:* Transfer into separate 250-ml plastic beakers 1.0, 2.0, 3.0, 5.0, 10.0, and 15.0 ml of the Sodium Fluoride Solution, add 50 ml of water, 5 ml of 1 N hydrochloric acid, 10 ml of 1 M sodium citrate, and 10 ml of 0.2 M disodium EDTA to each beaker, and mix. Transfer each solution into separate 100-ml volumetric flasks, dilute to volume with water, and mix. Transfer a 50-ml portion of each solution into separate 125-ml plastic beakers, and measure the potential of each solution with a suitable ion-selective electrode, using a suitable reference electrode. Plot the standard curve on two-cycle semi-logarithmic paper with  $\mu\text{g}$  F per 100 ml solution on the logarithmic scale.

#### Procedure

Transfer 1.00 g of the sample into a 150-ml glass beaker, add 10 ml of water, and while stirring continuously, add 20 ml of 1 N hydrochloric acid slowly to dissolve the sample. Boil rapidly for 1 min, then transfer into a 250-ml plastic beaker, and cool rapidly in ice water. Add 15 ml of 1 M sodium citrate and 10 ml of 0.2 M disodium EDTA, and mix. Adjust the pH to  $5.5 \pm 0.1$  with 1 N hydrochloric acid or 1 M sodium hydroxide, if necessary, then transfer into a 100-ml volumetric flask, dilute to volume with water, and mix. Transfer a 50-ml portion of this solution into a 125-ml plastic beaker and measure the potential of the solution with the apparatus described under Standard Curve. Determine the fluoride content, in  $\mu\text{g}$ , of the sample from the Standard Curve.

### Method IV - Ion-Selective Electrode Method C

#### Reagents

*Buffer Solution:* Dissolve 150 g of sodium citrate dehydrate and 10.3 g of disodium EDTA dihydrate in 800 ml of water, adjust the pH to 8.0 with 50% sodium hydroxide solution, and dilute to 1000 ml with water.

#### Fluoride Standard Solutions

- 1000 mg/kg Fluoride Standard: Transfer 2.2108 g of sodium fluoride, previously dried at 200° for 4 h, into a 1000-ml volumetric flask and dissolve in and dilute to volume with water. The resulting solution contains 1000 mg of fluoride per milliliter.
- 50 mg/kg Fluoride Standard: Pipet 50 ml of the 1000 mg/kg Fluoride Standard into a 1000-ml volumetric flask. Dilute to volume with water.
- 10 mg/kg Fluoride Standard: Pipet 100 ml of the 50 mg/kg Fluoride Standard into a 500-ml volumetric flask. Dilute to volume with water.

#### Fluoride Limit Solutions (for a 1-g sample)

- 50 mg/kg Fluoride Limit Solution (1 mg/kg fluoride standard): Pipet 50 ml of the 10 mg/kg Fluoride Standard into a 500-ml volumetric flask, and dilute to volume with water.
- 10 mg/kg Fluoride Limit Solution (0.2 mg/kg fluoride standard): Pipet 10 ml of the 10 mg/kg Fluoride Standard into a 500-ml volumetric flask, and dilute to volume with water.

#### Fluoride Limit Solutions (for a 2-g sample)

- 50 mg/kg Fluoride Limit Solution (2 mg/kg fluoride standard): Pipet 100 ml of the 10 mg/kg Fluoride Standard into a 500-ml volumetric flask, and dilute to volume with water.
- 10 mg/kg Fluoride Limit Solution (0.4 mg/kg fluoride standard): Pipet 20 ml of the 10 mg/kg Fluoride Standard into a 500-ml volumetric flask, and dilute to volume with water.

**Note:** Store all standard and limit solutions in plastic containers.

Sample Preparation: Accurately weigh the amount of sample specified in the monograph, transfer it into a 100-ml volumetric flask, and dissolve it in a minimal amount of water. Add 50.0 ml of the Buffer Solution, dilute to volume with water, and mix.

Electrode Calibration: Pipet 50 ml of the Buffer Solution into a plastic beaker. Place the fluoride ion and reference electrodes (or a combination fluoride electrode) into the plastic beaker and stir. At 5-min intervals, add 100  $\mu$ l and 1000  $\mu$ l of the 1000 mg/kg Fluoride Standard and read the potential, in millivolts, after each addition. The difference between the two readings is the slope of the fluoride electrode and should typically be in the range of 54 to 60 mV at 25°. If the difference in potential is not within this range, check, and, if necessary, replace the electrode, instrument, or solutions.

#### Procedure

Transfer the entire sample into a plastic beaker. Place the electrode into the beaker, allow the solution to equilibrate for 5 min with stirring, and read the potential, in millivolts. Remove and rinse the electrode(s) with water. In another beaker, using a pipet, add 50 ml of the Buffer Solution followed by 50 ml of the Fluoride Limit Solution that best reflects the fluoride limit of the sample. Place the electrode in the beaker, equilibrate for 3 min, and read the potential

in millivolts. If the potential of the Fluoride Limit Solution is less than that of the sample, the sample passes the test criterion for maximum acceptable fluoride level limit.

### **Iron Limit Test**

**Note:** *It is recommended to use an appropriate AAS/ICP method in the place of the limit test, if possible, for quantitative determination of iron.*

To 0.5 g of the sample, weighed to the nearest mg, add 2 ml of hydrochloric acid, and evaporate to dryness on a steam bath. Dissolve the residue in 2 ml of hydrochloric acid and 20 ml of water, and add a few drops of bromine TS. Boil the solution in a fume hood to remove the bromine, cool, dilute with water to 25 ml, and then add 50 mg of ammonium persulfate and 5 ml of ammonium thiocyanate TS. Any red colour produced should not exceed that of a control solution made the same way as the test solution but containing instead of the sample the amount of Iron Standard TS prescribed in the individual monograph.

### **Loss on Drying**

Loss on drying is the amount of volatile matter expelled under the conditions specified in the monograph. Because the volatile matter may include materials other than water, this test is designed for compounds in which the loss on drying may not definitely be attributable to water alone. The water content may be determined by a method such as *Karl Fischer* titration method

**Note:** *Suitable precautionary steps should be taken when weighing hygroscopic or deliquescent samples to ensure that they do not absorb moisture.*

Unless otherwise directed in the individual monograph, conduct the determination on 1 to 2 g of the substance, previously well mixed and accurately weighed. Reduce the sample to a fine powder when it occurs as crystals. Tare a glass-stoppered, shallow weighing bottle that has been dried for 30 min under the same conditions as will be employed in the determination. Transfer the sample into the bottle, replace the cover, and weigh the bottle and the sample. Distribute the sample as evenly as practicable to a depth of about 5 mm, and not over 10 mm in the case of bulky materials. Place the bottle with its contents in the drying chamber, removing the stopper and leaving it also in the chamber, and dry the sample at the temperature and for the time specified in the monograph. Upon opening the chamber, close the bottle promptly and allow it to come to room temperature in a desiccator before weighing.

If the substance melts at a lower temperature than that specified for the determination of *Loss on Drying*, prepare the sample as described above, then place it in a vacuum desiccator containing sulfuric acid. Evacuate the desiccator to 130 Pa (1 mm of mercury), maintain this vacuum for 24 h, and then weigh the dried sample.

### **Loss on Ignition**

Proceed as directed for Loss on Drying. However, unless otherwise directed, ignite the sample at a temperature of 450 - 550° and use a platinum, quartz or porcelain dish instead of the weighing bottle.

## Metallic Impurities

All the procedures for trace metals commence with dissolution of the sample and, if applicable, with destruction of organic matter in the sample. The trace metal content may then be determined by instrumental or chemical methods.

Atomic spectroscopy (atomic absorption as well as atomic emission) combines speed with accuracy and is widely used for the determination of metallic impurities.

Chemical methods depend on the formation of coloured compounds (complexes) with metal impurities under controlled conditions. The colour intensities of sample and standards are then compared visually or by using a spectrophotometer. Some of these methods lack in specificity and are subject to interferences from other trace elements.

### Instrumental Methods

#### *Principle*

The samples are dissolved in acid or digested in a mixture of sulfuric, nitric and, in some cases perchloric acids. Metals (barium, cadmium, lead, copper, chromium, and zinc) in solution are determined by suitable atomic absorption spectrophotometry (AAS) or inductively coupled plasma (ICP) methods. The choice of flame/furnace AAS or ICP methods depend on the concentration of the analyte in the prepared sample solution (its concentration in the sample and limitations associated with the sample preparation). Furnace technique, offers better sensitivity, may be preferred over flame technique, when dealing with low levels of impurities in complex matrices. Antimony and arsenic may be determined by using a hydride generation AAS or ICP. Alternatively, antimony may be determined by flame atomic absorption but the hydride generation technique is more sensitive.

#### *General precautions*

Because of the minute amounts of metals involved special care must be taken to reduce the reagent blanks to as low a value as possible. Contamination in the laboratory is a major concern in trace metal analysis. All apparatus should be thoroughly cleaned with a mixture of hot dilute acids (1 part hydrochloric acid, 1 part concentrated nitric acid, and 3 parts water) followed by thorough washing with water immediately before use. All operations involving acids shall be carried out in the specified fume cupboards. **Note:** *Special care must be taken while using perchloric acid.*

#### *Apparatus*

*Kjeldahl flasks*, of silica or borosilicate glass (nominal capacity 100 ml) fitted with an extension to the neck by means of a B24 ground joint, as shown in Figure 2. The extension serves to condense the fumes and carries a tap funnel through which the reagents are introduced.

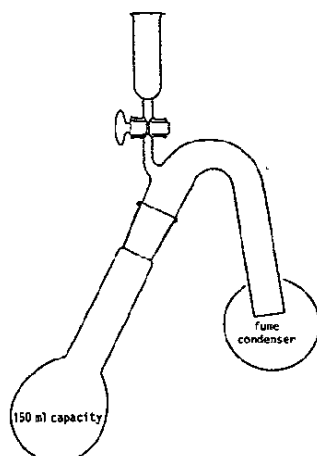


Figure 2. Modified Kjeldahl Flask (open type)

Atomic absorption spectrophotometer: Any commercial instrument operating in the absorption mode may be used providing it has required accessories (furnace and vapour generation) and facilities for the selection of the required oxidant/fuel combination from a choice of air, argon, nitrous oxide, hydrogen and acetylene and has a wavelength range from 180 to 600 nm.

All automated instruments have the facility of instrument control (selection of lamp, pre-warm up of lamp, wavelength and slit width and optimization) data acquisition and processing through a suitable software in a data station. However, with classical instruments, these need to be set manually and for operations in emission mode and measurements of absorption involving the generation of a gaseous hydride, a potentiometric recorder is necessary, preferably a multi-range type covering the range 1-20 mV.

Inductively coupled plasma-atomic emission spectrophotometer: Any commercial instrument, sequential or simultaneous system, operating in axial or radial mode may be used.

#### *Reagents*

Reagents shall be of an order of purity higher than accepted analytical reagent grade quality, preferably atomic spectroscopy grade. Metal-free water (Distilled water may be re-distilled from an all-glass apparatus or may be passed down a column of cation exchange resin, e.g., Amberlite IR 120(H), shall be used throughout. Deionized water (water subjected to reversed osmosis followed by passing ultra high quality deionisers) may also be used.

#### *Standards*

Preparations of atomic absorption standard solutions from pure metals or salts in the laboratory is cumbersome and subject to errors as large numbers of dilutions are involved. Single as well as mixed standard solutions in different concentration ranges are commercially available. Certified standards are also available for reference purpose. It is recommended to use commercially available standard solutions. The analyst must exercise proper care when diluting the stock standard solutions to not exceed a dilution factor of 20 in each step while diluting the concentrated solutions, in order to minimize dilution errors. Dilute the single standard stock solutions with 1% nitric acid to get the following solutions:

- (a) Standard copper solution: 50 µg/ml
- (b) Standard zinc solution: 10 µg/ml
- (c) Standard chromium solution: 50 µg/ml
- (d) Standard antimony solution: 200 µg/ml
- (e) Standard lead solution: 100 µg/ml
- (f) Standard barium solution: 200 µg/ml
- (g) Standard arsenic solution: 5 µg/ml
- (h) Standard cadmium solution: 10 µg/ml

#### *Preparation of test solutions*

Method I is applicable to substances soluble in dilute acids or mixtures of acids. Method II is used for other substances. The choice of method for the pre-treatment of a substance can also follow that given in the individual monograph.

#### *Method I*

Accurately weigh about 2.5 g of the sample and dissolve in a mixture of 4 ml of sulfuric acid and 5 ml of hydrochloric acid. Transfer the solution to a 50-ml volumetric flask. If barium is to be measured from the solution, add 0.0954 g of potassium chloride. Dilute to the mark with water. Mark this as Solution A.

#### *Method II*

**Note:** *Special care shall be taken while handling perchloric acid. All operations shall be carried out in a perchloric acid fume cupboard.*

Accurately weigh a known quantity of sample (about 2.5 g or based on the expected concentration of metal(s) in solution when made up to the volume, such that the concentration in solution will be higher than the first standard of the standard curve) into a 100 – 150-ml Kjeldahl flask, and add 5 ml of the dilute nitric acid. As soon as any initial reaction subsides, heat gently until further vigorous reactions cease and then cool. Add gradually 4 ml of sulfuric acid TS at such a rate as not to cause excessive frothing on heating (5-10 min is usually required) and then heat until the liquid darkens appreciably in colour, i.e., begins to char.

Add concentrated nitric acid slowly in small portions, heating between additions until darkening again takes place. Do not heat so strongly that charring is excessive, or loss of arsenic may occur; a small but not excessive amount of free nitric acid should be present throughout. Continue this treatment until the solution is only pale yellow in colour and fails to darken in colour on prolonged heating. If the solution is still coloured add 0.5 ml of the perchloric acid solution and a little concentrated nitric acid and heat for about 15 min, then add a further 0.5 ml of the perchloric acid solution and heat for a few minutes longer. Note the total amount of concentrated nitric acid used. Allow to cool somewhat and dilute with 10 ml of water. The solution should be quite colourless (if much iron is present it may be faintly yellow). Boil down gently, taking care to avoid bumping, until white fumes appear. Allow to cool, add a further 5 ml of water and again boil down gently to fuming. Finally, cool, add 10 ml 5 N hydrochloric acid and boil gently for a few minutes. Cool and transfer the solution to a 50-ml volumetric flask washing out the Kjeldahl flask with small portions of water. Add the washings to the graduated flask and dilute to the mark with water. If barium is to be measured



from the solution, add 0.0954 g of potassium chloride before dilution, as an ionizing buffer to prevent ionization of barium. Mark this as Solution A.

Prepare a reagent blank using the same quantities of reagents as used in the sample preparation.

***Measurement of Antimony, Barium, Cadmium, Chromium, Copper, Lead and Zinc by Atomic Absorption Flame Technique***

***Preparation of standard curve solutions***

To a series of 100-ml volumetric flasks pipette 0, 1, 2, 3, 4 and 5 ml of the appropriate standard solution [standards (a) to (f) and (h)] and dilute to about 50 ml. Add 8 ml concentrated sulfuric acid and 10 ml concentrated hydrochloric acid. In the case of barium [standard (f)], add 0.191 g of potassium chloride as an ionization buffer and shake to dissolve. Dilute to the mark with metal free water.

These solutions then contain 0, 1.0, 2.0, 3.0, 4.0 and 5.0 µg/ml for lead; 0, 2.0, 4.0, 6.0, 8.0 and 10.0 µg/ml for barium and antimony; , 0, 0.1, 0.2, 0.3, 0.4 and 0.5 µg/ml of cadmium and zinc or 0, 0.50, 1.0, 1.5, 2.0, 2.5 µg/ml for copper and chromium.

***Instrumental conditions***

Select the wavelengths and gases to be used for the particular element under consideration from the table below.

<b>Element</b>	<b>Wave length (nm)</b>	<b>Gases</b>
Antimony	217.6	Air/acetylene
Barium	553.6	Nitrous oxide/acetylene
Cadmium	228.8	Air/acetylene
Chromium	357.9	Nitrous oxide/acetylene
Copper	324.8	Air/acetylene
Lead	283.3	Air/acetylene
Zinc	213.9	Air/acetylene

The recommended settings for the various instrumental parameters vary from model to model, and certain parameters require optimization at the time of use to obtain the best results. Instruments should therefore be adjusted as described in the manufacturer's instructions using the type of flame and wavelength settings specified above.

***Procedure***

Set the atomic absorption spectrophotometer to the appropriate conditions. Aspirate the strongest standard containing the element to be determined and optimize the instrument settings to give full-scale or maximum deflection on the chart recorder. Measure the absorbances of the other standards and plot a graph showing the net absorbance against the concentration of the element in the standard solutions. Aspirate the solution A obtained from dissolution or the wet oxidation of the sample and the corresponding blank solution and

determine the net absorbance. If the concentration of the element in the solution is beyond the standard curve, dilute the solution as required and read the absorbance again. Using the graph prepared above, determine the concentration of the element in the sample solution.

#### Calculation

$$\text{Element in the sample [mg/kg]} = \frac{[\text{Concentration of element } (\mu\text{g/ml}) \times 50]}{[\text{Weight of sample taken (g)}]}$$

For certain elements flame atomic absorption method will not reach the required determination limits (e.g. a monograph specification limit for Pb of 1.0 mg/kg: A maximum of 5.0 grams of sample digested and made up to 50 ml will give a concentration of 0.1  $\mu\text{g/ml}$  in solution which cannot be read by flame technique). The analyst may choose to use an electro-thermal atomization method under such circumstances.

### ***Measurement of Antimony, Barium, Cadmium, Chromium, Copper, Lead and Zinc by Inductively Coupled Plasma (ICP) Technique***

#### Preparation of standard curve solutions

The standard curve solutions given below are nominal in nature. The concentration of standard curve solutions differ based upon the operation mode of the torch (axial or radial) of the ICP instrument. The analyst may alternatively prepare appropriate standard curve solutions following the instrument operation manual.

To a series of 100-ml volumetric flasks pipette 0, 1, 2, 3, 4 and 5 ml of the appropriate standard solution [standards (a) to (h)] and dilute to about 50 ml. Add 8 ml concentrated sulfuric acid and 10 ml concentrated hydrochloric acid. Dilute to the mark with metal free water. These solutions then contain 0, 1.0, 2.0, 3.0, 4.0 and 5.0  $\mu\text{g/ml}$  for lead; 0, 2.0, 4.0, 6.0, 8.0 and 10.0  $\mu\text{g/ml}$  for barium and antimony; , 0, 0.1, 0.2, 0.3, 0.4 and 0.5  $\mu\text{g/ml}$  of cadmium and zinc or 0, 0.50, 1.0, 1.5, 2.0, 2.5  $\mu\text{g/ml}$  for copper and chromium.

#### Instrumental conditions

Select appropriate emission wavelengths to be used with each element under consideration. The recommended settings for the various instrumental parameters vary from model to model, and certain parameters require optimization at the time of use to obtain the best results. Instruments should therefore be adjusted as described in the manufacturer's instructions.

#### Procedure

Set the ICP instrument as stated in the operation manual. Activate the method and key in the standards data into the data station of the ICP. Aspirate the blank solution and set the instrument to zero, aspirate the standards and determine a standard curve for each element with emission intensity plotted against the concentration of the element in the standard solutions. Aspirate the solution A obtained from dissolution or the wet oxidation of the sample. If the concentration of the element in the solution is beyond the standard curve, dilute the solution as required and read it again. Using the standard curve, determine the element in the sample.

#### Calculation

$$\text{Element in the sample [mg/kg]} = \frac{[\text{Concentration of element } (\mu\text{g/ml}) \times 50]}{[\text{Weight of sample taken (g)}]}$$

### ***Measurement of Lead and Cadmium by Atomic Absorption Electro-thermal Atomization (Furnace Atomization) Technique***

#### ***Chemical modifier solutions:***

Use of chemical modifier solutions in the furnace atomization allows use of higher ashing temperatures to reduce the background absorbance. These solutions must be of very high purity and are available commercially. One or more of the following modifiers may be used for the determination of lead and cadmium in different food additives.

- Palladium solution: 1000- 2000 µg/l
- Ascorbic acid: 5000 µg/l
- Monobasic ammonium phosphate: 5000 µg/l
- Orthophosphoric acid: 1000 µg/l

#### ***Preparation of standard curve solutions***

In a 100-ml volumetric flask, pipette 25 ml of lead and 10 ml cadmium standards (e and h) and dilute to the mark with water (standard solution A, 1 ml = 25 µg of pb and 1.0 µg of cd). Dilute 10 ml of A to 100 ml with water (standard solution B, 1 ml = 2.5 µg of pb and 0.1 µg Cd). Dilute 10 ml of B to 100 ml with water (standard solution C, 1 ml = 250 ng of pb and 10 ng Cd). Dilute 10 ml of C to 100 ml with water (working standard solution D, 1 ml = 25 ng of pb and 1 ng Cd).

#### ***Instrumental conditions***

General instrumental conditions are provided in the table below. The recommended settings for the various instrumental parameters vary from model to model, and certain parameters require optimization at the time of use to obtain the best results. Instruments should therefore be adjusted as described in the manufacturer's instructions.

Element	Wave length (nm)	Slit (nm)	Gases	Maximum Ashing Temperature		Atomization Temperature
				Without modifier	With modifier	
Cadmium	228.8	0.5	Argon	300	Argon	1800
Lead	283.3	0.5	Argon	400	Argon	2100

#### ***Procedure***

Place blank (1% nitric acid), working standard solution (solution D), a suitable modifier solution (if required) and sample solutions in the appropriate locations provided in the furnace auto sampler. Set up the furnace parameters following the instruction provided by the manufacturer to carry out triplicate injections. Clean the graphite tube and inject blank. Program the auto sampler to inject 5, 10, 15, 20 µl of standard (5 µl of modifier and remaining blank solution so as the total volume is 25 µl). Construct the standard curve from

the absorbance either from peak area or height. Inject 10 µl of sample solution and calculate the concentration in the samples as follows:

Injection volume of sample to furnace :	10 µl
Volume made up :	50 ml
Instrument reading (ng) :	R
Weight of sample, g :	W

$$\text{Concentration in sample (mg/kg)} = (R \times 5)/W$$

### ***Measurement of Arsenic and Antimony by Atomic Absorption Hydride Technique***

Arsenic and antimony are determined after preparation of their volatile hydrides which are collected either in the generation vessel itself or, in some designs, in a rubber balloon attached to the vessel. The gases are then expelled with Argon into a hydrogen flame.

#### Preparation of standard curve solution

Into a series of 100-ml volumetric flasks add from a burette, 0, 1, 2, 3, 4 and 5 ml of standard arsenic or antimony solution [Standards (g) and (d)] and dilute to about 50 ml with distilled water. Add 8 ml 98% sulfuric acid TS and 10 ml hydrochloric acid [1.18 specific gravity]. Shake to dissolve, and when solution is complete, dilute to the mark with distilled water.

#### Instrumental conditions

Using the atomic absorption spectrophotometer with the appropriate hollow cathode or electrode-less discharge lamp, select the wavelength for either arsenic (193.7 nm) or antimony (217.6 nm).

#### Procedure

Measure 5.0 ml of the strongest standard into the generation vessel, add 25 ml of water and 2 ml 5 N hydrochloric acid. Stopper the vessel and expel any air as described in the maker's instructions, filling the apparatus with Argon. Isolate the vessel from the atomizer using the by-pass valve. Remove the atomizer and then quickly add 1 pellet (about 0.2 g) of sodium borohydride and replace the stopper. Ensure that all the joints are secure.

When the reaction slows (20 - 30 sec) open the appropriate taps to allow the Argon to drive the generated hydride into the flame. When the hydride has all been expelled as shown by the recorder trace, return the taps to their original position and empty the vessel.

Optimize the instrument settings to give full scale deflection for the strongest standard. Measure the other standards, the sample and the blank solution using the same procedure.

Plot a graph relating peak height on the recorder to concentration of the arsenic or antimony in the standards. Using the net absorbance of the sample, read the concentration of arsenic or antimony in the solution from the graph .

#### Calculation

$$\text{Arsenic or antimony in the sample (mg/kg)} = \frac{[\text{Concentration of arsenic or antimony } (\mu\text{g/ml)} \times 50]}{[\text{Weight of sample taken (g)}]}$$

### ***Determination of Mercury by Cold Vapour Atomic Absorption Technique***

#### Principle

The sample is digested under closed conditions by heating under reflux with sulfuric and nitric acids. The oxidation is completed by addition of potassium permanganate solution. After

successive additions of hydroxylamine hydrochloride solution and stannous chloride solution, the mercury content is measured by cold vapour atomic absorption spectrometry. Alternatively, closed vessel microwave digestion system may be used for the digestion of samples.

#### Standards

Dilute commercially available mercury standard solution (e.g. 10 µg/ml) following a serial dilution technique (dilution factor in each dilution not to exceed 20) to get 0.02 µg Hg/ml

#### Apparatus

All the glassware must be cleaned with nitric acid (10% v/v) and washed thoroughly with water before use.

Mineralization apparatus fitted with reflux condenser (see figure 3).

Bubblers, with a ground glass stopper fitted with two tubes to permit entrainment of the mercury vapour and with a calibration mark at the required volume for measurement. The capacity of the bubbler and position of the mark depend on the atomic absorption spectrophotometer used. Clean the bubbler successively with chromic acid mixture (dissolve 4.0 g of potassium dichromate in 300 ml of 3.5 M sulfuric acid and make up to 1 litre with water), tap water and double distilled water before use.

(Alternatively, use the vapour generation accessory and follow operation instructions for its use)

Water vapour absorption apparatus, containing magnesium perchlorate.

Atomic absorption spectrophotometer suitable for the cold vapour determination of mercury in open or closed circuit, with a data station or recorder.



Figure 3. Mineralization Apparatus

### Procedure

#### Digestion of sample

Weigh out, to the nearest 2 mg, approximately 0.5 g sample containing not more than 0.5 µg total mercury. Introduce the sample into the receiver flask (M), and add a few glass beads. Connect the receiver flask to the condensate reservoir (D) and close the stopcock (R).

Introduce into the reservoir 25 ml of nitric acid (sp.gr. 1.40) followed by 10 ml sulfuric acid (sp.gr. 1.84). Mount and turn on the condenser (A). Open the stopcock carefully and allow small portions of the mixture of acids to run into the receiver flask. Interrupt the flow of acids if the reaction becomes too vigorous.

Empty the reservoir into the receiver flask, mix the contents of the latter well by careful shaking and leave the stopcock open.

Heat the receiver flask carefully. As soon as foaming has ceased, close the stopcock (R), continue heating and let the condensate collect in the reservoir.

Discontinue heating when the contents of the receiver flask begin to char. Allow a small portion of the condensate to run into the receiver flask, close the stopcock again and resume heating the receiver flask. Repeat this procedure for as long as the contents display charring when heated.

When charring has ceased, heat and add condensate as soon as white fumes appear. Continue alternately heating and adding condensate for one hour. Finally, heat the contents of the flask to white fumes.

Stop heating and allow to cool to approximately 40°. Open the stopcock and allow all the condensate to run into the receiver flask. Wash the apparatus out from the top of the condenser with 5 - 10 ml of water, collect the washings in the receiver flask and disconnect it from the reservoir.

#### Treatment of the Solution

Introduce the potassium permanganate solution (50.0 g/l) dropwise into the receiver flask, with agitation, until a pink colouration persists. Note the volume of permanganate solution used. (If this quantity exceeds 10 ml, repeat the procedure "Ashing" as above.)

Heat gently to boiling, then allow to cool.

Pour the contents of the receiver flask into a bubbler, wash the receiver flask with water and add the washings to the contents of the bubbler.

Measure the mercury content (see below) the same day as the treatment of the solution.

#### Measurement of Mercury Content

Introduce 5 ml of hydroxylamine hydrochloride (100 g/l) into the bubbler and make up to the mark either with double distilled water or with sulfuric acid (3.5 M solution) in the case of standard solutions. Add 5 ml of stannous chloride solution [prepare by dissolving 25.0 g of stannous chloride (SnCl<sub>2</sub>·2H<sub>2</sub>O) in 50 ml hydrochloric acid (sp. gr. 1.18), make up to 250 ml with water and bubble nitrogen through the solution. Store over a few granules of metallic tin], assemble the bubbler, connect it to the water vapour absorption apparatus and to the atomic absorption spectrophotometer. Set the latter in operation.

Mix the contents of the bubbler well by gentle shaking, pass air or nitrogen through, measure and record. Carry out measurements as quickly as possible after the addition of stannous chloride. If an open-circuit system is used, wait 30 sec before passing air or nitrogen.

#### Standard Curve

Introduce respectively 2-, 5-, 10-, 15- and 25-ml aliquots of the standard mercury solution (0.02 µg Hg/ml) into bubblers and 25 ml sulfuric acid (3.5 M) into a sixth bubbler. Add potassium permanganate solution dropwise, with agitation, to each bubbler until a colouration persists.

Measure the mercury content as described above.

Plot the standard curve with the measured absorption values as ordinates and the corresponding mercury contents in micrograms as abscissae. The working standards contain 0, 0.04, 0.10, 0.20, 0.30 and 0.50 µg of mercury, respectively.

#### Method of Addition

The method of addition may be used if an open-circuit system is used.

Place one of the working standard solutions in a bubbler and add an aliquot portion of the sample solution obtained after treatment. The quantity of mercury in the bubbler must lie in the range in which the photometer gives a linear response. Measure the mercury content as described above. If necessary, carry out several such determinations, using different working standard solutions.

#### Blank Determination

Carry out all the operations, from ashing to measurement, except for introduction of the sample. When treating the solution, add a quantity of potassium permanganate solution equal to that used for the experimental sample.

#### Calculation

Read off from the standard curve the quantities, in µg, of mercury corresponding to the measured absorption values.

Subtract the quantity of mercury found in the blank from that found in the sample.

$$\text{Net weight of mercury (}\mu\text{g)} / \text{sample weight (g)} = \text{mg/kg Hg in the sample}$$

### **Nickel in Polyols**

**Note:** *This method is also applicable for determination of nickel in polydextroses.*

#### Reagents

*Test solution:* Dissolve 20.0 g of the sample in a mixture of equal volumes of dilute acetic acid TS and water and dilute to 100 ml with the same mixture of solvents. Add 2.0 ml of a 1% w/v solution of ammonium pyrrolidinedithiocarbamate and 10 ml of methyl isobutyl ketone. Mix and allow the layers to separate and use the methyl isobutyl ketone layer for analysis.

*Standard solutions:* Prepare three standard solutions in the same manner as the test solution but adding 0.5 ml, 1.0 ml, and 1.5 ml, respectively, of a standard nickel solution containing 10 mg/kg Ni, in addition to the 20.0 g of the sample.

### Procedure

Set the instrument to zero using methyl isobutyl ketone prepared as described for the preparation of the test solution but omitting the substance to be examined. Use a nickel hollow-cathode lamp as source of radiation and an air-acetylene flame. The analysis wavelength for all solutions is 232.0 nm.

### **Nickel Limit Test**

**Note:** *It is recommended to use an appropriate AAS/ICP method in the place of the limit test, if possible, for quantitative determination of nickel.*

Dissolve 10 g of sample in sufficient water to produce 20 ml, add 3 ml bromine TS and 2 ml of a 20% w/v solution of citric acid, mix and add 10 ml of ammonia TS and 1 ml of dimethylglyoxime TS. Mix, dilute to 50 ml with water and allow to stand for 5 min; any colour produced is not more intense than that produced by similarly treating, at the same time, 1 ml of nickel standard solution [10 mg/kg Ni prepared by diluting 1.0 ml of a 0.401% w/v solution of nickel chloride ( $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$  analytical reagent grade) with water to 100.0 ml] diluted to 20 ml with water (0.5 mg/kg Ni).

### **Nitrogen Determination (Kjeldahl Method)**

**Caution:** *Provide adequate ventilation in the laboratory and do not permit accumulation of exposed mercury.*

**Note 1:** *The analyst may use commercially available automated Kjeldahl digestion and determination equipment for the determination of Kjeldahl nitrogen.*

**Note 2:** *All reagents should be nitrogen-free, where available, or otherwise very low in nitrogen content.*

### Method I

This method should be used unless otherwise directed in the individual monograph. It is not applicable for certain nitrogen-containing compounds that do not yield their entire nitrogen content upon digestion with sulfuric acid.

#### *Nitrites and Nitrates Absent*

Unless otherwise directed, transfer about 1 g of the substance, accurately weighed, to a 500-ml Kjeldahl flask of hard glass, wrapping the sample, if solid or semi-solid, in nitrogen-free filter paper to facilitate the transfer if desired. To the flask add 10 g of powdered potassium sulfate or anhydrous sodium sulfate, 500 mg of powdered cupric sulfate, and 20 ml of sulfuric acid. Gently heat the mixture, keeping the flask inclined at about a 45° angle, and after frothing has ceased, boil briskly until the solution has remained clear green in colour or almost colourless for 30 min. Cool, add 150 ml of water, mix, and cool again. Cautiously pour 100 ml of sodium hydroxide solution (2 in 5) down the inside of the flask so that it forms a layer under the acid solution, then add a few pieces of granulated zinc. Connect the flask to a distillation apparatus consisting of a Kjeldahl connecting bulb and a condenser, the delivery tube from which extends well beneath the surface of 50 ml of boric acid solution (1 in 25) contained in a 500-ml flask or bottle. Gently rotate the contents of the Kjeldahl flask to mix, and distil until about two-thirds of the solution has been collected in the receiving flask. To the receiving flask add methyl red/methylene blue TS, and titrate with 0.5 N sulfuric acid. Perform a blank determination substituting 2 g of sucrose for the sample and make the necessary corrections. Each ml of 0.5 N acid is equivalent to 7.003 mg of nitrogen.



**Note:** *If it is known that the substance to be determined has low nitrogen content, 0.1 N acid may be used in place of the 0.5 N solution, in which case each ml of 0.1 N acid is equivalent to 1.401 mg of nitrogen.*

#### *Nitrites and Nitrates Present*

Transfer to a 500-ml Kjeldahl flask of hard glass a quantity of the sample, accurately weighed, representing about 150 mg of nitrogen, add 25 ml of sulfuric acid in which 1 g of salicylic acid has been dissolved, mix, and allow to stand for 30 min, shaking frequently. Add 5 g of powdered sodium thiosulfate, mix, then add 500 mg of powdered cupric sulfate or mercuric oxide, and continue as directed as above, beginning with "Gently heat the mixture...". Prior to the digestion of substances known to have a nitrogen content exceeding 10%, add 500 mg to 1 g of benzoic acid to facilitate decomposition.

#### Method II (Semi-micro)

Transfer an accurately weighed or measured quantity of the sample equivalent to about 2 or 3 mg of nitrogen, to the digestion flask of a semi-micro Kjeldahl apparatus. Add 1 g of a powdered mixture of potassium sulfate and cupric sulfate (10 to 1), using a fine jet of water to wash down any material adhering to the neck of the flask, then pour 7 ml of sulfuric acid down the inside wall of the flask to rinse it. Add cautiously, down the inside of the flask, 1 ml of 30% hydrogen peroxide, swirling the flask during the addition (**Caution:** *Do not add any peroxide during the digestion.*)

Heat over a free flame or an electric heater until the solution has attained a clear blue colour and the walls of the flask are free from carbonized material. Cautiously add 20 ml of water, cool, then add through a funnel 30 ml of sodium hydroxide solution (2 in 5), and rinse the funnel with 10 ml of water. Connect the flask to a steam distillation apparatus and immediately begin the distillation with steam. Collect the distillate in 15 ml of boric acid solution (1 in 25) to which has been added 3 drops of methyl red/methylene blue TS and enough water to cover the end of the condensing tube. Continue passing the steam until 80 to 100 ml of distillate has been collected, then remove the absorption flask, rinse the end of the condenser tube with a small quantity of water, and titrate with 0.01 N sulfuric acid. Each ml of 0.01 N acid is equivalent to 0.140 mg (140 µg) of nitrogen.

When more than 2 to 3 mg of nitrogen is present in the measured quantity of the substance to be determined, 0.02 or 0.1 N sulfuric acid may be used in the titration if at least 15 ml of titrant is required. If the total dry weight of the material taken is greater than 100 mg, increase proportionately the quantities of sulfuric acid and sodium hydroxide added before distillation.

### **Non-Volatile Residue**

Unless otherwise indicated, transfer 100 ml of the sample into a tared 125-ml platinum evaporating dish, previously heated at 105° to constant weight, and evaporate the sample to dryness on a steam bath. Heat the dish at 105° for 30 min or to constant weight, cool in a desiccator, and weigh.

### **Phosphate Determination as P<sub>2</sub>O<sub>5</sub>**

#### Method I

Weigh accurately about 200 - 300 mg of sample, dissolve in 25 ml of water and 10 ml of diluted nitric acid TS and boil for 30 min. Filter if necessary, and wash any precipitate, then dissolve the precipitate by the addition of 1 ml diluted nitric acid TS. Adjust the temperature to about 50°, add 75 ml of ammonium molybdate TS, and maintain the temperature at about

50° for 30 min, stirring occasionally. Allow to stand for 16 h or overnight at room temperature. Decant the supernatant, through a filter paper, wash the precipitate once or twice with water by decantation using 30 to 40 ml each time, and pour the washings through the same filter. Transfer the precipitate to the same filter, and wash with potassium nitrate solution (1 in 100) until the filtrate is no longer acid to litmus paper. Transfer the precipitate with filter paper to the original precipitation vessel, add 50.0 ml of 1 N sodium hydroxide, agitate and stir until the precipitate is dissolved, add 3 drops of phenolphthalein TS and titrate the excess alkali with 1 N sulfuric acid. Each ml of 1 N sodium hydroxide consumed is equivalent to 3.088 mg of P<sub>2</sub>O<sub>5</sub>.

#### Method II

Weigh accurately Transfer about 1.5 g of the sample, transfer into accurately weighed, into a 500-ml volumetric flask beaker, add 100 ml of water and 25 ml of nitric acid, and boil for 10 min on a hot plate. Cool, quantitatively transfer into a 500- ml volumetric flask, dilute to volume with water and mix. Pipet 20.0 ml of this solution into a 500- ml Erlenmeyer flask, add 100 ml of water and heat just to boiling. Add with stirring 50 ml of quimociac TS, then cover with a watch glass and boil for 1 min in a well ventilated hood. Cool to room temperature, swirling occasionally while cooling, then filter through a tared crucible (or fritted glass crucible of medium porosity), and wash with five 25-ml portions of water. Dry at about 225° for 30 min, cool and weigh. Each mg of precipitate thus obtained is equivalent to 32.074 µg of P<sub>2</sub>O<sub>5</sub>.

### **Selenium Limit Test**

#### Reagents

*2,3-Diaminonaphthalene Solution:* On the day of use, dissolve 100 mg of 2,3-diaminonaphthalene (C<sub>10</sub>H<sub>10</sub>N<sub>2</sub>) and 500 mg of hydroxylamine hydrochloride (NH<sub>2</sub>OH·HCl) in sufficient 0.1 N hydrochloric acid to make 100 ml.

*Selenium Standard Solution:* Transfer 120.0 mg of powdered metallic selenium into a 1,000-ml volumetric flask, and dissolve in 100 ml of dilute nitric acid (1 in 2), warming gently on a steam bath to effect solution. Cool, dilute to volume with water, and mix. Transfer 5.0 ml of this solution into a 200-ml volumetric flask, dilute to volume with water, and mix. Each ml of this solution contains 3 µg of selenium (Se).

Alternatively, commercially available selenium standard solution may suitably be diluted to obtain a 3 µg/ml solution.

**Note:** *Method I referenced in older specifications is deleted – The colourimetric procedure described in Method II may be used, but it is recommended that, whenever possible, the determination of selenium is carried out using atomic absorption methods.*

#### Method II

*Preparation of Standard:* Transfer 2.0 ml of the Selenium standard solution into a 150-ml beaker, add 50 ml of 2 N hydrochloric acid, and mix.

*Sample Preparation:* Transfer into a 150-ml beaker the amount of sample specified in the individual monograph, dissolve in 25 ml of 4 N hydrochloric acid, swirling if necessary to effect solution, heat gently to boiling, and digest on a steam bath for 15 min. Remove from heat, add 25 ml of water, and allow to cool to room temperature.

### *Procedure*

Place the beakers containing the standard preparation and the sample preparation in a fume hood. Cautiously add 5 ml of ammonium hydroxide to each beaker and to a third beaker containing 50 ml of 2 N hydrochloric acid to serve as the blank. Allow the solutions to cool, and then adjust the pH of each solution to  $2.0 \pm 0.2$  with dilute ammonium hydroxide (1 in 2).

Add 200 mg of hydroxylamine hydrochloride to each beaker, swirl gently to dissolve, then without delay add 5 ml of 2,3-diaminonaphthalene solution to each solution, and mix. Cover each beaker with a watch glass, and allow to stand at room temperature for 100 min. Transfer the solutions into separate separators with the aid of about 10 ml of water, extract each solution with 5.0 ml of cyclohexane, shaking each separator vigorously for 2 min, and allow the layers to separate. Discard the aqueous phases, and centrifuge the cyclohexane extracts to remove any traces of water. Determine the absorbance of each extract in a 1-cm cell at the maximum at about 380 nm, with a suitable spectrophotometer, using the blank to set the instrument. The absorbance of the extract from the sample preparation is not greater than that from the standard solution when a 200-mg sample is tested, or not greater than one-half the absorbance of the extract from the standard solution when a 100-mg sample is tested.

### **Sulfates Limit Test**

Unless otherwise specified, place the prescribed quantity of the sample in a Nessler tube, dissolve it in about 30 ml of water, and neutralize with dilute hydrochloric acid TS if the solution is alkaline. Add 1 ml of dilute hydrochloric acid TS and dilute to 50 ml with water. If the use of a sample solution is prescribed, transfer the sample solution into a Nessler tube and dilute to 50 ml with water. Transfer the prescribed volume of 0.01 N sulfuric acid into another Nessler tube to serve as the standard, add 1 ml of dilute hydrochloric acid TS, and dilute to 50 ml with water.

If the solution containing the sample is not clear, filter both solutions under the same conditions. Add 2 ml of barium chloride TS to each solution, mix thoroughly, and allow to stand for 10 min. Compare the turbidity of the two solutions by observing the Nessler tubes from the sides and the tops against a black background. The turbidity of the sample does not exceed that of the standard.

### **Water-Insoluble Matter**

Treat 10 g of sample, accurately weighed, with 100 ml of hot water and filter through a tared filtering crucible. Wash the insoluble residue with hot water, dry at  $105^\circ$  for 2 h, cool and weigh.

### **Water Determination (Karl Fischer Titrimetric Method)**

**Note:** Determine the water content by the method below, unless otherwise specified in the individual monograph.

#### Principle

The Karl Fischer titrimetric determination of water is based on the quantitative reaction of water with an anhydrous solution of sulfur dioxide and iodine in the presence of a buffer that reacts with hydrogen ions. In the original titrimetric solution, known as Karl Fischer Reagent, the sulfur dioxide and iodine are dissolved in pyridine and methanol. Pyridine-free reagents are more commonly used now and are to be preferred in order to avoid the use of pyridine (a hazardous reagent).

The test specimen is titrated with the Karl Fischer Reagent directly. The stoichiometry of the reaction is not exact, and the reproducibility of the determination depends on such factors as the relative concentrations of the Karl Fischer Reagent ingredients, the nature of the inert solvent used to dissolve the test specimen, the apparent pH of the final mixture, and the technique used in the particular determination. Therefore, an empirically standardized technique is used to achieve the desired accuracy. Precision in the method is governed largely by the extent to which atmospheric moisture is excluded from the system.

The titration of water is usually carried out with the use of anhydrous methanol as the solvent for the test specimen; however, other suitable solvents may be used for special or unusual test specimens. Substances that may interfere with the test results are ferric ion, chlorine, and similar oxidizing agents, as well as significant amounts of strong acids or bases, phosgene, or anything that will reduce iodide to iodine, poison the reagent, and show the sample to be bone dry when water may be present (false negative). 8-Hydroxyquinoline may be added to the vessel to eliminate interference from ferric ion. Chlorine interference can be eliminated with SO<sub>2</sub> or unsaturated hydrocarbon. Excess pyridine or other amines may be added to the vessel to eliminate the interference of strong acids. Excess acetic acid or other carboxylic acids can be added to reduce the interference of strong bases. Aldehydes and ketones may react with the solution, showing the sample to be wet while the detector never reaches an endpoint (false positive).

#### Apparatus

Any apparatus may be used that provides for adequate exclusion of atmospheric moisture and for determination of the endpoint. In the case of a colorless solution that is titrated directly, the endpoint may be observed visually as a change in color from canary yellow to amber. More commonly, however, the endpoint is determined electrometrically with an apparatus employing a simple electrical circuit that serves to impress about 200 mV of applied potential between a pair of platinum electrodes (about 5 mm<sup>2</sup> in area and about 2.5 cm apart) immersed in the solution to be titrated. At the endpoint of the titration, a slight excess of the reagent increases the flow of current to between 50 and 150 microamperes for 30 s to 30 min, depending on the solution being titrated. The time is shortest for substances that dissolve in the Reagent. The longer times are required for solid materials that do not readily go into solution in the Karl Fischer Reagent. With some automatic titrators, the abrupt change in current or potential at the endpoint serves to close a solenoid-operated valve that controls the burette delivering the titrant. A commercially available apparatus generally comprises a closed system consisting of one or two automatic burettes and a tightly covered titration vessel fitted with the necessary electrodes and a magnetic stirrer. The air in the system is kept dry with a suitable desiccant such as phosphorus pentoxide, and the titration vessel may be purged by means of a stream of dry nitrogen or a current of dry air.

#### Karl-Fischer Reagent

A commercially available, stabilized solution of a Karl Fischer-type reagent may be used. Commercially available reagents containing solvents or bases other than pyridine and/or alcohols other than methanol also may be used. These may be single solutions or reagents formed in situ by combining the components of the reagents present in two discrete solutions. The diluted Karl Fischer Reagent called for in some monographs should be diluted as directed by the manufacturer. Either methanol, or another suitable solvent such as ethylene glycol monomethyl ether, may be used as the diluent.

**Note:** *If commercial solution is not available, prepare Karl Fischer Reagent as follows:*

**Caution:** *Pyridine is hazardous in nature. The analyst shall take proper care and all operations shall be carried out in fume cupboard.*

Add 125 g of iodine to a solution containing 670 ml of methanol and 170 ml of pyridine, and cool. Place 100 ml of pyridine in a 250-ml graduated cylinder, and keeping the pyridine cold in an ice bath, pass in dry sulfur dioxide until the volume reaches 200 ml. Slowly add this solution, with shaking, to the cooled iodine mixture. Shake to dissolve the iodine, transfer the solution to the apparatus, and allow the solution to stand overnight before standardizing. One milliliter of this solution, when freshly prepared, is equivalent to approximately 5 mg of water, but it deteriorates gradually; therefore, standardize it within 1 h before use, or daily in continual use. Protect the solution from light while in use. Store any bulk stock of the solution in a suitably sealed, glass-stoppered container, fully protected from light and under refrigeration.

#### Test Preparation

Unless otherwise specified in the individual monograph, use an accurately weighed or measured amount of the specimen under test estimated to contain 10 to 250 mg of water. Where the monograph specifies that the specimen under test is hygroscopic, accurately weigh a sample of the specimen into a suitable container. Use a dry syringe to inject an appropriate volume of methanol, or other suitable solvent, accurately measured, into the container and shake to dissolve the specimen. Dry the syringe, and use it to remove the solution from the container and transfer it to a titration vessel prepared as directed under Procedure. Repeat the procedure with a second portion of methanol, or other suitable solvent, accurately measured; add this washing to the titration vessel; and immediately titrate. Determine the water content, in milligrams, of a portion of solvent of the same total volume as that used to dissolve the specimen and to wash the container and syringe, and subtract this value from the water content, in milligrams, obtained in the titration of the specimen under test.

#### Standardization of the Reagent

Place enough methanol or other suitable solvent in the titration vessel to cover the electrodes, and add sufficient Karl Fischer Reagent to give the characteristic color or  $100 \pm 50$  microamperes of direct current at about 200 mV of applied potential. Pure methanol can make the detector overly sensitive, particularly at low mg/kg levels of water, causing it to deflect to dryness and slowly recover with each addition of reagent. This slows down the titration and may allow the system to actually pick up ambient moisture during the resulting long titration. Adding chloroform or a similar non-conducting solvent will retard this sensitivity and can improve the analysis. For determination of trace amounts of water (less than 1%), quickly add 25  $\mu$ l (25 mg) of pure water, using a 25- or 50- $\mu$ l syringe, and titrate to the endpoint.

The water equivalence factor  $F$ , in milligrams of water per milliliter of reagent, is given by the formula  $25/V$ , in which  $V$  is the volume, in milliliters, of the Karl Fischer Reagent consumed in the second titration. For the precise determination of significant amounts of water (more than 1%), quickly add between 25 and 250 mg (25 to 250  $\mu$ l) of pure water, accurately weighed by difference from a weighing pipet or from a precalibrated syringe or micropipet, the amount of water used being governed by the reagent strength and the burette size, as referred to under Volumetric Apparatus. Titrate to the endpoint.

Calculate the water equivalence factor,  $F$ , in milligrams of water per milliliter of reagent by the formula  $W/V$ , in which  $W$  is the weight, in milligrams, of the water, and  $V$  is the volume, in milliliters, of the Karl Fischer Reagent required.

Procedure

Unless otherwise specified, transfer 35 to 40 ml of methanol or other suitable solvent to the titration vessel, and titrate with the Karl Fischer Reagent to the electrometric or visual endpoint to consume any moisture that may be present. (Disregard the volume consumed because it does not enter into the calculations.) Quickly add the test preparation, mix, and again titrate with the Karl Fischer Reagent to the electrometric or visual endpoint.

Calculate the water content of the specimen, in milligrams, by the formula  $S \times F$ , in which S is the volume, in milliliters, of the Karl Fischer Reagent consumed in the second titration, and F is the water equivalence factor of the Karl Fischer Reagent.

## ORGANIC COMPONENTS

### Chlorinated Organic Compounds Limit Test

Weigh 0.25 g of the sample to the nearest mg, and dissolve in 10 ml of water. Acidify with nitric acid and filter off the precipitate. Mix the precipitate with 0.5 g of calcium carbonate, dry the mixture and then ignite. Take up the ignition residue in 20 ml of dilute nitric acid TS and filter. Mix the filtrate with 0.5 ml of 0.1 N silver nitrate. The turbidity should not be greater than that obtained by adding 0.5 ml of 0.1 N silver nitrate to a similar volume of dilute nitric acid TS containing the amount of 0.01 N hydrochloric acid prescribed in the individual monograph.

### Cyclohexylamine in Cyclamates

#### Reagents

*Methyl orange-boric acid solution:* Dissolve 200 mg of methyl orange and 3.5 g of boric acid in 100 ml of water, heating on a steam bath to effect solution. Allow to stand for at least 24 h, and filter before use.

*Standard solution:* Weigh accurately about 100 mg of cyclohexylamine in a 100-ml volumetric flask, dissolve in 50 ml of water and 0.5 ml of hydrochloric acid TS, dilute to volume with water, and mix. Transfer 5 ml of the solution into a second 100-ml volumetric flask, dilute to volume with water, and mix. Transfer 5 ml of the solution into a third 100-ml volumetric flask, dilute to volume with water, and mix. Each ml of this solution contains 2.5 µg of cyclohexylamine.

*Test preparation:* Prepare the test preparation as directed in the individual monograph.

#### Procedure

Transfer 10 ml each of the Standard solution and of the Test preparation into two separate 50-ml glass-stoppered centrifuge tubes, and transfer 10 ml of water to a third tube to serve as a blank. To each tube add 3.0 ml of disodium ethylenediaminetetraacetate solution (prepared by dissolving 10 g of disodium ethylenediaminetetraacetate and 3.4 g of sodium hydroxide in 100 ml of water) and 15 ml of a 20:1 mixture of chloroform and n-butanol, shake the tubes for 2 min, and centrifuge. Remove and discard the aqueous layer in each tube, and then transfer 10 ml of the chloroform solution from each tube into separate centrifuge tubes. To each tube add 2 ml of Methyl orange-boric acid solution, shake the tubes for 2 min, and centrifuge. Remove and discard the aqueous layer in each tube, then add to each tube 1 g of anhydrous sodium sulfate, shake well, and allow to settle. Transfer 5 ml of each clear chloroform solution into separate test tubes, add 0.5 ml of 50:1 mixture of methanol and sulfuric acid TS, and mix. Successively determine the absorbance of the solutions in 1 cm cells at 520 nm with a suitable spectrophotometer, using the blank to set the instrument at zero. The absorbance of the solution from the Test preparation does not exceed that from the Standard preparation.

### Dicyclohexylamine in Cyclamates

**Note:** *The procedure uses a packed column GC. In the absence of a packed column GC, capillary GC in the splitless mode, using an equivalent capillary column, may be used. GC conditions need to be established.*

*Standard solutions:* Weigh accurately about 100 mg of dicyclohexylamine (C<sub>12</sub>H<sub>23</sub>N, Refractive index (25, D): 1.480-1.488, specific gravity: d (25, 25): 0.905-0.915, boiling point: 254-256°) in a 100-ml volumetric flask, dissolve in chloroform, dilute to volume with chloroform and mix (standard A, 1.0 mg/ml). Transfer 10 ml standard A into a 100-ml volumetric flask, dilute to volume with chloroform and mix (standard B, 100 µg/ml). Into a series of 10 ml volumetric flasks, Transfer 0.0, 1.0, 2.0, 4.0, 6.0 and 8.0 ml of standard B solution. Dilute to volume with chloroform, and mix. The working standard solutions contain 10.0, 20.0, 40, 60 and 80.0 µg/ml, respectively.

#### Procedure

Dissolve 50 g of the sample in 300 ml of water, add 3 ml of sodium hydroxide TS, and extract with 50 ml and 30 ml of chloroform. Combine the extracts, add 2 g of anhydrous potassium carbonate and filter. Wash the container and the residue on the filter paper several times with 5 ml chloroform, combine the washings to the filtrate and concentrate in a rotary evaporator at 30° under vacuum, to about 0.5 ml, quantitatively transfer into a 2 ml volumetric flask, evaporate the solvent in the volumetric flask to about 0.5 ml under a stream of nitrogen, add 1 ml of nitrobenzene standard solution (100 mg in 500 ml chloroform) as an internal standard and make up to the mark with chloroform.

#### Gas Chromatographic Conditions

*Column:* Stainless steel, 1.5 m x 3-4 mm i.d., packed with 60-80 mesh diatomaceous earth (gas chromatographic grade) in a solution of methanolic potassium hydroxide. The final potassium hydroxide concentration should be about 3% of the diatomaceous support. Evaporate off the methanol, add a chloroform solution of polyethyleneglycol 6000, and evaporate the chloroform. The content of polyethyleneglycol 6000 should be about 10% of the diatomaceous support.

*Carrier gas:* Nitrogen or helium, flow rate should be set so that the retention time of nitrobenzene is about 7 min

*Injection port, column, and detector temperatures:* 225°, 130 -140°, and 250°, respectively.

*Standard curve:* Prepare a standard curve by mixing 1 ml of each of working standard solution with 1 ml of internal standard solution and analyze by gas chromatography using a flame ionization detector under the conditions described below. Prepare a standard curve by plotting concentration of dicyclohexylamine (in µg per ml), vs. the ratio of the dicyclohexylamine peak area to that of internal standard. Inject the sample solution and calculate the concentration of dicyclohexylamine (in µg per ml,) in the sample solution from the standard curve and calculate the dicyclohexylamine in the sample as follows.

Dicyclohexylamine (mg/kg) = Conc. in sample (µg/ml) x 0.02

### **1,4-Dioxane and Ethylene Oxide**

Determine by headspace gas chromatography using the following procedure:

*Stripped sample:* Place 3000 g of the sample into a 5000-ml, 4-neck, round-bottom flask equipped with a stirrer, a thermometer, a gas dispersion tube, a dry ice trap, a vacuum outlet, and a heating mantle. At room temperature, evacuate the flask carefully to a pressure of less than 1 mmHg, applying the vacuum slowly while observing for excessive foaming due to entrapped gases. After any foaming has subsided, spurge with nitrogen, allowing the pressure to raise to 10 mmHg. Heat the flask to 60° while increasing the pressure to about 60mmHg. Continue stripping for 4 h, then cool to room temperature. Shut off the vacuum pump, and



bring the flask pressure back to atmospheric while maintaining nitrogen sparging. Remove the sparging tube with the gas still flowing, then turn off the gas flow. Transfer the Stripped sample to a suitable nitrogen-filled container.

*Standard Preparations:* (Caution: Ethylene oxide and 1,4-dioxane are toxic and flammable. Prepare these solutions in a well-ventilated fume hood.) Add a suitable quantity of 1,4-dioxane to a known weight of organic-free water in a vial that can be sealed. Determine the amount added by weight difference. Using the special handling described in the following, complete the preparation. Ethylene oxide is a gas at room temperature. It is usually stored in a lecture-type gas cylinder or small metal pressure bomb. Chill the cylinder in a refrigerator before use. Transfer about 5 ml of the liquid ethylene oxide to a 100-ml beaker chilled in wet ice. Using a gastight gas chromatographic syringe that has been chilled in a refrigerator, transfer a suitable amount of the liquid ethylene oxide into the mixture. Immediately seal the vial, and shake. Determine the amount added by weight difference. By appropriate dilution with Stripped sample, prepare four solutions, covering the range from 1 to 20 mg/kg for the two components added to the matrix (e.g., 5, 10, 15, and 20 mg/kg). Transfer 10 ml of each of these solutions to separate 22-ml pressured headspace vials, seal each with a silicone septum, star spring, and pressure-relief safety aluminium sealing cap, and crimp the cap closed with a cap-sealing tool. Shake for 2 min.

*Sample Preparation:* Transfer  $10 \pm 0.01$  g of sample to a 22-mL pressure headspace vial, and seal, cap, and crimp as directed for the Standard Preparations.

#### Apparatus

Gas chromatograph equipped with a balanced pressure automatic headspace sampler and a flame-ionization detector.

Column:	50-m $\times$ 0.32-mm fused silica capillary column, or equivalent, bonded with a 5-mm film of 5% phenyl-95% ethylsiloxane, or equivalent.
Column temperature:	Program the column temperature from 70° to 250° at 10°/min
Transfer line temperature:	140°
Detector temperature:	250°
Carrier gas:	Helium
Flow rate:	app. 0.8 ml/min.
Performance:	On the two Calibration plots, no point digresses from its line by more than 10%.

*Calibration:* Place the vials containing the Standard Preparations in the automated sampler, and start the sequence so that each vial is heated at a temperature of 50° for 30 min before a suitable portion of its headspace is injected into the chromatograph. Set the automatic sampler for a needle withdrawal time of 0.3 min, a pressurization time of 1 min, an injection time of 0.08 min, and a vial pressure of 22 psig with the vial vent off. Obtain the peak areas for ethylene oxide and 1,4-dioxane, which have relative retention times of about 1.0 and 3.1, respectively. Plot the area versus milligram per kilogram on linear graph paper, and draw the best straight line through the points.

*Procedure:* Place the vial containing the Sample Preparation in the automatic sampler, and chromatograph its headspace as done for the Standard Preparations. Obtain the peak areas of each of the components, and read the concentrations directly from the Calibration plots.

## Fumaric and Maleic Acid

Determine by HPLC using the following conditions:

Mobile Phase: Filtered, degassed solution of 0.01 N sulfuric acid in water.

**Note:** *For all reference standards, do not dry before use, and keep the containers tightly closed and protected from light. Determine the water content of Fumaric Acid Reference Standard titrimetrically before use, and make the necessary correction in preparing the Standard Preparation.*

Standard Preparation: Transfer about 5 mg of Fumaric Acid Reference Standard (USP or equivalent) and about 2mg of Maleic Acid Reference Standard (USP or equivalent), both accurately weighed, into a 1000-ml volumetric flask, dilute to volume with Mobile Phase, and mix.

Sample Preparation: Transfer about 100 mg of sample, accurately weighed, into a 100-ml volumetric flask, dilute to volume with Mobile Phase, and mix.

Resolution Solution: Transfer about 1 g of sample, about 10 mg of Fumaric Acid Reference Standard, and about 4 mg of Maleic Acid Reference Standard, all accurately weighed, into a 1000-ml volumetric flask, dilute to volume with Mobile Phase, and mix.

### Apparatus

Liquid chromatograph

Column: 30-cm × 6.5-mm (i.d.) column, or equivalent, packed with a strong cation exchange resin consisting of sulfonated cross-linked styrene–divinylbenzene copolymer in the hydrogen form (Polypore H from Brownlee Lab, or equivalent)

Detection: 210-nm

Column temperature:  $37 \pm 1^\circ$

Flow rate: App. 0.6 ml/min

### Performance

Inject a portion of the Resolution Solution, and obtain the chromatogram. Record the peak responses from the chromatogram. The resolution of the maleic acid and sample peaks is not less than 2.5; the resolution of the fumaric acid and sample peaks is not less than 7.0; and the relative standard deviation of the Sample Solution peak for replicate injections is not more than 2.0%.

### Procedure

Separately inject about 20  $\mu$ l each of the Standard Preparation and the Test Preparation into the chromatograph, record the chromatograms, and measure the peak responses. The relative retention times are approximately 0.6 for maleic acid, approximately 1.0 for malic acid, and approximately 1.5 for fumaric acid. Calculate the quantities, in milligrams, of maleic acid and fumaric acid, in the portion of the sample taken by the formula  $100C \times (rU/rS)$ , in which C is the concentration, in milligrams per milliliter, of the corresponding Reference Standard in the Standard Preparation, and rU and rS are the responses of the corresponding peaks from the Test Preparation and the Standard Preparation, respectively.

## Gum Constituents Identification

Boil a mixture of 200 mg of the sample and 20 ml of 10% sulfuric acid for 3 h. Allow to cool and add excess barium carbonate, mixing with a magnetic stirrer until the solution is pH 7, and filter. Evaporate the filtrate in a rotatory evaporator at 30 - 50° under vacuum until a crystalline (or syrupy) residue is obtained. Dissolve in 10 ml of 40% methanol. This is the hydrolysate. Place 1 to 5 µl spots of hydrolysate on the starting line of two Silica Gel G thin layer plates. On the same plates apply 1 to 10 µg of the reference standards specified in the individual monograph.

Develop one plate in solvent A and one plate in solvent B:

A. A mixture of formic acid, methyl ethyl ketone, tertiary butanol and water (15/30/40/15 by volume) and

B. A mixture of glacial acetic acid/chloroform/water (74/65/11 by volume).

After development spray with a solution of 1.23 g anisidine and 1.66 g phthalic acid in 100 ml ethanol and heat the plates at 100° for 10 min. A greenish yellow colour is produced with hexoses, a red colour with pentoses and a brown colour with uronic acids. Compare sample spots with those for the solutions of the reference standards and identify the constituents specified in the individual monograph.

## Norbixin

Determine by HPLC using the following:

### Reagents

Dimethylformamide

Acetonitrile

0.1 M NaOH

Methanol

Acetic acid

Norbixin (purity 99 % or higher; prepare according to the procedure in Scotter *et al.* (1994, 1998) as it is not currently available commercially)

**Note:** *all solvents should be HPLC-grade*

### Apparatus:

HPLC system with a suitable pump, injector, and integrator

Column: Stainless steel; 250 x 4.6 mm

Stationary phase: Mixed C8/C18 bonded phase, 5 µm or similar

Detector: UV/visible

### HPLC conditions:

Column temperature: 35°

Mobile phase: Isocratic 65 % Solution A; 35 % Solution B

Solution A: acetonitrile; Solution B: 2 % acetic acid (v/v)

Flow rate: 1.0 ml/min

Injection: 10 µl

Detection: 460 nm

Run time: 40 min

**Note:** *The retention time of norbixin is approximately 10 min*

**Procedure:**

*Standard solution:* Weigh accurately about 25 - 50 mg of the norbixin standard and dissolve in 5 ml of 0.1 M NaOH solution. Transfer quantitatively to a 50 ml volumetric flask and dilute to volume with methanol.

*Sample solution:*

*Oil-soluble samples:* Weigh accurately about 25 - 50 mg of the sample and dissolve in 3 to 5 ml of dimethylformamide. Transfer quantitatively to a 50 ml volumetric flask and dilute to volume with acetonitrile.

*Water-soluble samples:* Weigh accurately about 25 - 50 mg of the sample and dissolve in 5 ml of 0.1 M NaOH solution. Transfer quantitatively to a 50 ml volumetric flask and dilute to volume with methanol.

**Calculation:**

$$\text{Norbixin (\%)} = \frac{A_s \times W_{St} \times P_{St}}{A_{St} \times W_s} \times 100$$

Where:

- As is the peak area of the sample solution
- ASt is the peak area of the standard solution
- PSt is the purity of the standard expressed as a proportion of Norbixin in the norbixin standard (for example, 0.99 if the standard is 99% pure).
- WSt is the weight of the standard (mg)
- Ws is the weight of the sample (mg)

**References:**

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**Oxalate Limit Test**

Dissolve 0.5 g of sample in 4 ml of water, add 3 ml concentrated hydrochloric acid and then 1 g of granulated zinc. Heat for 1 min in a boiling water bath. Let stand for 2 min at room temperature; decant the supernatant solution into a test tube containing 0.25 ml of a 1% solution of phenylhydrazine hydrochloride. Mix, heat to boiling and cool immediately. Transfer the solution into a glass cylinder with a ground glass stopper and add an equal volume of concentrated hydrochloric acid. Add 0.25 ml of a 5% solution of potassium hexacyanoferrate (III), mix well and let stand for 30 min. The colour of the solution is not more intense than that of a standard solution prepared in the same manner and containing 4.0 ml of a solution of 0.005% oxalic acid in water.

**Polyols: Thin Layer Chromatography**

Examine by thin layer chromatography (TLC) using silica gel as the coating substance, and using standard and test solutions described in the individual monograph.

Reagents

4-Aminobenzoic acid reagent: Prepare a solution by dissolving 1 g of 4-aminobenzoic acid in a solvent mixture composed of 18 ml acetic acid, 20 ml water and 1 ml phosphoric acid. Prepare this reagent immediately before use.

Sodium periodate reagent: 0.2% w/v sodium periodate in water

Procedure

Apply 2  $\mu$ l of each of the standard and test solution to the bottom of the TLC plate. Develop the chromatogram over a path of 17 cm using as the mobile phase a mixture of 70 volumes of propanol, 20 volumes of ethyl acetate and 10 volumes of water. Allow the plate to dry in air and spray with a mixture of 2 volumes of 4-aminobenzoic acid reagent with 3 volumes of acetone. Heat at 100° for 15 min. Spray with the sodium periodate reagent. Heat at 100° for 15 min. The principal spot in the chromatogram obtained from the test solution corresponds in position, colour and size to the principal spot obtained from the standard solution.

**Pyrrolidone carboxylic acid**

Proceed as directed under thin-layer chromatography (see Analytical Techniques) as follows:

Sample: 2  $\mu$ l of a 0.5 in 100 solution of the sample

Reference: 2  $\mu$ l of a 0.5 in 100 solution of monosodium L-glutamate containing 2.5 mg of pyrrolidone carboxylic acid

Solvent: A mixture of 2 volumes of n-butanol, 1 volume of glacial acetic acid and 1 volume of water.

Adsorbent: Silica gel

Potassium iodide-starch solution: Stir and heat 0.5 g of starch in about 50 ml of water until it gelatinizes; after cooling add 0.5 g of potassium iodide and water to make up to 100 ml.

Stop the development when the solvent front has advanced about 10 cm from the point of the application dry the plate for 30 min in air.

At the same time, prepare a similar chamber to that used for developing; place a 50-ml beaker containing about 3 g of sodium hypochlorite in the chamber; slowly add 1 ml of hydrochloric acid into the beaker to generate chlorine gas; put on the lid and allow to stand for 30 sec to fill the chamber with chlorine. Place the dried plate in this chamber, put on the lid and allow to stand for 20 min. Take out the plate, keep for 10 min in air and spray with ethanol. After drying, spray with potassium iodide-starch solution and observe the plate under natural light immediately after the standard spot has appeared.

No spot corresponding to pyrrolidone carboxylic acid standard is detected in the sample (sensitivity = 0.2%).

**Readily Carbonizable Substances**Procedure

Unless otherwise directed, add the specified quantity of the substance, finely powdered if in solid form, in small portions to the comparison container, which is made of colourless glass resistant to the action of sulfuric acid and contains the specified volume of sulfuric acid TS.

Stir the mixture with a glass rod until solution is complete, allow the solution to stand for 15 min, unless otherwise directed, and compare the colour of the solution with that of the

specified matching fluid in a comparison container which also is of colourless glass and has the same internal and cross-section dimensions. View the fluids transversely against a background of white porcelain or white glass.

When heat is directed in order to effect solution of the substance in the sulfuric acid TS, mix the sample and the acid in a test tube, heat as directed, cool, and transfer the solution to the comparison container for matching.

#### Matching Fluids

For purposes of comparison, a series of twenty matching fluids, each designated by a letter of the alphabet, is provided, the composition of each being as indicated in the following table. To prepare the matching fluid specified, pipet the prescribed volumes of the colorimetric test solutions (TSC) and water into one of the matching containers, and mix the solutions in the container.

Matching Fluid	Parts of Cobaltous Chloride TSC	Parts of Ferric Chloride TSC	Parts of Cupric Sulfate TSC	Parts of Water
A	0.1	0.4	0.1	4.4
B	0.3	0.9	0.3	8.5
C	0.1	0.6	0.1	4.2
D	0.3	0.6	0.4	3.7
E	0.4	1.2	0.3	3.1
F	0.3	1.2	0.0	3.5
G	0.5	1.2	0.2	3.1
H	0.2	1.5	0.0	3.3
I	0.4	2.2	0.1	2.3
J	0.4	3.5	0.1	1.0
K	0.5	4.5	0.0	0.0
L	0.8	3.8	0.1	0.3
M	0.1	2.0	0.1	2.8
N	0.0	4.9	0.1	0.0
O	0.1	4.8	0.1	0.0
P	0.2	0.4	0.1	4.3
Q	0.2	0.3	0.1	4.4
R	0.3	0.4	0.2	4.1
S	0.2	0.1	0.0	4.7
T	0.5	0.5	0.4	3.6

**Note:** Solutions A-D are very light brownish-yellow.; solutions E-L are yellow through reddish-yellow; solutions M-O are greenish-yellow; and solutions P-T are light pink.

## Reducing Substances (as Glucose)

### Method I (Volumetric)

Transfer about 1 g of the sample, accurately weighed, into a 250-ml Erlenmeyer flask, dissolve in 10 ml of water, and add 25 ml of alkaline cupric citrate TS and cover the flask with a small beaker. Boil gently for exactly 5 min and cool rapidly to room temperature. Add 25 ml of 10% acetic acid solution, 10.0 ml of 0.1 N iodine, 10 ml of dilute hydrochloric acid TS and 3 ml of starch TS, and titrate with 0.1 N sodium thiosulfate to the disappearance of the blue colour. Calculate the content of reducing substances (as D-glucose) by the formula:

$$\% \text{ Reducing substances (as D-glucose)} = [(V_1N_1 - V_2N_2) \times 2.7] / \text{Sample wt. (g)}$$

Where:

$V_1$  and  $N_1$  are the volume (ml) and normality, respectively, of the iodine solution,  
 $V_2$  and  $N_2$  are the volume (ml) and normality, respectively, of the sodium thiosulfate solution,  
 and 2.7 is an empirically determined equivalence factor for D-glucose.

### Method II (Gravimetric)

Dissolve 7 g of the sample in 35 ml of water in a 400-ml beaker and mix. Add 25 ml of cupric sulfate TS and 25 ml of alkaline tartrate TS. Cover the beaker with glass, heat the mixture at such a rate that it comes to a boil in approximately 4 min and boils for exactly 2 min. Filter the precipitated cuprous oxide through a tared Gooch crucible previously washed with hot water, ethanol, and ether, and dried at 100° for 30 min. Thoroughly wash the collected cuprous oxide on the filter with hot water, then with 10 ml of ethanol and finally with 10 ml of ether, and dry at 100° for 30 min. The weight of the cuprous oxide does not exceed that prescribed in the individual monograph.

## Related Foreign Substances in Flavour Enhancers

Proceed as directed under "Thin-layer chromatography" (see Analytical Techniques) using a sample of the solution described under Method of Assay in the monograph. Use a mixture of 80 volumes of a saturated solution of ammonium sulfate, 18 volumes of a 13.6% w/v solution of sodium acetate and 2 volumes of isopropanol as the developing solvent. Use microcrystalline cellulose as the absorbent. Stop the development when the solvent front has advanced about 10 cm from the point of the application, dry the plate in air, and observe under ultraviolet light (about 254 nm) in a dark place. Only a spot of 5'-guanylic acid or 5'-inosinic acid is detected.

### Residual solvents

The solvents listed in the table below can be determined by this method based on headspace gas chromatography. The method may also be used for the determination of isobutyl acetate and methyl acetate. However, information on the approximate retention time for these two solvents is not available.

Solvent	Approximate retention times (min)	Solvent	Approximate retention times (min)
Ethanal	2.81	Ethyl acetate	10.05
Methanol	2.93	Chloroform	10.33
Ethanol	4.09	2-Methyl-1-propanol	11.05
Ethanenitrile	4.55	1-Butanol	12.79
Propanone	4.76	Hexamethyldisiloxane	14.42
2-Propanol	5.23	Propyl acetate	14.97
Ethoxyethane	5.67	4-Methyl-2-pentanone	16.18
2-Methyl-2-propanol	6.21	Pyridine	16.39
Dichloromethane	6.45	3-methyl-2-pentanone	16.90
1-Propanol	7.78	Toluene	18.25
Trimethylsilanol	8.41	Butyl acetate	20.61
2-Butanol	9.61		

Reagents:

Blank: sample with very low solvent content

Internal standard: 3-methyl-2-pentanone

Methanol

Demineralised water

Method I (Determination carried out in water)

*Internal standard solution:* Add 50.0 ml water to a 50 ml injection vial and seal. Accurately weigh and inject 15 µl 3-methyl-2-pentanone through the septum and reweigh to within 0.01 mg.

*Blank solution:* Weigh accurately 0.20 g of the blank into an injection vial. Add 5.0 ml of water and 1.0 ml of the internal standard solution. Heat at 60° for 10 min and shake vigorously for 10 sec.

*Samples:* Weigh accurately 0.20 g sample into an injection vial. Add 5.0 ml water and add 1.0 ml of the internal standard solution. Heat at 60° for 10 min and shake vigorously for 10 sec.

*Calibration solution:* Weigh accurately 0.20 g of the blank into an injection vial. Add 5.0 ml of the water and 1.0 ml of the internal standard solution. Weigh the vial accurately to within 0.01 mg. Inject a known volume of the component of interest through the septum and again reweigh the vial. Heat at 60° for 10 min and shake vigorously for 10 sec.

Method II (Determination carried out in methanol)

*Internal standard solution:* Add 50.0 ml methanol to a 50 ml injection vial and seal. Accurately weigh and inject 15 µl 3-methyl-2-pentanone through the septum and reweigh to within 0.01 mg.

*Blank solution:* Weigh accurately 0.20 g of the blank into an injection vial. Add 5.0 ml of methanol and 1.0 ml of the internal standard solution. Heat at 60° for 10 min and shake vigorously for 10 sec.

*Samples:* Weigh accurately 0.20 g sample into an injection vial. Add 5.0 ml methanol and add 1.0 ml of the internal standard solution. Heat at 60° for 10 min and shake vigorously for 10 sec.



*Calibration solution:* Solution A: Add 50.0 ml methanol to a 50 ml vial and seal. Accurately weigh, to within 0.01 mg, the vial and inject 50 µl of the component of interest through the septum. Reweigh the vial. Mix well.

Weigh into an injection vial, a known amount of blank (0.20 g), add 4.9 ml of methanol and 1.0 ml internal standard solution. Introduce 0.1 ml of Solution A into the injection vial. Mix well and heat at 60° for 10 min and shake vigorously for 10 sec.

Procedure:

Place the sample, blank and calibration samples in the sample tray of the head-space gas chromatograph – FID system. Analyse using the following analytical conditions.

*Column:* Fused silica, length 0.8 m, i.d. 0.53mm, coated with DB-wax, film thickness 1 µm

*Coupled with:* Fused silica, length 30 m, i.d. 0.53 mm, coated with DB-1, film thickness 5 µm

*Conditions:*

Carrier gas: Helium

Flow rate: 208 kPa, 5 ml/min

Detector: FID

Temperatures

Injector: 140°

Oven conditions: 35° for 5 min, then 5°/min to 90°, then 6 min at 90°

Detector: 300°

Head space sampler

Sample heating temperature: 60°

Sample heating period: 10 min

Syringe temperature: 70°

Transfer temperature: 80°

Sample gas injection: 1.0 ml in split mode

Calculation

$$A \times B \times C / 50 = \text{mg component per injection vial}$$

Where:

A = relative peak area of the component concerned

B = mg internal standard

C = calibration factor

*Determination of calibration factors*

Method 1:

$$C = D \times 50 / (E \times (F - G))$$

Method 2:

$$C = D / (E \times (F - G) \times 10)$$

Where:

D = mg component weighed

E = mg internal standard

F = relative peak area of component for the calibration solution

G = relative peak area of the same component for the blank solution

## Toluenesulfonamides in Saccharines

Determine by gas chromatography (see Analytical Techniques)

### Standard and test solutions

*Methylene chloride:* Use a suitable chromatography grade (or pure solvent obtained by distillation in all-glass apparatus from analytical grade).

*Internal standard stock solution:* Weigh accurately, about 100 mg of 95% n-tricosane into a 10 ml volumetric flask, dissolve in n-heptane, dilute to volume with the same solvent and mix.

*Stock standard preparation:* Weigh accurately 20 mg each of reagent grade o-toluenesulfonamide and p-toluenesulfonamide into a 10 ml volumetric flask, dissolve in methylene chloride, dilute to volume with the same solvent, and mix.

*Dilute standard preparations:* Pipet into five 10-ml volumetric flasks 0.1, 0.25, 1.0, 2.5 and 5 ml, respectively, of the "Stock standard preparation". Pipet 0.25 ml of the "Internal standard stock solution" into each flask, dilute each to volume with methylene chloride, and mix. These solutions contain 250 µg of n-tricosane, plus respectively, 20, 50, 200, 500 and 1000 µg per ml of each toluenesulfonamide.

*Test preparation:* Dissolve 2 g of the sample in 8.0 ml of sodium carbonate TS. Mix the solution thoroughly with 10 g of chromatographic siliceous earth (Celite 545 or equivalent). Transfer the mix into a 25 x 250-mm chromatographic tube having a fritted glass disk and a Teflon stopcock at the bottom, and a reservoir at the top. Pack the contents of the tube by tapping the column on a padded surface, and then by tamping firmly from the top. Place 100 ml of methylene chloride in the reservoir, and adjust the stopcock so that 50 ml of eluate is collected in 20-30 min. To the eluate add 25 µl of "Internal standard stock solution". Mix, and then concentrate the solution to a volume of 1 ml in a suitable concentrator tube fitted with a modified Snyder column, using a Kontes tube heater maintained at 90°.

### Procedure

**Note:** *The procedure uses a packed column GC. In the absence of a packed column GC, capillary GC in the splitless mode, using an equivalent capillary column, may be used. GC conditions need to be established.*

Inject 2.5 µl of the "Test preparation" into a suitable gas chromatograph equipped with a flame-ionization detector. The column is of glass, approximately 3 m in length and 2 mm in inside diameter, and it is packed with 3% methyl phenyl silicone in 100 to 120 mesh silanized calcined diatomaceous silica (**Caution:** *The glass column should extend into the injector for on-column injection and into the detector base to avoid contact with metal*). The carrier is helium flowing at a rate of 30 ml per min. The injection port, column, and detector are maintained at 225°, 180°, and 250°, respectively. The instrument attenuation setting should be such that 2.5 µl of the "Dilute standard preparation" containing 200 µg per ml of each toluene sulfonamide gives a response of 40-80% of full-scale deflection. Record the chromatogram, note the peaks for o-toluene sulfonamide, p-toluene sulfonamide, and the n-tricosane internal standard, and calculate the areas of each peak by suitable means. The retention times for o-toluene sulfonamide, p-toluene sulfonamide, and n-tricosane are about 5, 6, and 15 min, respectively.

In a similar manner, obtain the chromatograms for 2.5-µl portions of each of the five "Dilute standard preparations", and for each solution determine the areas of the o-toluene sulfonamide, p-toluene sulfonamide, and n-tricosane peaks. From the values thus obtained,

prepare standard curves by plotting concentration of each toluene sulfonamide, in  $\mu\text{g}$  per ml, vs. the ratio of the respective toluene sulfonamide peak area to that of n-tricosane. From the standard curve determine the concentration, in  $\mu\text{g}$  per ml, of each toluene sulfonamide in the "Test preparation". Divide each value by 2 to convert the result mg/kg of the toluene sulfonamide in 2 g sample taken for analysis.

**Note:** *If the toluene sulfonamide content of the sample is greater than about 500 mg/kg, the impurity may crystallize out of the methylene chloride concentrate (see "Test preparation"). Although this level of impurity exceeds that permitted by the specifications, the analysis may be completed by diluting the concentrate (usually 1:10 is satisfactory) with methylene chloride containing 250  $\mu\text{g}$  of n-tricosane per ml, and by applying appropriate dilution factors in the calculation. Care must be taken to re-dissolve completely any crystalline toluene sulfonamide to give a homogeneous solution.*

### **Triphenylphosphine oxide**

Determine by HPLC using the following:

#### Reagents

Hexane

Isopropanol

Tetrahydrofuran (THF)

Triphenylphosphine oxide (TPPO) (purity 99% or higher; ACROS 14043-0250 or equivalent)

**Note:** *all solvents should be HPLC-grade*

#### Apparatus:

HPLC system with a suitable pump, injector, and integrator

Column: Stainless steel; 150 x 4.6 mm

Stationary phase: Supelcosil LC-Si, 5  $\mu\text{m}$  or similar

Detector: UV

#### HPLC conditions:

Column temperature: 20°

Mobile phase: Isopropanol:hexane (1:24 v/v)

Flow rate: 1.5 ml/min

Injection: 50  $\mu\text{l}$

Detection: 210 nm

Run time: 10 min

**Note:** *The retention time of TPPO is approximately 8.1 min*

#### Procedure:

**Standard solution:** Weigh accurately about 10 mg of the TPPO standard and dissolve in THF. Transfer quantitatively to a 1000-ml volumetric flask and dilute to volume with THF.

**Sample solution:** Accurately weigh about 1000 mg of the sample and dissolve in THF. Transfer quantitatively to a 100-ml volumetric flask and dilute to volume with THF.

#### Calculation:

$$\text{TPPO (\%)} = \frac{A_s \times W_{\text{St}} \times P_{\text{St}} \times 100}{A_{\text{St}} \times W_s \times 1000} \times 100$$

Where:

$A_s$  is the peak area of the sample solution

$A_{\text{St}}$  is the peak area of the standard solution

$P_{\text{St}}$  is the purity of the standard expressed as a proportion of TPPO in the TPPO standard (for example, 0.99 if the standard is 99% pure).

$W_{\text{St}}$  is the weight of the standard (mg)

$W_s$  is the weight of the sample (mg)

## MICROBIOLOGICAL ANALYSES

**Note:** *All methods in this Section reference media and reagents, which are prepared as detailed in the Section entitled "Media, Reagents and Solutions".*

### Total (Aerobic) Plate Count

#### Equipment and materials

1. Work area, level table with ample surface in clean, well-lighted (100 foot-candles at working surface) and well-ventilated room that is reasonably free of dust and drafts. The microbial density, measured in fallout pour plates taken during plating, of air in working area should not exceed 15 colonies /per plate during 15 min exposure.
2. Petri dishes, glass (15 x 100 mm) or plastic (15 x 90 mm).
3. Pipets, 1, 5, and 10 ml, graduated in 0.1 ml units.
4. Dilution bottles, 6 oz (160 ml), borosilicate-resistant glass, with rubber stoppers or plastic screw caps.
5. Water bath, for tempering agar, thermostatically controlled to  $45 \pm 1^\circ$ .
6. Incubator,  $35 \pm 1^\circ$ .
7. Colony counter, dark-field, Quebec, or equivalent, with suitable light source and grid plate.
8. Tally register.
9. Thermometers appropriate range; accuracy checked.
10. Dilution blanks,  $90 \pm 1$  ml Butterfield's phosphate-buffered dilution water

#### Media and reagents

1. Butterfield's phosphate-buffered dilution water.
2. Plate count agar.

#### Procedure

Using separate sterile pipets, prepare decimal dilutions of  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ , and others as appropriate, of sample homogenate by transferring 10 ml of previous dilution to 90 ml of diluent. Avoid sampling foam. Shake all dilutions 25 times in 30 cm (1 ft) arc within 7 sec. Pipet 1 ml of each dilution into separate, duplicate, appropriately marked petri dishes. Reshake dilution bottle 25 times in 30 cm arc within 7 sec if dilution stands more than 3 min before pipeting test portion into petri dish. Add 12-15 ml plate count agar (cooled to  $44-46^\circ$ ) to each plate within 15 min of original dilution. Add agar immediately to petri dishes when sample diluent contains hygroscopic materials. Pour agar and dilution water control plates for each series of samples. Immediately mix sample dilutions and agar medium thoroughly and uniformly by alternate rotation and back-and-forth motion of plates on flat level surface. Let agar solidify, invert petri dishes, and incubate promptly for  $48 \pm 2$  h at  $35^\circ$ .

After incubation, count duplicate plates in suitable range (25-250 colonies), using colony counter and tally register; record results per dilution plate counted. Duplicate plates of at least 1 of 3 dilutions should be in 25-250 colony range. When only 1 dilution is in appropriate range, compute average count per g for dilution and report as total plate count per g (see Table 1, Sample No. 1). When 2 dilutions are in appropriate range, determine average count per dilution before averaging 2 dilution counts to obtain total plate count per g (see Table 1, Sample No. 2). If none or only one of duplicate plates of required dilution yields 25-250 colonies, proceed as in "Guidelines", below. Round off counts to two significant figures only at time of conversion to total plate counts. When rounding off numbers, raise second digit to

next higher number only when third digit from left is 5 or greater, and replace dropped digit with zero. If third digit is 4 or less, replace third digit with zero and leave second digit the same.

Guidelines for calculating and reporting total plate counts in uncommon cases

Report all total plate counts computed from duplicate plates containing less than 25 or more than 250 colonies as estimated counts. Use the following as a guide:

- *Plates with fewer than 25 colonies.* When duplicate plates of lowest dilution have fewer than 25 colonies, count actual number on each duplicate of that dilution, average the number of colonies per plate, and multiply by dilution factor to obtain estimated total plate count. Mark total plate count with asterisk to denote that it was estimated from counts outside 25-250 per plate range (see Table 1, Sample No. 3).
- *Plates with more than 250 colonies.* When number of colonies per plate exceeds 250, count colonies in those portions of plate that are representative of colony distribution. Mark calculated total plate count with asterisk to denote that it was estimated from counts outside 25-250 per plate range (see Table 1, Sample No. 4).
- *Spreaders.* Spreading colonies are usually of 3 distinct types: 1) a chain of colonies, not too distinctly separated, that appears to be caused by disintegration of a bacterial clump; 2) one that develops in film of water between agar and bottom of dish; and 3) one that forms in film of water at edge or on surface of agar. If plates prepared from sample have excessive spreader growth such that (a) area covered by spreaders, including total area of repressed growth, exceeds 50% of plate area, or (b) area of repressed growth exceeds 25% of plate area, report plates as spreader. Determine average count for each dilution; report arithmetic average of these values as total plate count. (See Table 1, Sample No. 5). When it is necessary to count plates containing spreaders not eliminated by (a) or (b) above, count each of the 3 distinct spreader types as one source. For the first type, if only one chain exists, count it as a single colony. If one or more chains appear to originate from separate sources, count each source as one colony. Do not count each individual growth in such chains as a separate colony. Types 2 and 3 usually result in distinct colonies and are counted as such. Combine the spreader count and the colony count to compute the total plate count.
- *Duplicate plates, one with 25-250 colonies, the other with more than 250 colonies.* When one plate contains 25-250 colonies and the duplicate contains more than 250 colonies, count both plates and include the plate with more than 250 colonies in computing total plate count (see Table 1, Sample No. 6).
- *Duplicate plates, one plate of each dilution with 25-250 colonies.* When one plate of each dilution contains 25-250 colonies and the duplicate contains more than 250 colonies or fewer than 25 colonies, count all 4 plates and include plates with more than 250 or fewer than 25 colonies in computing the total plate count (see Table 1, Sample No. 7).
- *Duplicate plates, both plates of one dilution with 25-250 colonies and only one duplicate of the other dilution with 25-250 colonies.* When both plates of one dilution contain 25-250 colonies and only one duplicate of the other dilution contains 25-250 colonies, count all 4 plates and include the plate with fewer than 25 or the plate with more than 250 colonies in computing aerobic plate count (see Table 1, Sample No. 8).

- *Plates with no CFU.* When plates from all dilutions have no colonies, report APC as less than 1 times the corresponding lowest dilution used. Mark calculated APC with asterisk to denote that it was estimated from counts outside the 25-250 per plate range. When plate(s) from a sample are known to be contaminated or otherwise unsatisfactory, record the result(s) as laboratory accident (LA).

Table 1. Examples of computation of total plate count (2 plates/dilution poured)

Sample No.	Colonies counted			
	1:100	1:1,000	1:10,000	Aerobic plate count/g
1	TNTC	<u>175</u>	16	190,000
	TNTC	<u>208</u>	17	
2	TNTC	<u>224</u>	<u>25</u>	250,000
	TNTC	<u>245</u>	<u>30</u>	
3	<u>18</u>	2	0	1,600*
	<u>14</u>	0	0	
4	TNTC	TNTC	<u>523</u>	5,200,000*
	TNTC	TNTC	<u>487</u>	
5	TNTC	<u>245</u>	<u>35</u>	290,000
	TNTC	<u>230</u>	Spreader	
6	TNTC	<u>245</u>	23	260,000
	TNTC	<u>278</u>	20	
7	TNTC	<u>225</u>	<u>21</u>	270,000
	TNTC	<u>255</u>	<u>40</u>	
8	TNTC	<u>210</u>	<u>18</u>	230,000
	TNTC	<u>240</u>	<u>28</u>	
	TNTC	<u>260</u>	<u>30</u>	270,000
	TNTC	<u>230</u>	<u>28</u>	

\*(Asterisk) : estimated count

TNTC : Too numerous to count. Colony count is significantly beyond count range of 250 colonies.

Underlined numbers are used to calculate aerobic plate count.

### Spiral Plate Count Method (Alternative Method)

The spiral plate count (SPLC) method for microorganisms uses a mechanical plater to inoculate a rotating agar plate with liquid sample. The sample volume dispensed decreases as

the dispensing stylus moves from the center to the edge of the rotating plate. The microbial concentration is determined by counting the colonies on a part of the petri dish where they are easily countable and dividing this count by the appropriate volume. One inoculation determines microbial densities between 500 and 500,000 microorganisms/ml. Additional dilutions may be made for suspected high microbial concentrations.

#### Equipment and materials

1. Spiral plater
2. Spiral colony counter with special grid for relating deposited sample volumes to specific portions of petri dishes
3. Vacuum trap for disposal of liquids (2-4 liter vacuum bottle to act as vacuum reservoir and vacuum source of 50-60 cm Hg)
4. Disposable micro beakers, 5 ml
5. Petri dishes, plastic or glass, 150 x 15 mm or 100 x 15 mm
6. Plate count agar (standard methods)
7. Calculator (optional), inexpensive electronic hand calculator is recommended
8. Polyethylene bags for storing prepared plates
9. Commercial sodium hypochlorite solution, about 5% NaOCl (bleach)
10. Sterile dilution water
11. Syringe, with Luer tip for obstructions in stylus; capacity not critical
12. Work area, storage space, refrigerator, thermometers, tally, incubator, as described for Conventional Plate Count Method, above.
13. Sodium hypochlorite solution (5.25%). Available commercially.

#### Preparation of agar plates

Automatic dispenser with sterile delivery system is recommended to prepare agar plates. Agar volume dispensed into plates is reproducible and contamination rate is low compared to hand-pouring of agar in open laboratory. When possible, use laminar air flow hood along with automated dispenser. Pour same quantity of agar into all plates so that same height of agar will be presented to spiral plater stylus tip to maintain contact angle. Agar plates should be level during cooling.

The following method is suggested for pre-pouring agar plates: Use automatic dispenser or pour constant amount (about 15 ml/100 mm plate; 50 ml/150 mm plate) of sterile agar at 60-70° into each petri dish. Let agar solidify on level surface with poured plates stacked no higher than 10 dishes. Place solidified agar plates in polyethylene bags, close with ties or heat-sealer, and store inverted at 0-4.4°. Bring pre-poured plates to room temperature before inoculation.

#### Preparation of samples.

Samples are prepared as described under Procedures.

#### Description of spiral plater

Spiral plater inoculates surface of prepared agar plate to permit enumeration of microorganisms in solutions containing between 500 and 500,000 microorganisms per ml. An operator with minimum training can inoculate 50 plates per h. Within the range stated, dilution bottles or pipets and other auxiliary equipment are not required. Required bench space is minimal, and time to check instrument alignment is less than 2 min. Plater deposits decreasing amount of sample in Archimedean spiral on surface of pre-poured agar plate. Volume of sample on any portion of plate is known. After incubation, colonies appear along line of spiral. If colonies on a portion of plate are sufficiently spaced from each other, count



them on special grid which associates a calibrated volume with each area. Estimate number of microorganisms in sample by dividing number of colonies in a defined area by volume contained in same area.

#### Plating procedure

Check stylus tip angle daily and adjust if necessary. (Use vacuum to hold microscope cover slip against face of stylus tip; if cover slip plane is parallel at about 1 mm from surface of platform, tip is properly oriented). Liquids are moved through system by vacuum. Clean stylus tip by rinsing for 1 s with sodium hypochlorite solution followed by sterile dilution water for 1 s before sample introduction. This rinse procedure between processing of each sample minimizes cross-contamination. After rinsing, draw sample into tip of Teflon tubing by vacuum applied to 2-way valve. When tubing and syringe are filled with sample, close valve attached to syringe. Place agar plate on platform, place stylus tip on agar surface, and start motor. During inoculation, label petri plate lid. After agar has been inoculated, stylus lifts from agar surface and spiral plater automatically stops. Remove inoculated plate from platform and cover it. Move stylus back to starting position. Vacuum-rinse system with hypochlorite and water, and then introduce new sample. Invert plates and promptly place them in incubator for  $48 \pm 3$  h at  $35 \pm 1^\circ$ .

#### Sterility controls

Check sterility of spiral plater for each series of samples by plating sterile dilution water.

**Caution:** *Pre-poured plates should not be contaminated by a surface colony or be below room temperature (water can well-up from agar). They should not be excessively dry, as indicated by large wrinkles or glazed appearance. They should not have water droplets on surface of agar or differences greater than 2 mm in agar depth, and they should not be stored at 0-4.4° for longer than 1 month. Reduced flow rate through tubing indicates obstructions or material in system. To clear obstructions, remove valve from syringe, insert hand-held syringe with Luer fitting containing water, and apply pressure. Use alcohol rinse to remove residual material adhering to walls of system. Dissolve accumulated residue with chromic acid. Rinse well after cleaning.*

#### Counting grid

*Description:* Use same counting grid for both 100 and 150 mm petri dishes. A mask is supplied for use with 100 mm dishes. Counting grid is divided into 8 equal wedges; each wedge is divided by four arcs labelled 1, 2, 3, and 4 from outside grid edge. Other lines within these arcs are added for ease of counting. A segment is the area between two arc lines within a wedge. Number of areas counted (e.g., 3) means number of segments counted within a wedge. The spiral plater deposits sample on agar plate in the same way each time. The grid relates colonies on spiral plate to the volume in which they were contained. When colonies are counted with grid, sample volume becomes greater as counting starts at outside edge of plate and proceeds toward the center of plate.

**Calibration:** The volume of sample represented by various parts of the counting grid is shown in the operator's manual that accompanies a spiral plater. Grid area constants have been checked by the manufacturer and are accurate. To verify these values, prepare 11 bacterial concentrations in range of  $10^6$ - $10^3$  cells/ml by making 1:1 dilutions of bacterial suspension (use a nonspreader). Plate all. Incubate both sets of plates for  $48 \pm 3$  h at  $35 \pm 1^\circ$ . Calculate concentrations for each dilution. Count spiral plates over grid surface, using counting rule of 20 (described below), and record number of colonies counted and grid area over which they were counted. Each spiral colony count for a particular grid area, divided by aerobic count/ml

for corresponding spirally plated bacterial concentrations, indicates volume deposited on that particular grid area. Use the following formula:

$$\text{Volume (ml) for grid area} = \frac{\text{Spiral colonies counted in area}}{\text{Bacterial count / ml (APC)}}$$

Example:

$$\begin{aligned} \text{Volume (ml)} &= \frac{31 + 30 \text{ colonies}}{4.1 \times 10^4 \text{ bacteria/ml}} \\ &= 0.0015 \text{ ml} \end{aligned}$$

#### Examination and reporting of spiral plate counts.

*Counting rule of 20.* After incubation, center spiral plate over grid by adjusting holding arms on viewer. Choose any wedge and begin counting colonies from outer edge of first segment toward center until 20 colonies have been counted. Complete by counting remaining colonies in segment where 20th colony occurs. Any count irregularities in sample composition are controlled by counting the same segments in the opposite wedge and recording results. Two segments of each wedge were counted on opposite sides of plate with 31 and 30 colonies, respectively. The sample volume contained in the darkened segments is 0.0015 ml. To estimate number of microorganisms, divide count by volume contained in all segments counted.

If 20 CFU are not within the 4 segments of the wedge, count CFU on entire plate. If the number of colonies exceeds 75 in second, third, or fourth segment, which also contains the 20th colony, the estimated number of microorganisms will generally be low because of coincidence error associated with crowding of colonies. In this case, count each circumferentially adjacent segment in all 8 wedges, counting at least 50 colonies, e.g., if the first 2 segments of a wedge contain 19 colonies and the third segment contains the 20th and 76th (or more), count colonies in all circumferentially adjacent first and second segments in all 8 wedges. Calculate contained volume in counted segments of wedges and divide into number of colonies.

When fewer than 20 colonies are counted on the total plate, report results as "less than 500 estimated SPLC per ml." If colony count exceeds 75 in first segment of wedge, report results as "greater than 500,000 estimated SPLC per ml." Do not count spiral plates with irregular distribution of colonies caused by dispensing errors. Report results of such plates as laboratory accident (LA). If spreader covers entire plate, discard plate. If spreader covers half of plate area, count only those colonies that are well distributed in spreader-free areas.

Compute SPLC unless restricted by detection of inhibitory substances in sample, excessive spreader growth, or laboratory accidents. Round off counts as described above. Report counts as SPLC or estimated SPLC per ml.

### **Coliforms and *E. coli***

#### Equipment and materials

1. Covered water bath, with circulating system to maintain temperature of  $45.5 \pm 0.2^\circ$ . Water level should be above that of medium in immersed tubes
2. Immersion-type thermometer,  $1-55^\circ$ , about 55 cm long, with  $0.1^\circ$  subdivisions, National Bureau of Standards certified, or equivalent
3. Incubator,  $35 \pm 1^\circ$

4. Balance with capacity of  $\geq 2$  kg and sensitivity of 0.1 g
5. Blender and blender jar
6. Sterile graduated pipets, 1.0 and 10.0 ml
7. Sterile utensils for sample handling
8. Dilution bottles made of borosilicate glass, with stopper or polyethylene screw caps equipped with Teflon liners. Commercially prepared dilution bottles containing sterile Butterfield's phosphate buffer can also be used.
9. Colony counter
10. pH meter

#### Media and reagents

1. Brilliant green lactose bile (BGLB) broth, 2%
2. Lauryl tryptose (LST) broth
3. EC broth
4. Levine's eosin-methylene blue (L-EMB) agar
5. Tryptone (tryptophane) broth
6. MR-VP broth
7. Koser's citrate broth
8. Plate count agar (PCA)
9. Butterfield's phosphate-buffered dilution water
10. Kovacs' reagent
11. Voges-Proskauer (VP) reagents
12. Gram stain reagents
13. Methyl red indicator.

#### Presumptive test for coliform bacteria

Aseptically weigh 10 g sample into sterile, screw-cap jar. Add 90 ml diluent and shake vigorously (50 times through 30 cm arc) to obtain  $10^{-1}$  dilution. Let stand 3-5 min and shake to re-suspend (5 times through 30 cm arc) just before making serial dilutions and inoculations.

Prepare all decimal dilutions with 90 ml sterile dilution water plus 10 ml from previous dilution unless otherwise specified. The dilutions to be prepared depend on the anticipated coliform density. Shake all suspensions 25 times in 30 cm arc for 7 sec. Do not use pipets to deliver  $<10\%$  of their total volume. Transfer 1 ml portions to 3 LST tubes for each dilution for 3 consecutive dilutions. Hold pipet at angle so that its lower edge rests against tube. Let pipet drain 2-3 sec. Not more than 15 min should elapse from time sample is blended until all dilutions are in appropriate media.

Incubate tubes  $48 \pm 2$  h at  $35^\circ$ . Examine tubes at  $24 \pm 2$  h for gas, i.e., displacement of medium in fermentation vial or effervescence when tubes are gently agitated. Re-incubate negative tubes for additional 24 h. Examine a second time for gas. Perform a confirmation test on all presumptive positive (gassing) tubes.

#### Confirmation test for coliforms

Gently agitate each gassing LST tube. Hold the LST tube at angle and insert a loop to avoid transfer of pellicle (if present). Transfer one loopful of suspension to a tube of BGLB broth. Incubate BGLB tubes  $48 \pm 2$  h at  $35^\circ$ . Examine for gas production and record. Calculate most probable number (MPN) of coliforms based on proportion of confirmed gassing LST tubes for three consecutive dilutions.

### Confirmation test for *E. coli*

Gently agitate each gassing LST tube and transfer a loopful of each suspension to tube of EC broth. Incubate EC tubes  $48 \pm 2$  h at  $45.5 \pm 0.2^\circ$ . Examine for gas production at  $24 \pm 2$  h; if negative, examine again at  $48 \pm 2$  h. Streak a loopful of suspension from each gassing tube to L-EMB agar. It is essential that 1 portion of plate exhibit well-separated colonies. Incubate 18-24 h at  $35^\circ$ . Examine plates for suspicious *E. coli* colonies, i.e., dark centered with or without metallic sheen. Pick two suspicious colonies from each L-EMB plate and transfer them to PCA agar slants for morphological and biochemical tests. Incubate PCA slants 18-24 h at  $35^\circ$ . If typical colonies are not present, pick 5-10 or more colonies deemed most likely to be *E. coli*, from every plate.

Perform gram stain. Examine all cultures appearing as gram-negative short rods or cocci for the following biochemical activities (the first four tests are collectively termed IMViC):

- *Indole production.* Inoculate tube of tryptone broth and incubate  $24 \pm 2$  h at  $35^\circ$ . Test for indole by adding 0.2-0.3 ml Kovacs' reagent. Appearance of distinct red colour in the upper layer is positive test.
- *Voges-Proskauer-reactive compounds.* Inoculate tube of MR-VP broth and incubate  $48 \pm 2$  h at  $35^\circ$ . Transfer 1 ml to 13 x 100 mm tube. Add 0.6 ml alpha-naphthol solution and 0.2 ml 40% KOH, and shake. Add a few crystal of creatine. Shake and let stand 2 h. Test is positive if eosin pink colour develops.
- *Methyl red-reactive compounds.* Incubate MR-VP tube additional  $48 \pm 2$  h at  $35^\circ$  after Voges-Proskauer test. Add 5 drops methyl red solution to each tube. A distinct red colour is a positive test. Yellow is a negative reaction.
- *Use of citrate.* Lightly inoculate tube of Koser's citrate broth; avoid detectable turbidity. Incubate  $96 \pm 2$  h at  $35^\circ$ . Development of distinct turbidity is positive reaction.
- *Production of gas from lactose.* Inoculate tube of LST broth and incubate  $48 \pm 2$  h at  $35^\circ$ . Displacement of medium from inner vial or effervescence after gentle agitation is a positive reaction.

*Interpretation.* All cultures that (a) ferment lactose with production of gas within 48 h at  $35^\circ$ , (b) appear as Gram-negative non-sporeforming rods or cocci, and (c) give IMViC (the first four tests.) patterns of ++-- (biotype 1) or +--- (biotype 2) are considered to be *E. coli*.

Alternatively, MPN determination of *E. coli*, fecal coliforms and coliforms protocols are given below.

**Note:** *Alternatively, instead of performing the IMViC tests, use commercially prepared biochemical strip tests. Use growth from PCA slants to perform these assays.*

### **MPN method (Alternative Method)**

#### MPN - Presumptive test for coliforms, fecal coliforms and *E. coli*

Weigh 50 g into sterile high-speed blender jar. Add 450 ml of Butterfield's phosphate-buffered water and blend for 2 min. If <50 g of sample are available, weigh portion that is equivalent to half of the sample and add sufficient volume of sterile diluent to make a 1:10 dilution. The total volume in the blender jar should completely cover the blades.

Prepare decimal dilutions with sterile Butterfield's phosphate diluent. The number of dilutions to be prepared depends on anticipated coliform density. Shake all suspensions 25

times in 30 cm arc or vortex mix for 7 s. Do not use pipets to deliver <10% of their total volume. Transfer 1 ml portions to three LST tubes for each dilution for at least three consecutive dilutions. Hold pipet at angle so that its lower edge rests against the tube. Let pipet drain 2-3 s. Not more than 15 min should elapse from time the sample is blended until all dilutions are inoculated in appropriate media.

Incubate LST tubes at 35°. Examine tubes and record reactions at 24 ± 2 h for gas, i.e., displacement of medium in fermentation vial or effervescence when tubes are gently agitated. Re-incubate gas-negative tubes for an additional 24 h and examine and record reactions again at 48 ± 2 h. Perform confirmed test on all presumptive positive (gas) tubes.

#### MPN - Confirmed test for coliforms

From each gassing LST tube, transfer a loopful of suspension to a tube of BGLB broth, avoiding pellicle if present. Incubate BGLB tubes at 35° and examine for gas production at 48 ± 2 h. Calculate most probable number (MPN) of coliforms based on proportion of confirmed gassing LST tubes for 3 consecutive dilutions.

#### MPN - Confirmed test for fecal coliforms and *E. coli*

From each gassing LST tube from the Presumptive test, transfer a loopful of suspension to a tube of EC broth (a sterile wooden applicator stick may also be used for these transfers). Incubate EC tubes 24 ± 2 h at 45.5° and examine for gas production. If negative, re-incubate and examine again at 48 ± 2 h. Use results of this test to calculate fecal coliform MPN. To continue with *E. coli* analysis, follow protocol for Completed test for *E. coli* (below).

**Note:** *Fecal coliform analyses are done at 45.5 ± 0.2°.*

#### MPN - Completed test for *E. coli*.

Gently agitate each gassing EC tube and streak for isolation, a loopful to a L-EMB agar plate and incubate for 18-24 h at 35°. Examine plates for suspicious *E. coli* colonies, i.e., dark centered and flat, with or without metallic sheen. Transfer up to five suspicious colonies from each L-EMB plate to PCA slants incubate for 18-24 h at 35° and use for further testing.

**Note:** *Identification of any one of the five colonies as *E. coli* is sufficient to regard that EC tube as positive; hence, not all five isolates may need to be tested.*

Perform Gram stain. All cultures appearing as Gram-negative short rods, should be tested for the IMViC reactions above and also re-inoculated back into LST to confirm gas production.

## ***Salmonella***

### Equipment and materials

1. Blender and sterile blender jars
2. Sterile, 16 oz (500 ml) wide-mouth, screw-cap jars, sterile 500 ml Erlenmeyer flasks, sterile 250 ml beakers, sterile glass or paper funnels of appropriate size.
3. Sterile, bent glass or plastic spreader rods
4. Balance, with weights; 2000 g capacity, sensitivity of 0.1 g
5. Balance, with weights; 120 g capacity, sensitivity of 5 mg
6. Incubator, 35 ± 2°
7. Refrigerated incubator or laboratory refrigerator, 4 ± 2°
8. Water bath, 49 ± 1°
9. Water bath, circulating, thermostatically-controlled, 43 ± 0.2°
10. Water bath, circulating, thermostatically-controlled, 42 ± 0.2°

11. Sterile spoons or other appropriate instruments for transferring food samples
12. Sterile culture dishes, 15 x 100 mm, glass or plastic
13. Sterile pipets, 1 ml, with 0.01 ml graduations; 5 and 10 ml, with 0.1 ml graduations
14. Inoculating needle and inoculating loop (about 3 mm id or 10 5l), nichrome, platinum-iridium, chromel wire, or sterile plastic
15. Sterile test or culture tubes, 16 x 150 mm and 20 x 150 mm; serological tubes, 10 x 75 mm or 13 x 100 mm
16. Test or culture tube racks
17. Vortex mixer
18. Sterile shears, large scissors, scalpel, and forceps
19. Lamp (for observing serological reactions)
20. Fisher or Bunsen burner
21. pH test paper (pH range 6-8) with maximum graduations of 0.4 pH units per color change
22. pH meter

#### Media and reagents

1. Lactose broth
2. Nonfat dry milk (reconstituted)
3. Selenite cystine (SC) broth
4. Tetrathionate (TT) broth
5. Rappaport-Vassiliadis (RV) medium
6. Xylose lysine desoxycholate (XLD) agar
7. Hektoen enteric (HE) agar
8. Bismuth sulfite (BS) agar
9. Triple sugar iron agar (TSI)
10. Tryptone (tryptophane) broth
11. Trypticase (tryptic) soy broth
12. Trypticase soy broth with ferrous sulfate
13. Lauryl tryptose (LST) broth
14. Trypticase soy-tryptose broth
15. MR-VP broth
16. Simmons citrate agar
17. Urea broth
18. Urea broth (rapid)
19. Malonate broth
20. Lysine iron agar (LIA) (Edwards and Fife)
21. Lysine decarboxylase broth
22. Motility test medium (semisolid)
23. Potassium cyanide (KCN) broth
24. Phenol red carbohydrate broth
25. Purple carbohydrate broth
26. MacConkey agar
27. Nutrient broth
28. Brain heart infusion (BHI) broth
29. Papain solution, 5%
30. Cellulase solution, 1%
31. Tryptose blood agar base
32. Universal preenrichment broth
33. Buffered peptone water
34. Potassium sulfite powder, anhydrous

35. Chlorine solution, 200 mg/kg, containing 0.1% sodium dodecyl sulfate
36. Ethanol, 70%
37. Kovacs' reagent
38. Voges-Proskauer (VP) test reagents
39. Creatine phosphate crystals
40. Potassium hydroxide solution, 40%
41. 1 N Sodium hydroxide solution
42. 1 N Hydrochloric acid
43. Brilliant green dye solution, 1%
44. Bromocresol purple dye solution, 0.2%
45. Methyl red indicator
46. Sterile distilled water
47. Tergitol Anionic 7
48. Triton X-100
49. Physiological saline solution, 0.85% (sterile)
50. Formalinized physiological saline solution
51. *Salmonella* polyvalent somatic (O) antiserum
52. *Salmonella* polyvalent flagellar (H) antiserum
53. *Salmonella* somatic group (O) antisera: A, B, C<sub>1</sub>, C<sub>2</sub>, C<sub>3</sub>, D<sub>1</sub>, D<sub>2</sub>, E<sub>1</sub>, E<sub>2</sub>, E<sub>3</sub>, E<sub>4</sub>, F, G, H, I, Vi, and other groups, as appropriate
54. *Salmonella* Spicer-Edwards flagellar (H) antisera

### Procedure

#### *Pre-enrichment*

Aseptically weigh 25 g sample into sterile, wide-mouth, screw-cap jar (500 ml) or other appropriate container. Add 225 ml sterile lactose broth and mix well, essentially preparing a 1:9 sample/broth ratio. Cap jar securely and let stand  $60 \pm 5$  min at room temperature. Mix well by swirling and determine pH with test paper. Adjust pH, if necessary, to  $6.8 \pm 0.2$ . Loosen jar cap about 1/4 turn and incubate  $24 \pm 2$  h at  $35^\circ$ .

#### *Special cases*

In some cases, the analysis of samples may be hampered by the viscosity of thickening agents. Additional treatment may be required.

- For gum ghatti, 0.1% of NaCl (final concentration) in lactose broth pre-enrichment medium, adjusted to pH 6.5, is added.
- For the analysis of gelatin, add 5 ml of a 5% papain solution (final concentration of 0.1%) in lactose broth pre-enrichment medium, mix well. Cap jar securely and incubate at  $35^\circ$  for  $60 \pm 5$  min. Mix by swirling and adjust to pH 6.8, if necessary. Loosen jar cap about 1/4 turn and incubate  $24 \pm 2$  h at  $35^\circ$ .
- For carob bean gum and guar gum, aseptically weigh 25 g sample into sterile beaker (250 ml) or other appropriate container. Prepare a 1.0% mannan endo-1,4-betamannosidase (EC 3.2.178) solution (add 1 g mannosidase to 99 ml sterile distilled water). Dispense into 150 ml bottles. Mannosidase solution may be stored at  $2-5^\circ\text{C}$  for up to 2 weeks. Add 225 ml sterile lactose broth and 2.25 ml sterile 1% mannosidase solution to sterile, wide-mouth, screw-cap jar (500 ml) or other appropriate container. While vigorously stirring the mannosidase/lactose broth with magnetic stirrer, pour 25 g analytical unit quickly through sterile glass funnel into the mannosidase/lactose broth. Cap jar securely and let stand  $60 \pm 5$  min at room

temperature. Incubate loosely capped container without pH adjustment, for  $24 \pm 2$  h at  $35^\circ$ .

#### *Enrichment*

For Carob Bean gum and Guar gum. Transfer 1 ml mixture to 10 ml selenite cystine (SC) broth and another 1 ml mixture to 10 ml TT broth. Vortex. Incubate SC and TT broths  $24 \pm 2$  h at  $35^\circ$ .

For all other samples. Transfer 0.1 ml mixture to 10 ml Rappaport-Vassiliadis (RV) medium and another 1 ml mixture to 10 ml tetrathionate (TT) broth. Vortex.

1. Incubate selective enrichment media as follows:

High microbial load. Incubate RV medium  $24 \pm 2$  h at  $42 \pm 0.2^\circ$  (circulating, thermostatically-controlled, water bath). Incubate TT broth  $24 \pm 2$  h at  $43 \pm 0.2^\circ$  (circulating, thermostatically-controlled, water bath).

Low microbial load (except carob bean gum and guar gum). Incubate RV medium  $24 \pm 2$  h at  $42 \pm 0.2^\circ\text{C}$  (circulating, thermostatically controlled, water bath). Incubate TT broth  $24 \pm 2$  h at  $35 \pm 2.0^\circ$ .

2. Mix (vortex, if tube) and streak 3 mm loopful ( $10 \mu\text{l}$ ) incubated TT broth on bismuth sulfite (BS) agar (prepare BS plates the day before streaking and store in the dark at room temperature), xylose lysine desoxycholate (XLD) agar, and Hektoen enteric (HE) agar.
3. Repeat with 3 mm loopful ( $10 \mu\text{l}$ ) of RV medium (for samples of high and low microbial load foods) and of SC broth (for guar gum).
4. For options of refrigerating incubated sample pre-enrichments and incubated sample selective enrichments (SC and TT broths only) of low moisture foods, see 994.04 in *Official Methods of Analysis*, AOAC International.
5. Incubate plates  $24 \pm 2$  h at  $35^\circ$ .
6. Examine plates for presence of colonies that may be *Salmonella*.

#### *Colony screening*

Examine plates as follows:

1. Typical *Salmonella* colony morphology

- Hektoen enteric (HE) agar: Blue-green to blue colonies with or without black centers. Many cultures of *Salmonella* may produce colonies with large, glossy black centers or may appear as almost completely black colonies. Atypically, a few *Salmonella* species produce yellow colonies with or without black centers.
- Bismuth sulfite (BS) agar: Typical *Salmonella* colonies may appear brown, grey, or black; sometimes they have a metallic sheen. Surrounding medium is usually brown at first, but may turn black in time with increased incubation, producing the so-called halo effect. Some strains may produce green colonies with little or no darkening of surrounding medium.
- Xylose lysine desoxycholate (XLD) agar: Pink colonies with or without black centers. Many cultures of *Salmonella* may have large, glossy black centers or may appear as almost completely black colonies. Atypically, a few *Salmonella* species produce yellow colonies with or without black centers.

2. Atypical *Salmonella* colony morphology



In the absence of typical or suspicious *Salmonella* colonies, search for atypical *Salmonella* colonies as follows:

- HE and XLD agars. Atypically a few *Salmonella* cultures produce yellow colonies with or without black centers on HE and XLD agars. In the absence of typical *Salmonella* colonies on HE or XLD agars after  $24 \pm 2$  h incubation, then pick two or more atypical *Salmonella* colonies.
- BS agar. Atypically some strains produce green colonies with little or no darkening of the surrounding medium. If typical or suspicious colonies are not present on BS agar after  $24 \pm 2$  h, then do not pick any colonies but re-incubate an additional  $24 \pm 2$  h. If typical or suspicious colonies are not present after  $48 \pm 2$  h incubation, then pick two or more atypical colonies.

### 3. Suggested control cultures

In addition to the positive control cultures (typical *Salmonella*), three additional *Salmonella* cultures are recommended to assist in the selection of atypical *Salmonella* colony morphology on selective agars. These cultures are a lactose-positive, H<sub>2</sub>S-positive *S. diarizonae* (ATCC 12325) and a lactose-negative, H<sub>2</sub>S-negative *S. abortus equi* (ATCC 9842); OR a lactose-positive, H<sub>2</sub>S-negative *S. diarizonae* (ATCC 29934). These cultures may be obtained from the American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209.

### *Agar slant analysis*

1. Select two or more colonies typical or suspected to be *Salmonella* from each selective agar. Inoculate into triple sugar iron (TSI) agar and lysine iron agar (LIA). If BS agar plates have no colonies typical or suspected to be *Salmonella* or no growth whatsoever, incubate them an additional 24 h. Lightly touch the very center of the colony to be picked with sterile inoculating needle and inoculate TSI agar slant by streaking slant and stabbing butt. Without flaming, inoculate LIA by stabbing butt twice and then streaking slant. Since lysine decarboxylation reaction is strictly anaerobic, the LIA slants must have deep butt (4 cm). Store picked selective agar plates at 5-8°.
2. Incubate TSI agar and LIA slants at 35° for  $24 \pm 2$  h. Cap tubes loosely to maintain aerobic conditions while incubating slants to prevent excessive H<sub>2</sub>S production. *Salmonella* in culture typically produces alkaline (red) slant and acid (yellow) butt, with or without production of H<sub>2</sub>S (blackening of agar) in TSI agar. In LIA, *Salmonella* typically produces alkaline (purple) reaction in butt of tube. Consider only distinct yellow in butt of tube as acidic (negative) reaction. Do not eliminate cultures that produce discoloration in butt of tube solely on this basis. Most *Salmonella* cultures produce H<sub>2</sub>S in LIA. Some non-*Salmonella* cultures produce a brick-red reaction in LIA slants.
3. If typical colonies are present on the BS agar after  $24 \pm 2$  h incubation, then pick two or more colonies. Irrespective of whether or not BS agar plates are picked at  $24 \pm 2$  h, re-incubate BS agar plates an additional  $24 \pm 2$  h. After  $48 \pm 2$  h incubation, pick two or more typical colonies, if present, from the BS agar plates, only if colonies picked from the BS agar plates incubated for  $24 \pm 2$  h give atypical reactions in triple sugar iron agar (TSI) and lysine iron agar (LIA) that result in culture being discarded as not being *Salmonella*.
4. All cultures that give an alkaline butt in LIA, regardless of TSI reaction, should be retained as potential *Salmonella* isolates and submitted for biochemical and serological tests. Cultures that give an acid butt in LIA and an alkaline slant and acid butt in TSI

should also be considered potential *Salmonella* isolates and should be submitted for biochemical and serological tests. Cultures that give an acid butt in LIA and an acid slant and acid butt in TSI may be discarded as not being *Salmonella*. Test retained, presumed-positive TSI cultures as directed below, to determine if they are *Salmonella* species, including *S. arizonae*. If TSI cultures fail to give typical reactions for *Salmonella* (alkaline slant and acid butt) pick additional suspicious colonies from selective medium plate not giving presumed-positive culture and inoculate TSI and LIA slants as described above.

5. Apply biochemical tests to:

- Three presumptive TSI agar cultures recovered from set of plates streaked from RV medium, if present, and presumptive TSI agar cultures recovered from plates streaked from tetrathionate broth, if present.
- If three presumptive-positive TSI cultures are not isolated from one set of agar plates, test other presumptive-positive TSI agar cultures, if isolated, by biochemical and serological tests. Examine a minimum of six TSI cultures for each 25 g analytical unit.

*Biochemical and Serological Testing for Salmonella*

1. Mixed cultures:

Streak TSI agar cultures that appear to be mixed on MacConkey agar, HE agar, or XLD agar. Incubate plates  $24 \pm 2$  h at  $35^\circ$ . Examine plates for presence of colonies suspected to be *Salmonella*, as follows:

- a. MacConkey agar. Typical colonies appear transparent and colourless, sometimes with dark center. Colonies of *Salmonella* will clear areas of precipitated bile caused by other organisms sometimes present.
- b. Hektoen enteric (HE) agar. See ‘*Typical Salmonella colony morphology*’, above, for procedure.
- c. Xylose lysine desoxycholate (XLD) agar. See ‘*Typical Salmonella colony morphology*’, above, for procedure.

Transfer at least two colonies suspected to be *Salmonella* to TSI agar and LIA slants as described above, and continue as under ‘*Agar slant analysis*’.

2. Pure cultures:

- a. Urease test (conventional). With sterile needle, inoculate growth from each presumed-positive TSI agar slant culture into tubes of urea broth. Since occasional, uninoculated tubes of urea broth turn purple-red (positive test) on standing, include uninoculated tube of this broth as control. Incubate  $24 \pm 2$  h at  $35^\circ$ .
- b. Optional urease test (rapid). Transfer two 3 mm loopfuls of growth from each presumed-positive TSI agar slant culture into tubes of rapid urea broth. Incubate 2 h in  $37 \pm 0.5^\circ$  water bath. Discard all cultures giving positive test. Retain for further study all cultures that give negative test (no change in colour of medium).

3. Serological polyvalent flagellar (H) test:

- a. Perform the polyvalent flagellar (H) test at this point, or later, as described below. Inoculate growth from each urease-negative TSI agar slant into 1) brain heart infusion broth and incubate 4-6 h at  $35^\circ$  until visible growth occurs (to test on same day); or 2)

trypticase soy-tryptose broth and incubate  $24 \pm 2$  h at  $35^\circ$  (to test on following day). Add 2.5 ml formalinized physiological saline solution to 5 ml of either broth culture.

b. Select two formalinized broth cultures and test with *Salmonella* polyvalent flagellar (H) antisera. Place 0.5 ml of appropriately diluted *Salmonella* polyvalent flagellar (H) antiserum in 10 x 75 mm or 13 x 100 mm serological test tube. Add 0.5 ml antigen to be tested. Prepare saline control by mixing 0.5 ml formalinized physiological saline solution with 0.5 ml formalinized antigen. Incubate mixtures in  $48-50^\circ$  water bath. Observe at 15 min intervals and read final results in 1 h.

Positive - agglutination in test mixture and no agglutination in control.

Negative - no agglutination in test mixture and no agglutination in control.

Nonspecific - agglutination in both test mixture and control. Test the cultures giving such results with Spicer-Edwards antisera, below.

4. Spicer-Edwards serological test:

Use this test as an alternative to the polyvalent flagellar (H) test. It may also be used with cultures giving non-specific agglutination in polyvalent flagellar (H) test. Perform Spicer-Edwards flagellar (H) antisera test as described above. Perform additional biochemical tests (below) on cultures giving positive flagellar test results. If both formalinized broth cultures are negative, perform serological tests on four additional broth cultures (above). If possible, obtain two positive cultures for additional biochemical testing. If all urease-negative TSI cultures from sample give negative serological flagellar (H) test results, perform additional biochemical tests.

5. Testing of urease-negative cultures:

a. Lysine decarboxylase broth. If LIA test was satisfactory, it need not be repeated. Use lysine decarboxylase broth for final determination of lysine decarboxylase if culture gives doubtful LIA reaction. Inoculate broth with small amount of growth from TSI agar slant suspicious for *Salmonella*. Replace cap tightly and incubate  $48 \pm 2$  h at  $35^\circ$  but examine at 24 h intervals. *Salmonella* species cause alkaline reaction indicated by purple colour throughout medium. Negative test is indicated by yellow colour throughout medium. If medium appears discoloured (neither purple nor yellow) add a few drops of 0.2% bromocresol purple dye and re-read tube reactions.

b. Phenol red dulcitol broth or purple broth base with 0.5% dulcitol. Inoculate broth with small amount of growth from TSI agar culture. Replace cap loosely and incubate  $48 \pm 2$  h at  $35^\circ$ , but examine after 24 h. Most *Salmonella* species give positive test, indicated by gas formation in inner fermentation vial and acid pH (yellow) of medium. Production of acid should be interpreted as a positive reaction. Negative test is indicated by no gas formation in inner fermentation vial and red (with phenol red as indicator) or purple (with bromocresol purple as indicator) colour throughout medium.

c. Tryptone (or tryptophane) broth. Inoculate broth with small amount of growth from TSI agar culture. Incubate  $24 \pm 2$  h at  $35^\circ$  and proceed as follows:

1) Potassium cyanide (KCN) broth. Transfer 3 mm loopful of 24 h tryptophane broth culture to KCN broth. Heat rim of tube so that good seal is formed when tube is stoppered with wax-coated cork. Incubate  $48 \pm 2$  h at  $35^\circ$  but examine after 24 h. Interpret growth (indicated by turbidity) as positive. Most *Salmonella* species do not grow in this medium, as indicated by lack of turbidity.

2) Malonate broth. Transfer 3 mm loopful of 24 h tryptone broth culture to malonate broth. Since occasional uninoculated tubes of malonate broth turn blue (positive test) on standing, include uninoculated tube of this broth as control. Incubate  $48 \pm 2$  h at  $35^\circ$ , but examine after 24 h. Most *Salmonella* species cultures give negative test (green or unchanged colour) in this broth.

3) Indole test. Transfer 5 ml of 24 h tryptophane broth culture to empty test tube. Add 0.2-0.3 ml Kovacs' reagent. Most *Salmonella* cultures give negative test (lack of deep red colour at surface of broth). Record intermediate, varying shades of orange and pink as  $\pm$ .

4) Serological flagellar (H) tests for *Salmonella*. If either polyvalent flagellar (H) test (above) or the Spicer-Edwards flagellar (H) test tube test (above) has not already been performed, either test may be performed here.

5) Discard as not *Salmonella* any culture that shows either positive indole test and negative serological flagellar (H) test, or positive KCN test and negative lysine decarboxylase test.

6. Serological somatic (O) tests for *Salmonella*:

**Note:** Pre-test all antisera to *Salmonella* with known cultures.

a. Polyvalent somatic (O) test.

Using wax pencil, mark off two sections about 1 x 2 cm each on inside of glass or plastic petri dish (15 x 100 mm). Commercially available sectioned slides may be used. Emulsify 3 mm loopful of culture from 24-48 h TSI slant or, preferably, tryptose blood agar base (without blood) with 2 ml 0.85% saline. Add 1 drop of culture suspension to upper portion of each rectangular crayon-marked section. Add 1 drop of saline solution to lower part of one section only. Add 1 drop of *Salmonella* polyvalent somatic (O) antiserum to other section only. With clean sterile transfer loop or needle, mix culture suspension with saline solution for one section and repeat for other section containing antiserum. Tilt mixtures in back-and-forth motion for 1 min and observe against dark background in good illumination. Consider any degree of agglutination a positive reaction. Classify polyvalent somatic (O) test results as follows:

Positive - agglutination in test mixture; no agglutination in saline control.

Negative - no agglutination in test mixture; no agglutination in saline control.

Nonspecific - agglutination in test and in control mixtures. Perform further biochemical and serological tests as described in *Edwards and Ewing's Identification of Enterobacteriaceae* (Ewing, W.H. 1986. Edwards and Ewing's Identification of Enterobacteriaceae, 4th ed. Elsevier, New York).

b. Somatic (O) group tests:

Test as in 6a, above, using individual group somatic (O) antisera including Vi, if available, in place of *Salmonella* polyvalent somatic (O) antiserum. For special treatment of cultures giving positive Vi agglutination reaction, refer to sec. 967.28B in *Official Methods of Analysis* (AOAC International). Record cultures that give positive agglutination with individual somatic (O) antiserum as positive for that group. Record cultures that do not react with individual somatic (O) antiserum as negative for that group.

Table 2. Summary of biochemical and serological reactions of *Salmonella*

Test or substrate	Positive	Negative	species reactions <sup>a</sup>
1. Glucose (TSI)	yellow butt	red butt	+
2. Lysine decarboxylase (LIA)	purple butt	yellow butt	+
3. H <sub>2</sub> S (TSI and LIA)	blackening	no blackening	+
4. Urease	purple-red colour	no colour change	-
5. Lysine decarboxylase broth	purple colour	yellow colour	+
6. Phenol red dulcitol broth	yellow colour and/or gas	no gas; no colour change	+ <sup>b</sup>
7. KCN broth	growth	no growth	-
8. Malonate broth	blue colour at surface	no colour change	- <sup>c</sup>
9. Indole test	deep red colour at surface	yellow colour at surface	-
10. Polyvalent flagellar test	agglutination	no agglutination	+
11. Polyvalent somatic test	agglutination	no agglutination	+
12. Phenol red lactose broth	yellow colour and/or gas	no gas; no colour change	- <sup>c</sup>
13. Phenol red sucrose broth	yellow colour and/or gas	no gas; no colour change	-
14. Voges-Proskauer test	pink-to-red colour	no colour change	-
15. Methyl red test	diffuse red colour	diffuse yellow colour	+
16. Simmons citrate	growth; blue colour	no growth; no colour change	v

<sup>a</sup>+ is 90% or more positive in 1 or 2 days; - is 90% or more negative in 1 or 2 days; v is variable.

<sup>b</sup>Majority of *S. arizonae* cultures are negative.

<sup>c</sup>Majority of *S. arizonae* cultures are positive.

Classify as *Salmonella* those cultures which exhibit typical *Salmonella* reactions for test Nos. 1-11, shown in Table 2, above. If one TSI culture from 25 g sample is classified as *Salmonella*, further testing of other TSI cultures from the same 25 g sample is unnecessary. Cultures that contain demonstrable *Salmonella* antigens as shown by positive *Salmonella* flagellar (H) test but do not have biochemical characteristics of *Salmonella* should be purified and retested.

Table 3. Criteria for discarding non-*Salmonella* cultures

Test or substrate	Results
1. Urease	positive (purple-red colour)
2. Indole test and Polyvalent flagellar (H) test or Spicer-Edwards flagellar test	positive (violet colour at surface) negative (no agglutination)
3. Lysine decarboxylaseKCN broth	negative (yellow colour) positive (growth)
4. Phenol red lactose broth	positive (yellow colour and/or gas) <sup>a, b</sup>
5. Phenol red sucrose broth	positive (yellow colour and/or gas) <sup>b</sup>
6. KCN broth Voges-Proskauer test Methyl red test	positive (growth) positive (pink-to-red colour) negative (diffuse yellow colour)

<sup>a</sup> Test malonate broth positive cultures further to determine if they are *Salmonella arizonae*.

<sup>b</sup> Do not discard positive broth cultures if corresponding LIA cultures give typical *Salmonella* reactions; test further to determine if they are *Salmonella* species.

#### *Atypical Salmonella Colony Testing*

Perform the following additional biochemical tests on cultures that do not give typical *Salmonella* reactions for test Nos. 1-11 in Table 12, above, and that consequently do not classify as *Salmonella* (see Table 23, also above).

##### a. Phenol red lactose broth or purple lactose broth

1) Inoculate broth with small amount of growth from unclassified 24-48 h TSI agar slant. Incubate  $48 \pm 2$  h at  $35^\circ$ , but examine after 24 h. Positive--acid production (yellow colour) and gas production in inner fermentation vial. Consider production of acid only as positive reaction. Most cultures of *Salmonella* give negative test result, indicated by no gas formation in inner fermentation vial and red (with phenol red as indicator) or purple (with bromcresol purple as indicator) colour throughout medium.

2) Discard as not *Salmonella* cultures that give positive lactose tests, except cultures that give acid slants in TSI and positive reactions in LIA or cultures that give positive malonate broth reactions. Perform further tests on these cultures to determine if they are *S. arizonae*.

##### b. Phenol red sucrose broth or purple sucrose broth

Follow procedure described as directly above. Discard as not *Salmonella*, cultures that give positive sucrose tests, except those that give acid slants in TSI and positive reactions in LIA.

##### c. MR-VP broth

Inoculate medium with small amount of growth from each unclassified TSI slant suspected to contain *Salmonella*. Incubate  $48 \pm 2$  h at  $35^\circ$ .

1) Perform Voges-Proskauer (VP) test at room temperature as follows: Transfer 1 ml 48 h culture to test tube and incubate remainder of MR-VP broth an additional 48 h at  $35^\circ$ . Add 0.6 ml  $\alpha$ -naphthol and shake well. Add 0.2 ml 40% KOH solution and shake. To intensify and speed reaction, add a few crystals of creatine. Read results after 4 h; development of pink-to-ruby red color throughout medium is positive test. Most cultures

of *Salmonella* are VP-negative, indicated by absence of development of pink-to-red color throughout broth.

2) Perform methyl red test as follows: To 5 ml of 96 h MR-VP broth, add 5-6 drops of methyl red indicator. Read results immediately. Most *Salmonella* cultures give positive test, indicated by diffuse red color in medium. A distinct yellow color is negative test. Discard, as not *Salmonella*, cultures that give positive KCN and VP tests and negative methyl red test.

d. Simmons citrate agar

Inoculate this agar, using needle containing growth from unclassified TSI agar slant. Inoculate by streaking slant and stabbing butt. Incubate  $96 \pm 2$  h at 35°C. Read results as follows:

Positive - presence of growth, usually accompanied by color change from green to blue. Most cultures of *Salmonella* are citrate-positive.

Negative - no growth or very little growth and no color change.

*Alternative Method for Identification of Salmonella*

As alternative to conventional biochemical tube system, use any of 5 commercial biochemical systems (API 20E, Enterotube II, *Enterobacteriaceae* II, MICRO-ID, or Vitek GNI) for presumptive generic identification of *Salmonella*. Choose a commercial system based on a demonstration in the analyst's own laboratory of adequate correlation between commercial system and the biochemical tube system outlined in this identification section.

Commercial biochemical kits should not be used as a substitute for serological tests.

Assemble supplies and prepare reagents required for the kit. Inoculate each unit according to Method 978.24 (API 20E, Enterotube II, and *Enterobacteriaceae* II), sec. 989.12 (MICRO-ID), and Method 991.13 (Vitek GNI) in *Official Methods of Analysis*, incubating for time and temperature specified. Add reagents, observe, and record results. For presumptive identification, classify cultures, according to *Official Methods of Analysis* (AOAC International) as *Salmonella* or not *Salmonella*.

For confirmation of cultures presumptively identified as *Salmonella*, perform the *Salmonella* serological somatic (O) test and the *Salmonella* serological flagellar (H) test or the Spicer-Edwards flagellar (H) test and classify cultures according to the following guidelines:

- a. Report as *Salmonella* those cultures classified as presumptive *Salmonella* with commercial biochemical kits when the culture demonstrates positive *Salmonella* somatic (O) test and positive *Salmonella* (H) test.
- b. Discard cultures presumptively classified as not *Salmonella* with commercial biochemical kits when cultures conform to AOAC criteria for classifying cultures as not *Salmonella*.
- c. For cultures that do not conform to a or b, classify according to additional tests specified above, or additional tests as specified by Ewing, or send to a reference typing laboratory for definitive serotyping and identification.

*Treatment of cultures giving negative flagellar (H) test.*

If biochemical reactions of certain flagellar (H)-negative culture strongly suggest that it is *Salmonella*, the negative flagellar agglutination may be the result of non-motile organisms or insufficient development of flagellar antigen. Proceed as follows: Inoculate motility test

medium in petri dish, using small amount of growth from TSI slant. Inoculate by stabbing medium once about 10 mm from edge of plate to depth of 2-3 mm. Do not stab to bottom of plate or inoculate any other portion. Incubate 24 h at 35°C. If organisms have migrated 40 mm or more, retest as follows: Transfer 3 mm loopful of growth that migrated farthest to trypticase soy-tryptose broth. Repeat either polyvalent flagellar (H) or Spicer-Edwards serological tests. If cultures are not motile after the first 24 h, incubate an additional 24 h at 35°C; if still not motile, incubate up to 5 days at 25°C. Classify culture as non-motile if above tests are still negative.

### ***Staphylococcus aureus***

#### **Direct Plate Count Method**

**Note:** *This method is suitable for the analysis in which more than 100 S. aureus cells/g may be expected. If the analyst suspects that the number of S. aureus cells is below this limit, then the MPN method should be used. If unknown, both procedures can be used.*

#### Equipment and materials

1. Drying cabinet, laminar air flow or a well-ventilated room that is free of dust and draft with microbial density of the air in working area not exceeding 15 colonies per plate during a 15-minute exposure.
2. Petri dishes, plastic (15 x 90 mm) or glass (15 x 100 mm)
3. Pipets, 1, 5, and 10 ml, graduated in 0.1 ml units
4. Incubator, 35 ± 1°
5. Sterile bent glass streaking rods or hockey stick, 3-4 mm diameter, 15-20 cm long with an angled spreading surface 45-55 mm long
6. Colony counter, dark-field, Quebec or equivalent, with suitable light source and grid plate.
7. Tally register
8. Sterile test tubes (13 x 100 mm)

#### Media and reagents

1. Trypticase (tryptone) soy agar (TSA)
2. Baird-Parker medium
3. Sterile coagulase plasma (rabbit) with EDTA (commercially available)
4. Lysostaphin solution
5. Hydrogen peroxide (3%, v/v)
6. Tolidine blue-deoxyribonucleic (DNA) acid agar
7. 0.02 M phosphate-saline buffer containing 1% NaCl
8. Trypticase (tryptic) soy broth (TSB) containing 10% NaCl and 1% sodium pyruvate
9. Paraffin oil, sterile
10. Phenol Red Carbohydrate Broth

#### Sample preparation

Under aseptic conditions, prepare serial dilutions of sample by transferring 10 ml of previous dilution to 90 ml of diluent using separate pipets. Avoid sample foam. Shake all dilutions 25 times in 30 cm (1 ft) arc within 7 seconds.

#### Isolation



For each dilution to be plated, aseptically transfer 1 ml sample suspension to 3 plates of Baird-Parker agar, distributing 1 ml of inoculum equitably to 3 plates (e.g., 0.4 ml, 0.3 ml, and 0.3 ml). Spread inoculum over surface of agar plate, using sterile bent glass streaking rod. Retain plates in upright position until inoculum is absorbed by agar (about 10 min on properly dried plates). If inoculum is not readily adsorbed, place plates upright in incubator for about 1 h. Invert plates and incubate 45-48 h at 35°. Select plates containing 20-200 colonies, unless only plates at lower dilutions (>200 colonies) have colonies with typical appearance of *S. aureus*. Colonies of *S. aureus* are circular, smooth, convex, moist, 2-3 mm in diameter on uncrowded plates, grey to jet-black, frequently with light-coloured (off-white) margin, surrounded by opaque zone and frequently with an outer clear zone; colonies have buttery to gummy consistency when touched with inoculating needle. Occasionally from various foods and dairy products, nonlipolytic strains of similar appearance may be encountered, except that surrounding opaque and clear zones are absent. Strains isolated from frozen or desiccated foods that have been stored for extended periods frequently develop less black coloration than typical colonies and may have rough appearance and dry texture.

#### Enumeration

Count and record colonies. If several types of colonies are observed which appear to be *S. aureus* on selected plates, count number of colonies of each type and record counts separately. When plates of the lowest dilution contain <20 colonies, these may be used. If plates containing >200 colonies have colonies with the typical appearance of *S. aureus* and typical colonies do not appear at higher dilutions, use these plates for the enumeration of *S. aureus*, but do not count nontypical colonies. Select > 1 colony of each type counted and test for coagulase production. Add number of colonies on triplicate plates represented by colonies giving positive coagulase test and multiply by the sample dilution factor. Report this number as number of *S. aureus*/g of food tested.

#### Identification of *S. aureus*

##### *a. Coagulase test*

Transfer suspect *S. aureus* colonies into small tubes containing 0.2-0.3 ml TSB containing 10% NaCl and 1% sodium pyruvate broth and emulsify thoroughly. Inoculate agar slant of suitable maintenance medium, e.g., TSA, with loopful of TSB suspension. Incubate TSB culture suspension and slants 18-24 h at 35°. Retain slant cultures at room temperature for ancillary or repeat tests in case coagulase test results are questionable. Add 0.5 ml reconstituted coagulase plasma with EDTA (B-4, above) to the TSB culture and mix thoroughly. Incubate at 35° and examine periodically over a 6 h period for clot formation. Only firm and complete clot that stays in place when tube is tilted or inverted is considered positive for *S. aureus*. Partial clotting, formerly 2+ and 3+ coagulase reactions, must be tested further (for this method see: Sperber, W.H. and Tatini, S.R. 1975. Interpretation of the tube coagulase test for identification of *Staphylococcus aureus*. Appl. Microbiol. 29:502-505). Test known positive and negative cultures simultaneously with suspect cultures of unknown coagulase activity. Stain all suspect cultures with Gram reagent and observe microscopically. A latex agglutination test (AUREUS TEST™, Trisum Corp., Taipei, Taiwan) may be substituted for the coagulase test if a more rapid procedure is desired.

#### Ancillary identification tests

##### *a. Catalase test*

On a clean glass slide or spot plate, emulsify growth from TSA slant in 3% hydrogen peroxide. Production of gas bubbles shows a positive reaction. Include known positive and negative cultures.

b. *Anaerobic utilization of glucose*

Inoculate tube of Phenol Red Carbohydrate broth containing glucose (0.5%).

Immediately inoculate each tube heavily with wire loop. Make certain inoculum reaches bottom of tube. Cover surface of agar with layer of sterile paraffin oil at least 25 mm thick. Incubate 5 days at 35°. Acid is produced anaerobically if indicator changes to yellow throughout tube, indicating presence of *S. aureus*. Run controls simultaneously (positive and negative cultures and medium controls).

c. *Anaerobic utilization of mannitol*

Repeat b, above, using mannitol as carbohydrate in medium. *S. aureus* is usually positive but some strains are negative. Run controls simultaneously.

d. *Lysostaphin sensitivity*

Transfer isolated colony from agar plate with inoculating loop to 0.2 ml phosphate-saline buffer, and emulsify. Transfer half of suspended cells to another tube (13 x 100 mm) and mix with 0.1 ml phosphate-saline buffer as control. Add 0.1 ml lysostaphin (dissolved in 100 ml of 0.02 M phosphate-saline buffer containing 1% NaCl for a final concentration of 25 µg lysostaphin/ml) to original tube. Incubate both tubes at 35° for not more than 2 h. If turbidity clears in test mixture, test is considered positive. If clearing has not occurred in 2 h, test is negative. *S. aureus* is generally positive.

e. *Thermostable nuclease production*

This test is claimed to be as specific as the coagulase test but less subjective, because it involves a colour change from blue to bright pink. It is not a substitute for the coagulase test but rather is a supportive test, particularly for 2+ coagulase reactions. Prepare microslides by spreading 3 ml toluidine blue-deoxyribonucleic acid agar on the surface of each microscope slide. When agar has solidified, cut 2 mm diameter wells (10-12 per slide) in agar and remove agar plug by aspiration. Add about 0.01 ml of heated sample (15 min in boiling water bath) of broth cultures used for coagulase test to well on prepared slide. Incubate slides in moist chamber 4 h at 35°. Development of bright pink halo extending at least 1 mm from periphery of well indicates a positive reaction.

### Characteristics

Some typical characteristics of two species of staphylococci and the micrococci, which may be helpful in their identification, are listed in Table 1.

<b>Table 1. Typical characteristics of <i>S. aureus</i>, <i>S. epidermidis</i>, and Micrococci<sup>(a)</sup></b>			
<b>Characteristic</b>	<b><i>S. aureus</i></b>	<b><i>S. epidermidis</i></b>	<b>Micrococci</b>
Catalase activity	+	+	+
Coagulase production	+	-	-
Thermonuclease production	+	-	-
Lysostaphin sensitivity	+	+	-
<b>Anaerobic utilization of</b>			
glucose	+	+	-
mannitol	+	-	-
<sup>a</sup> +, Most (90% or more) strains are positive; -, most (90% or more) strains are negative.			

### **MPN method**

**Note:** *The Most Probable Number (MPN) method is recommended for routine surveillance of products in which small numbers of *S. aureus* are expected and in foods expected to contain a large population of competing species.*

Equipment and materials - Same as for Direct Plate Count Method.

Media and reagents - Same as for Direct Plate Count Method. Also required: Trypticase (tryptic) soy broth (TSB) containing 10% NaCl and 1% sodium pyruvate.

Preparation of sample - Same as for Direct Plate Count Method.

#### Determination of MPN

Inoculate 3 tubes of TSB containing 10% NaCl and 1% sodium pyruvate with 1 ml portions of decimal dilutions of each sample. Highest dilution must give negative endpoint. Incubate tubes  $48 \pm 2$  h at 35°. Using 3 mm loop, transfer 1 loopful from each tube showing growth (turbidity) to plate of Baird-Parker medium with properly dried surface. Vortex-mix tubes before streaking if growth is visible only on bottom or sides of tubes. Streak inoculum to obtain isolated colonies. Incubate plates 48 h at 35°. From each plate showing growth, transfer at least 1 colony suspected to be *S. aureus* to TSB broth (see C of Direct Plate Count Method above). Continue procedure for identification and confirmation of *S. aureus* (see d of Direct Plate Count, above).

### **Enumeration of Yeasts and Moulds**

#### Equipment and materials

1. Basic equipment (and appropriate techniques) for preparation of sample homogenate
2. Equipment for plating samples
3. Incubator, 25°
4. Arnold steam chest

5. pH meter
6. Water bath,  $45 \pm 1^\circ$

### Media and reagents

1. Potato dextrose agar (PDA)
2. Malt extract agar
3. Malt agar (MA)
4. Plate count agar (PCA), standard methods; add 100 mg chloramphenicol/liter when this medium is used for yeast and mould enumeration. This medium is not efficient when "spreader" moulds are present.
5. Antibiotic solutions
6. Dichloran rose bengal chloramphenicol (DRBC) agar
7. Dichloran 18% glycerol (DG18) agar

### Procedures

#### *Antibiotics*

Antibiotics are added to mycological media to inhibit bacterial growth. Chloramphenicol is the antibiotic of choice, because it is stable under autoclave conditions. Therefore, media preparation is easier and faster due to the elimination of the filtration step. The recommended concentration of this antibiotic is 100 mg/liter medium. If bacterial overgrowth is apparent, prepare media by adding 50 mg/liter chloramphenicol before autoclaving and 50 mg/liter filter-sterilized chlortetracycline when the media have been tempered, right before pouring plates.

Prepare stock solution by dissolving 0.1 g chloramphenicol in 40 ml distilled water; add this solution to 960 ml medium mixture before autoclaving. When both chloramphenicol and chlortetracycline are used, add 20 ml of the above chloramphenicol stock solution to 970 ml medium before autoclaving. Then, prepare chlortetracycline stock solution by dissolving 0.5 g antibiotic in 100 ml distilled water and filter sterilize. Use 10 ml of this solution for each 990 ml of autoclaved and tempered medium. Refrigerate in the dark and re-use remaining stock solutions for up to a month. Stock solutions should be brought to room temperature before adding to tempered medium.

#### *Sample Preparation*

Analyze 25-50 g from each subsample; generally, larger sample sizes increase reproducibility and lower variance compared with small samples. Add appropriate amount of 0.1% peptone water to the weighed sample to achieve  $10^{-1}$  dilution, then homogenize in a stomacher for two min. Alternatively, blending for 30-60 sec can be used but is less effective. Make appropriate 1:10 (1+9) dilutions in 0.1% peptone water. Dilutions of  $10^{-6}$  should suffice.

#### *Plating and incubation of samples*

Spread-plate method: Aseptically pipet 0.1 ml of each dilution on pre-poured, solidified DRBC agar plates and spread inoculum with a sterile, bent glass rod. DG18 is preferred when the water activity of the analyzed sample is less than 0.95. Plate each dilution in triplicate.

Pour-plate method: Use sterile cotton-plugged pipet to place 1 ml portions of sample dilutions into prelabelled 15 x 100 mm petri plates (plastic or glass), and immediately add 20-25 ml tempered DG18 agar medium. Mix contents by gently swirling plates clockwise then counter clockwise, taking care to avoid spillage on dish lid. Add agar within 1-2 min after adding dilution. Otherwise, dilution may begin to adhere to dish bottom (especially if sample

is high in starch content and dishes are plastic) and may not mix uniformly. Plate each dilution in triplicate, using wide bore pipets. From preparation of first sample dilution to pouring of final plate, no more than 20 min, preferably 10 min, should elapse.

Incubate plates in dark at 25°. Do not stack plates higher than 3 and do not invert. Let plates remain undisturbed until counting.

#### *Counting of plates*

Count plates after 5 days of incubation. If there is no growth at 5 days, re-incubate for another 48 h. Do not count colonies before the end of the incubation period because handling of plates could result in secondary growth from dislodged spores, making final counts invalid. Count plates containing 10-150 colonies. If mainly yeasts are present, plates with 150 colonies are usually countable. However, if substantial amounts of mould are present, depending on the type of mold, the upper countable limit may have to be lowered at the discretion of the analyst. Report results in colony forming units (CFU)/g or CFU/ml based on average count of triplicate set. Round off counts to two significant figures. If third digit is 6 or above, round off to digit above (e.g., 456 = 460); if 4 or below, round off to digit below (e.g., 454 = 450). If third digit is 5, round off to digit below if first two digits are an even number (e.g., 445 = 440); round off to digit above if last two digits are an odd number (e.g., 455 = 460). When plates from all dilutions have no colonies, report mould and yeast counts (MYC) as less than 1 times the lowest dilution used.



## SPECIFIC METHODS

### ENZYME PREPARATIONS

#### **$\alpha$ -Amylase Activity (Bacterial)**

##### Application and Principle

This procedure is used to determine the  $\alpha$ -amylase activity, expressed as bacterial amylase units (BAU), of enzyme preparations derived from *Bacillus subtilis* var., *Bacillus licheniformis* var., and *Bacillus stearothermophilus*. It is not applicable to products that contain  $\beta$ -amylase. The assay is based on the time required to obtain a standard degree of hydrolysis of a starch solution at  $30 \pm 0.1^\circ$ . The degree of hydrolysis is determined by comparing the iodine colour of the hydrolysate with that of a standard.

##### Apparatus

Use the Reference Colour Standard, the Comparator, and the Comparator Tubes as described under  $\alpha$ -Amylase Activity, Fungal, but use either daylight or daylight-type fluorescent lamps as the light source for the Comparator. (Incandescent lamps give slightly lower results.)

##### Reagents and Solutions

*pH 6.6 Buffer*: Dissolve 9.1 g of potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) in sufficient water to make 1000 ml (Solution A). Dissolve 9.5 g of dibasic sodium phosphate ( $\text{Na}_2\text{HPO}_4$ ) in sufficient water to make 1000 ml (Solution B). Add 400 ml of Solution A to 600 ml of Solution B, mix, and adjust the pH to 6.6, if necessary, by the addition of Solution A or Solution B as required.

*Dilute Iodine Solution*: Prepare as directed under  $\alpha$ -Amylase Activity, Fungal.

*Special Starch*: Use the material described under  $\alpha$ -Amylase Activity, Fungal.

*Starch Substrate Solution*: Disperse 10.0 g (dry-weight basis) of Special Starch in 100 ml of cold water, and slowly pour the mixture into 300 ml of boiling water. Boil and stir for 1 to 2 min, and then cool while continuously stirring. Quantitatively transfer the mixture into a 500 ml volumetric flask with the aid of water, add 10 ml of pH 6.6 Buffer, dilute to volume with water, and mix.

*Sample Preparation*: Prepare a solution of the sample so that 10 ml of the final dilution will give an endpoint between 15 and 35 min under the conditions of the assay.

##### Procedure

Pipet 5.0 ml of Dilute Iodine Solution into a series of  $13 \times 100$ -mm test tubes, and place them in a water bath maintained at  $30 \pm 0.1^\circ$ , allowing 20 tubes for each assay. Pipet 20.0 ml of the Starch Substrate Solution into a 50 ml Erlenmeyer flask, stopper, and equilibrate for 20 min in the water bath at  $30^\circ$ . At zero time, rapidly pipet 10.0 ml of the Sample Preparation into the equilibrated mixture, and continue as directed in the Procedure under  $\alpha$ -Amylase Activity, Fungal, beginning with “. . . mix immediately by swirling, stopper the flask. . . .”

##### Calculation

One bacterial amylase unit (BAU) is defined as that quantity of enzyme that will dextrinize starch at the rate of 1 mg/min under the specified test conditions.

Calculate the  $\alpha$ -amylase activity of the sample, expressed as BAU, by the formula

$$\text{BAU/g} = 40F/T,$$

in which 40 is a factor (400/10) derived from the 400 mg of starch (20 ml of a 2% solution) and the 10 ml aliquot of Sample Preparation used;  $F$  is the dilution factor (total dilution volume/sample weight, in grams); and  $T$  is the dextrinizing time, in min.

## **$\alpha$ -Amylase Activity (Fungal)**

### Application and Principle

This procedure is used to determine the  $\alpha$ -amylase activity of enzyme preparations derived from *Aspergillus niger* var.; *Aspergillus oryzae* var.; *Rhizopus oryzae* var.; (and barley malt). The assay is based on the time required to obtain a standard degree of hydrolysis of a starch solution at  $30 \pm 0.1^\circ$ . The degree of hydrolysis is determined by comparing the iodine colour of the hydrolysate with that of a standard.

### Apparatus

*Reference Colour Standard:* Use a special  $\alpha$ -Amylase Color Disk (Orbeco Analytical Systems, 185 Marine Street, Farmingdale, NY 11735, Catalog No. 620-S5 or similar). Alternatively, prepare a colour standard by dissolving 25.0 g of cobaltous chloride ( $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ) and 3.84 g of potassium dichromate in 100 ml of 0.01 N hydrochloric acid. This standard is stable indefinitely when stored in a stoppered bottle or comparator tube.

*Comparator:* Use either the standard Hellige comparator (Orbeco, Catalog No. 607) or the pocket comparator with prism attachment (Orbeco, Catalog No. 605AHT) or similar. The comparator should be illuminated with a 100-W frosted lamp placed 6 in. from the rear opal glass of the comparator and mounted so that direct rays from the lamp do not shine into the operator's eyes.

*Comparator Tubes:* Use the precision-bored square tubes with a 13-mm viewing depth that are supplied with the Hellige comparator. Suitable tubes are also available from other apparatus suppliers.

### Reagents and Solutions

*Buffer Solution (pH 4.8):* Dissolve 164 g of anhydrous sodium acetate in about 500 ml of water, add 120 ml of glacial acetic acid, and adjust the pH to 4.8 with glacial acetic acid. Dilute to 1000 ml with water, and mix.

*$\beta$ -Amylase Solution:* Dissolve into 5 ml of water a quantity of  $\beta$ -amylase, free from  $\alpha$ -amylase activity (Sigma Chemical Co., Catalog No. A7005 or equivalent), equivalent to 250 mg of  $\beta$ -amylase with 2000° diastatic power.

*Special Starch:* Use starch designated as "Starch (Lintner) Soluble" (Baker Analyzed Reagent, Catalog No. 4010 or equivalent). Before using new batches, test them in parallel with previous lots known to be satisfactory. Variations of more than  $\pm 3^\circ$  diastatic power in the averages of a series of parallel tests indicate an unsuitable batch.

*Buffered Substrate Solution:* Disperse 10.0 g (dry-weight basis) of Special Starch in 100 ml of cold water, and slowly pour the mixture into 300 ml of boiling water. Boil and stir for 1 to 2 min, then cool, and add 25 ml of Buffer Solution, followed by all of the  $\beta$ -Amylase Solution. Quantitatively transfer the mixture into a 500 ml volumetric flask with the aid of water saturated with toluene, dilute to volume with the same solvent, and mix. Store the solution at  $30^\circ \pm 2^\circ$  for not less than 18 h but not more than 72 h before use. (This solution is also known as "buffered limit dextrin substrate.")



*Stock Iodine Solution:* Dissolve 5.5 g of iodine and 11.0 g of potassium iodide in about 200 ml of water, dilute to 250 ml with water, and mix. Store in a dark bottle, and make a fresh solution every 30 days.

*Dilute Iodine Solution:* Dissolve 20 g of potassium iodide in 300 ml of water, and add 2.0 ml of Stock Iodine Solution. Quantitatively transfer the mixture into a 500 ml volumetric flask, dilute to volume with water, and mix. Prepare daily.

#### Sample Preparation

Prepare a solution of the sample so that 5 ml of the final dilution will give an endpoint between 10 and 30 min under the conditions of the assay.

For barley malt, finely grind 25 g of the sample in a Miag-Seck mill (Buhler-Miag, Inc., P.O. Box 9497, Minneapolis, MN 55440 or similar). Quantitatively transfer the powder into a 1000 ml Erlenmeyer flask, add 500 ml of a 0.5% solution of sodium chloride, and allow the infusion to stand for 2.5 h at  $30^{\circ} \pm 0.2^{\circ}$ , agitating the contents by gently rotating the flask at 20-min intervals.

**Caution:** *Do not mix the infusion by inverting the flask. The quantity of the grist left adhering to the inner walls of the flask as a result of agitation must be as small as possible.*

Filter the infusion through a 32-cm fluted filter of Whatman No. 1, or equivalent, paper on a 20-cm funnel, returning the first 50 ml of filtrate to the filter. Collect the filtrate until 3 h have elapsed from the time the sodium chloride solution and the sample were first mixed. Pipet 20.0 ml of the filtered infusion into a 100 ml volumetric flask, dilute to volume with the 0.5% sodium chloride solution, and mix.

#### Procedure

Pipet 5.0 ml of Dilute Iodine Solution into a series of  $13 \times 100$ -mm test tubes, and place them in a water bath maintained at  $30^{\circ} \pm 0.1^{\circ}$ , allowing 20 tubes for each assay.

Pipet 20.0 ml of the Buffered Substrate Solution, previously heated in the water bath for 20 min, into a 50 ml Erlenmeyer flask, and add 5.0 ml of 0.5% sodium chloride solution, also previously heated in the water bath for 20 min. Place the flask in the water bath.

At zero time, rapidly pipet 5.0 ml of the Sample Preparation into the equilibrated substrate, mix immediately by swirling, stopper the flask, and place it back in the water bath. After 10 min, transfer 1.0 ml of the reaction mixture from the 50 ml flask into one of the test tubes containing the Dilute Iodine Solution, shake the tube, then pour its contents into a Comparator Tube, and immediately compare with the Reference Colour Standard in the Comparator, using a tube of water behind the colour disk.

**Note:** *Be certain that the pipet tip does not touch the iodine solution as carry-back of iodine to the hydrolyzing mixture will interfere with enzyme action and will affect the results of the determination.*

In the same manner, repeat the transfer and comparison procedure at accurately timed intervals until the  $\alpha$ -amylase colour is reached, at which time record the elapsed time. In cases where two comparisons 30 s apart show that one is darker and the other lighter than the Reference Colour Standard, record the endpoint to the nearest quarter min. Shake out the 13-mm Comparator Tube between successive readings. Minimize slight differences in colour discrimination between operators by using a prism attachment and by maintaining a 15- to 25-cm. distance between the Comparator and the operator's eye.

### Calculation

One  $\alpha$ -amylase dextrinizing unit (DU) is defined as the quantity of  $\alpha$ -amylase that will dextrinize soluble starch in the presence of an excess of  $\beta$ -amylase at the rate of 1 g/h at 30°.

Calculate the  $\alpha$ -amylase dextrinizing units in the sample as follows:

$$\text{DU (solution)} = 24/(W \times T), \text{ and}$$

$$\text{DU (dry basis)} = \text{DU (solution)} \times 100/(100 - M),$$

in which W is the weight, in grams, of the enzyme sample added to the incubation mixture in the 5 ml aliquot of the Sample Preparation used; T is the elapsed dextrinizing time, in minutes; 24 is the product of the weight of the starch substrate (0.4 g) and 60 min; and M is the percent moisture in the sample, determined by suitable means.

## **Antibacterial Activity**

### Scope

This procedure is designed for the determination of antibacterial activity in enzyme preparation derived from microbial sources.

### Principle

The assay is based on the measurement of inhibition of bacterial growth under specific circumstances.

### Culture Plates

Six organisms are tested: *Staphylococcus aureus* (ATCC 6538); *Escherichia coli* (ATCC 11229); *Bacillus cereus* (ATCC 2); *Bacillus circulans* (ATCC 4516); *Streptococcus pyrogenes* (ATCC 12344); and *Serratia marcescens* (ATCC 14041).

Make a test plate of each organism by preparing a 1:10 dilution of a 24 h Trypticase Soya Broth culture in Trypticase Agar (TSA) (for *Streptococcus pyrogenes* a 1:20 dilution).

Pour 15 ml of plain TSA into a Petri dish and allow the medium to harden. Overlay with 10 ml of seeded TSA and allow to solidify. Place a paper disk prepared according to Disk Preparation of the tested enzyme on each of the six inoculated plates.

### Disk Preparation

Make a 10% solution of the enzyme by adding 1 g of enzyme to 9 ml of sterile, distilled water.

Mix thoroughly with a Vortex mixer to obtain a homogeneous suspension. Autoclave suitable paper disks (for instance, S & S Analytical Filter Papers No. 740-E, 12.7 mm in diameter), then saturate them with the enzyme by application of 0.1 ml (about 3 drops) of a 10% solution of the enzyme to the disk surface. Prepare six disks (one for each of the six organisms) for each enzyme: place one disk on the surface of the six inoculated agar plates.

### Incubation

Keep the six plates in the refrigerator overnight to obtain proper diffusion. Incubate the plates at 37° for 24 h. Examine the plates for any inhibition zones that may have been caused by the enzyme preparation.

### Interpretation

A visually clear zone around a disk (total diameter: 16 mm) indicates the presence of antibacterial components in the enzyme preparation. If an enzyme preparation shows obvious antibacterial activity against three (or more) organisms, it is concluded that antimicrobial agents are present.

## **Catalase Activity**

### Scope

This procedure is designed for the determination of catalase activity, expressed as Baker Units.

### Principle

The assay is an exhaustion method based on the breakdown of hydrogen peroxide by catalase, and the simultaneous breakdown of the catalase by the peroxide, under controlled conditions.

### Reagents and Solutions

*0.250 N Sodium thiosulfate:* Dissolve 62.5 g of sodium thiosulfate,  $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$  in 750 ml of recently boiled and cooled water, add 3.0 ml of 0.2 N sodium hydroxide as a stabilizer, dilute to 1,000 ml with water, and mix. Standardize as directed for 0.1 N Sodium thiosulfate (Volumetric Solutions), and adjust to exactly 0.250 N if necessary.

*Peroxide substrate solution:* Dissolve 25.0 g of anhydrous dibasic sodium phosphate ( $\text{Na}_2\text{HPO}_4$ ), or 70.8 g of  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ , in about 1,500 ml of water, and adjust to  $\text{pH } 7.0 \pm 0.1$  with 85% phosphoric acid. Cautiously add 100 ml of 30% hydrogen peroxide, dilute to 2,000 ml, in a graduate, and mix. Store in a clean amber bottle, loosely stoppered. The solution is stable for more than one week if kept at  $5^\circ$  in a full container.

**Note:** *With freshly prepared substrate, the blank will require about 16 ml of 0.250 N sodium thiosulfate. If the blank requires less than 14 ml, the substrate solution is unsuitable and should be prepared fresh again. It is essential that the sample titration is between 50% and 80% of that required for the blank.*

### Procedure

Pipet an aliquot of not more than 1.0 ml of the sample, previously diluted to contain approximately 3.5 Baker Units of catalase, into a 200-ml beaker. Rapidly add 100 ml of Peroxide Substrate Solution, previously adjusted to  $25^\circ$ , and stir immediately for 5 to 10 sec. Cover the beaker, and incubate at  $25 \pm 1^\circ$  until the reaction is completed. Stir vigorously for 5 sec and then pipet 4.0 ml from the beaker into a 50-ml Erlenmeyer flask. Add 5 ml of 2 N sulfuric acid to the flask, mix, then add 5.0 ml of 40% potassium iodide, freshly prepared, and 1 drop of 1% ammonium molybdate and mix. While continuing to mix, titrate rapidly to a colourless endpoint with 0.250 N Sodium thiosulfate, recording the required volume, in ml, as S. Perform a blank determination with 4.0 ml of Peroxide Substrate Solution, and record the required volume, in ml, as B.

**Note:** *When preparations derived from beef liver are tested, the reaction is complete within 30 min. Preparations derived from Aspergillus and other sources may require up to 1 h. In assaying an enzyme of unknown origin, a titration should be run after 30 min and then at 10 min intervals thereafter. The reaction is complete when two consecutive titrations are the same.*

### Calculation

One Baker Unit is that amount of catalase that will decompose 266 mg of hydrogen peroxide under the conditions of the assay. Calculate the activity of the sample by the formula:

$$\text{Baker Units per g or ml} = 0.4 (B - S) \times (1/C)$$

in which C is the ml of aliquot of original enzyme preparation added to each 100 ml of Peroxide Substrate Solution, or, when 1 ml of diluted enzyme is used, C is the dilution factor.

### **Cellulase Activity**

#### Application and Principle

This assay is based on the enzymatic hydrolysis of the interior  $\beta$ -1,4-glucosidic bonds of a defined carboxymethylcellulose substrate at pH 4.5 and at 40°. The corresponding reduction in substrate viscosity is determined with a calibrated viscometer.

#### Apparatus

*Calibrated Viscometer:* Use a size 100 Calibrated Cannon-Fenske Type Viscometer, or its equivalent (Scientific Products, Catalog No. P2885-100).

*Constant-Temperature Glass Water Bath:* (40  $\pm$  0.1°) Use a constant-temperature glass water bath, or its equivalent

*Stopwatches:* Use two stopwatches, Stopwatch No. 1, calibrated in 1/10 min for determining the reaction time (Tr), and Stopwatch No. 2, calibrated in 1/5 s for determining the efflux time (Tt).

*Waring Blender:* Use a two-speed Waring blender, or its equivalent (Scientific Products, Catalog No. 58350-1).

#### Reagents and Solutions

*Acetic Acid Solution (2 N):* While agitating a 1 l beaker containing 800 ml of water, carefully add 116 ml of glacial acetic acid. Cool to room temperature. Quantitatively transfer the solution to a 1 l volumetric flask, and dilute to volume with water.

*Sodium Acetate Solution (2 N):* Dissolve 272.16 g of sodium acetate trihydrate in approximately 800 ml of water contained in a 1 liter beaker. Quantitatively transfer to a 1 liter volumetric flask, and dilute to volume with water.

*Acetic Acid Solution (0.4 N):* Transfer 200 ml of Acetic Acid Solution (2 N) into a 1 liter volumetric flask, and dilute to volume with water.

*Sodium Acetate Solution (0.4 N):* Transfer 200 ml of Sodium Acetate Solution (2 N) into a 1 liter volumetric flask, and dilute to volume with water.

*Acetate Buffer (pH 4.5):* Using a standardized pH meter, add Sodium Acetate Solution (0.4 N) with continuous agitation to 400 ml of Acetic Acid Solution (0.4 N) in a suitable flask until the pH is 4.5  $\pm$  0.05.

*Sodium Carboxymethylcellulose:* Use sodium carboxymethylcellulose (Hercules, Inc., CMC Type 7HF or equivalent).

*Sodium Carboxymethylcellulose Substrate (0.2% w/v):* Transfer 200 ml of water into the bowl of the Waring blender. With the blender on low speed, slowly disperse 1.0 g (moisture-free basis) of the Sodium Carboxymethylcellulose into the bowl, being careful not to splash out any of the liquid. Using a rubber policeman to assist, wash down the sides of the glass

bowl with water. Place the top on the bowl and blend at high speed for 1 min. Quantitatively transfer to a 500-ml volumetric flask, and dilute to volume with water. Filter the substrate through gauze before use.

#### Sample Preparation

Prepare an enzyme solution so that 1 ml of the final dilution will produce a relative fluidity change between 0.18 and 0.22 in 5 min under the conditions of the assay. Weigh the enzyme, and quantitatively transfer it to a glass mortar. Triturate with water and quantitatively transfer the mixture to an appropriate volumetric flask. Dilute to volume with water, and filter the enzyme solution through Whatman No. 1 filter paper before use.

#### Procedure

Place the Calibrated Viscometer in the  $40 \pm 0.1^\circ$  water bath in an exactly vertical position. Use only a scrupulously clean viscometer. (To clean the viscometer, draw a large volume of detergent solution followed by water through the viscometer by using an aspirator with a rubber tube connected to the narrow arm of the viscometer.) Pipet 20 ml of filtered Sodium Carboxymethylcellulose Substrate and 4 ml of Acetate Buffer into a 50-ml Erlenmeyer flask. Allow at least two flasks for each enzyme sample and one flask for a substrate blank. Stopper the flasks, and equilibrate them in the water bath for 15 min. At zero time, pipet 1 ml of the enzyme solution into the equilibrated substrate. Start stopwatch no. 1, and mix the solution thoroughly. Immediately pipet 10 ml of the reaction mixture into the wide arm of the viscometer. After approximately 2 min, apply suction with a rubber tube connected to the narrow arm of the viscometer, drawing the reaction mixture above the upper mark into the driving fluid head. Measure the efflux time by allowing the reaction mixture to freely flow down past the upper mark. As the meniscus of the reaction mixture falls past the upper mark, start stopwatch no. 2. At the same time, record the reaction time, in minutes, from stopwatch no. 1 ( $T_r$ ). As the meniscus of the reaction mixture falls past the lower mark, record the time, in seconds, from stopwatch no. 2 ( $T_t$ ).

Repeat the final step until a total of four determinations are obtained over a reaction time ( $T_r$ ) of not more than 15 min.

Prepare a substrate blank by pipetting 1 ml of water into 24 ml of buffered substrate. Pipet 10 ml of the reaction mixture into the wide arm of the viscometer. Determine the time ( $T_s$ ) in seconds required for the meniscus to fall between the two marks. Use an average of five determinations for ( $T_s$ ).

Prepare a water blank by pipetting 10 ml of equilibrated water into the wide arm of the viscometer. Determine the time ( $T_w$ ) in seconds required for the meniscus to fall between the two marks. Use an average of five determinations for ( $T_w$ ).

#### Calculations

One Cellulase Unit (CU) is defined as the amount of activity that will produce a relative fluidity change of 1 in 5 min in a defined carboxymethylcellulose substrate under the conditions of the assay.

Calculate the relative fluidities ( $F_r$ ) and the ( $T_n$ ) values for each of the four efflux times ( $T_t$ ) and reaction times ( $T_r$ ) as follows:

$$F_r = (T_s - T_w)/(T_t - T_w),$$

$$T_n = 1/2(T_t/60 \text{ s/min}) + T_r = (T_t/120) + T_r,$$

in which

Fr is the relative fluidity for each reaction time;

Ts is the average efflux time, in seconds, for the substrate blank;

Tw is the average efflux time, in seconds, for the water blank;

Tt is the efflux time, in seconds, of reaction mixture;

Tr is the elapsed time, in minutes, from zero time, that is, the time from addition of the enzyme solution to the buffered substrate until the beginning of the measurement of efflux time (Tt);

Tn is the reaction time, in minutes (*Tr*), plus one-half of the efflux time (*Tt*), converted to minutes.

Plot the four relative fluidities (Fr) as the ordinate against the four reaction times (Tn) as the abscissa. A straight line should be obtained. The slope of this line corresponds to the relative fluidity change per minute and is proportional to the enzyme concentration. The slope of the best line through a series of experimental points is a better criterion of enzyme activity than is a single relative fluidity value. From the graph, determine the Fr values at 10 and 5 min. They should have a difference in fluidity of not more than 0.22 or less than 0.18. Calculate the activity of the enzyme unknown as follows:

$$CU/g = [1000(Fr_{10} - Fr_5)]/W,$$

in which

Fr5 is the relative fluidity at 5 min of reaction time;

Fr10 is the relative fluidity at 10 min of reaction time;

1000 is the milligrams per gram;

W is the weight, in mg of enzyme added to the reaction mixture in a 1-ml aliquot of enzyme solution.

## Ethylenimine Limit Test

### Scope

This procedure is designed to detect the presence of ethylenimine in immobilized enzyme preparations containing poly(ethylenimine).

### Principle

The principle of the method is to react any free ethylenimine which may be present in a sample of immobilized enzyme preparation with an aqueous solution of 1,2-naphthoquinone-4-sulfonate (Folin's reagent) to produce 4-(1-aziridiny)-1,2-naphthoquinone. This reaction product is extracted into chloroform and the extract analyzed by high performance liquid chromatography (HPLC).

### Apparatus

- High performance liquid chromatograph equipped with an ultraviolet detector (254 nm), injection valve and Lichrosorb DIOL column, 5 nm, 4.6-mm i.d. x 25-cm (or equivalent)
- Glass syringe 10  $\mu$ l
- Separatory funnel, 100 ml
- Pipettes of convenient volumes for the preparation of standard solutions.

### Reagents and Solutions

*Chloroform* with 1% ethanol as a stabilizer, UV grade, distilled in glass

*Hexane*, UV grade, distilled in glass

*2-propanol*, UV grade, distilled in glass

*Methyl alcohol*, UV grade, distilled in glass

*Acetone*, UV grade, distilled in glass

*1,2-naphthoquinone-4-sulfonic acid, sodium salt*

*0.1 N sodium hydroxide (NaOH)*

*0.1 M Potassium dihydrogen phosphate (KH<sub>2</sub>PO<sub>4</sub>)*

*Buffer Solution:* pH 7.7; mix 200 ml of 0.1 M KH<sub>2</sub>PO<sub>4</sub> with 93.4 ml of 0.1 N NaOH.

*Folin's Reagent:* Dissolve 0.40 g of 1,2-naphthoquinone-4-sulfonic acid sodium salt in 100 ml of buffer solution. Dilute to 500 ml with distilled water in a volumetric flask. Wrap the flask in aluminium foil and store in the refrigerator. Discard the reagent after five days.

#### *4-(1-Aziridinyl)-1,2-naphthoquinone*

A standard sample of known purity is required. If a commercial source for this standard is not readily available, the substance may be synthesized by the following procedure:

Wrap a separatory funnel with aluminium foil and add 2 g of the sodium salt of 1,2-naphthoquinone-4-sulfonic acid dissolved in 250 ml of distilled water.

Add 25 ml of 0.5 M trisodium phosphate, shake and check that the pH is between 10.5 and 11.5. Add 0.3 ml ethylenimine and shake intermittently for 10 min.

**Caution:** *Ethylenimine has been identified as a carcinogen. Appropriate precautions must be taken in handling the compound to avoid personnel exposure and area contamination.*

Extract the 4-(aziridinyl)-1,2-naphthoquinone formed with six 200-ml portions of chloroform.

Place the combined extracts in a 2-liter beaker wrapped in aluminium foil in which three holes have been made.

Evaporate the chloroform at room temperature with a nitrogen purge. Transfer the dry residue to a 50-ml beaker wrapped in aluminium foil.

Add 35 ml of methyl alcohol and 1 ml of chloroform to the residue and stir briefly. Not all of the residue will dissolve.

Place the beaker in an ice-water bath for 10 min and then filter the precipitate through Whatman 42 filter paper.

Rinse the precipitate in the filter with 4 ml of chilled methyl alcohol and discard the filtrates.

Dry the precipitate with a nitrogen purge, transfer it to a brown glass bottle and purge again. Dry the compound overnight in a desiccator containing Drierite. The melting point of the compound is 173-175°. The compound is to be used for making standard solutions for calibration purposes. The compound should be stored in a freezer until standard solutions are to be prepared.

*0.5 g/l Standard Solution:* Accurately weigh about 125 mg of 4-(1-aziridinyl)-1,2-naphthoquinone into a 250 ml volumetric flask [low actinic glass] and add chloroform to the mark.

*0.1 mg/l Standard Solution:* By appropriate dilution(s) of the 0.5 g/l Standard Solution, prepare a standard solution which contains 0.1 mg/L (0.1 ng/μl).

### Analysis

Accurately weigh a sample of immobilized enzyme preparation containing about 10 g of dry matter into an aluminium foil-covered beaker. Add 50 ml of Folin's Reagent and agitate the mixture for several minutes. Decant the Folin's Reagent into a separatory funnel and extract with 2 ml of chloroform. Analyze a 20  $\mu$ l portion of the chloroform extract by the following chromatographic conditions:

Column: Lichrosorb DIOL 5 nm (or equivalent)

Mobile phase: hexane:chloroform (with 1% ethanol) : isopropanol = 59.5 : 40.0 : 0.5 (v/v)

Flow rate: 2 ml/min.

Inject a 20  $\mu$ l portion of the 0.1 mg/L Standard Solution. The sample response is not greater than that of the 0.1 mg/L Standard Solution. (Another sample containing a standard addition of 4-(1-aziridinyl)-1,2-naphthoquinone to immobilized enzyme preparation should be analyzed to verify that the chromatographic response does not contain interfering substances.)

## **$\beta$ -Galactosidase (Lactase) Activity**

### Application and Principle

This procedure is used to determine  $\beta$ -Galactosidase activity of enzyme preparations derived from *Aspergillus oryzae* var. The assay is based on a 15-min hydrolysis of an *o*-nitrophenyl-b-D-galactopyranoside substrate at 37° and pH 4.5.

### Reagents and Solutions

**2.0 N Acetic Acid:** Dilute 57.5 ml of glacial acetic acid to 500 ml with water. Mix well, and store in a refrigerator.

**4.0 N Sodium Hydroxide:** Dissolve 40.0 g of sodium hydroxide in sufficient water to make 250 ml.

**Acetate Buffer:** Combine 50 ml of 2.0 N Acetic Acid and 11.3 ml of 4.0 N Sodium Hydroxide in a 1000-ml volumetric flask, and dilute to volume with water. Verify that the pH is 4.50  $\pm$  0.05, using a pH meter, and adjust, if necessary, with 2.0 N Acetic Acid or 4.0 N Sodium Hydroxide.

**2.0 mM *o*-Nitrophenol Stock:** Transfer 139.0 mg of *o*-nitrophenol to a 500-ml volumetric flask, dissolve in 10 ml of USP alcohol (95% ethanol) by swirling, and dilute to volume with 1% sodium carbonate.

### *o*-Nitrophenol Standards

**0.10 mM Standard Solution:** Pipet 5.0 ml of the 2.0 mM *o*-Nitrophenol Stock solution into a 100-ml volumetric flask, and dilute to volume with 1% sodium carbonate solution.

**0.14 mM Standard Solution:** Pipet 7.0 ml of the 2.0 mM *o*-Nitrophenol Stock solution into a 100-ml volumetric flask, and dilute to volume with 1% sodium carbonate solution.

**0.18 mM Standard Solution:** Pipet 9.0 ml of the 2.0 mM *o*-Nitrophenol Stock solution into a 100-ml volumetric flask, and dilute to volume with 1% sodium carbonate solution.



*Substrate:* Transfer 370.0 mg of *o*-nitrophenyl- $\beta$ -D-galactopyranoside to a 100-ml volumetric flask, and add 50 ml of Acetate Buffer. Swirl to dissolve, and dilute to volume with Acetate Buffer.

**Note:** Perform the assay procedure within 2 h of Substrate preparation.

#### Test Preparation

Prepare a solution from the test sample preparation such that 1 ml of the final dilution will contain between 0.15 and 0.65 lactase unit. Weigh, and quantitatively transfer the enzyme to a volumetric flask of appropriate size. Dissolve the enzyme in water, swirling gently, and dilute with water if necessary.

**Note:** Perform the assay procedure within 2 h of dissolution of the Test Preparation.

#### System Suitability

Determine the absorbance of the three *o*-Nitrophenol Standards at 420 nm in a 1-cm cell, using a suitable spectrophotometer. Use water to zero the instrument. Calculate the millimolar extinction,  $M$ , for each of the *o*-Nitrophenol Standards (0.10, 0.14, and 0.18 mM) by the equation

$$e = A_n/C,$$

in which  $A_n$  is the absorbance of each *o*-Nitrophenol Standard at 420 nm and  $C$  is the corresponding concentration of *o*-nitrophenol in the standard.  $M$  for each standard should be approximately 4.60/mM. Perform a linear regression analysis of the absorbance readings of the three *o*-Nitrophenol Standards versus the *o*-nitrophenol concentration in each (0.10, 0.14, and 0.18 mM). The  $r^2$  should not be less than 0.99. Determine the mean  $M$  of the three *o*-Nitrophenol Standards for use in the calculations below.

#### Procedure

For each sample or blank, pipet 2.0 ml of the Substrate solution into a 25  $\times$  150-mm test tube, and equilibrate in a water bath maintained at  $37.0 \pm 0.1^\circ$  for approximately 10 min. At zero time, rapidly pipet 0.5 ml of the Test Preparation (or 0.5 ml of water as a blank) into the equilibrated substrate, mix by brief (1 s) vortex, and immediately return the tubes to the water bath. After exactly 15 min of incubation, rapidly add 2.5 ml of 10% sodium carbonate solution, and vortex the tube to stop the enzyme reaction. Dilute the samples and blanks to 25.0 ml by adding 20.0 ml of water, and thoroughly mix. Determine the absorbance of the diluted samples and blanks at 420 nm in a 1-cm cell, using a suitable spectrophotometer. Use water to zero the instrument.

#### Calculation

One lactase unit (ALU) is defined as that quantity of enzyme that will liberate *o*-nitrophenol at a rate of 1 mmol/min under the conditions of the assay. Calculate the activity (lactase activity per gram) of the enzyme preparation taken for analysis as follows:

$$\text{ALU/g} = [(A_S - B)(25)]/[(\epsilon)(15)(W)],$$

in which,

$A_S$  is the average of absorbance readings for the Test Preparation;

$B$  is the average of absorbance readings for the blank;

25 is the final volume, in ml, of the diluted incubation mixture;

$\epsilon$  is the mean absorptivity of the *o*-Nitrophenol Standards per micromole;

15 is the incubation time, in minutes; and

W is the weight, in grams, of original enzyme preparation contained in the 0.5-ml aliquot of Test Preparation used in the incubation.

## **Glucoamylase Activity (Amyloglucosidase Activity)**

### Application and Principle

This procedure is used to determine the glucoamylase activity of preparations derived from *Aspergillus niger* var., but it may be modified to determine preparations derived from *Aspergillus oryzae* var. and *Rhizopus oryzae* var. (as indicated by the variations in the text below). The sample hydrolyzes *p*-nitrophenyl- $\alpha$ -D-glucopyranoside (PNPG) to *p*-nitrophenol (PNP) and glucose at pH 4.3 and 50°.

Use the quantity of PNP liberated per unit of time to calculate the enzyme activity. Measure the PNP liberated against a quantity of a standard preparation of PNP by measuring the absorbance of the solutions at 400 nm after adjusting the pH of the reaction mixture to pH 8.0.

**Note:** Use a pH of 5.0 when testing preparations derived from *Aspergillus oryzae* var. or *Rhizopus oryzae* var.

### Apparatus

*Water Bath:* Use an open, circulating water bath with control accuracy of at least  $\pm 0.1^\circ$ .

*Spectrophotometer:* Use a spectrophotometer suitable for measuring absorbances at 400 nm.

*Cuvettes:* Use 10-mm light-path fused quartz.

*Thermometer:* Use a partial immersion thermometer with a suitable range, graduated in  $1/10^\circ$ .

*Timer:* Use a solid-state timer, model 69240 (GCS Corporation, Precision Scientific Group), or equivalent, accurate to  $\pm 0.01$  min in 240 min.

*Vortex Mixer:* Use a standard variable-speed mixer.

### Reagents and Solutions

*p-Nitrophenol Stock Solution (PNP) (0.001 M):* Dissolve 139.11 mg of *p*-nitrophenol previously dried (60°, maximum 4 h) into water, and dilute to 1000 ml.

**Caution:** Avoid contact with skin. If contact occurs, wash the affected area with water. Work in a well-ventilated area.

*Acetate Buffer Solution:* (0.1 M) Dissolve 4.4 g of sodium acetate trihydrate ( $\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$ ) in approximately 800 ml of water, add 4.5 ml of acetic acid ( $\text{C}_2\text{H}_4\text{O}_2$ ). Adjust to pH  $4.5 \pm 0.05$  by adding either sodium acetate or glacial acetic acid as required. Dilute to 1 l.

**Note:** Use a pH of 5.0 when testing preparations derived from *Aspergillus oryzae* var. or *Rhizopus oryzae* var.

*Sodium Carbonate Solution (0.3 M):* Dissolve 15.9 g of sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) in water, and dilute to 500 ml.

*p-Nitrophenyl- $\alpha$ -D-glucopyranoside Solution (PNPG):* Dissolve 100.0 mg of PNPG (Sigma Chemical Co., Catalog No. N1377 or equivalent) in acetate buffer, and dilute to 100 ml.

Standards

Dilute three portions of PNP Stock Solution to produce standards for the standard curve. Add 3 ml of the PNP Stock Solution to 125 ml of Sodium Carbonate Solution, and dilute to 500 ml with water to produce the first standard, containing 0.006 mmol/ml. Add 2 ml of PNP Stock Solution to 25 ml of Sodium Carbonate Solution, and dilute to 100 ml with water to produce the second standard, containing 0.02 mmol/ml. Add 5 ml of PNP Stock Solutions to 25 ml of Sodium Carbonate Solution, and dilute to 100 ml with water to produce the third standard, containing 0.05 mmol/ml.

Sample Solution

Dilute  $1.00 \pm 0.01$  g of sample in sufficient Acetate Buffer Solution to produce a solution that contains between 0.1 and 0.3 glucoamylase units of activity per ml.

Procedure

Measure absorbances of each of the three PNP Standard Solutions to calculate the molar extinction coefficient. Equilibrate the PNPG Solution in a 50° water bath for at least 15 min. For active samples, transfer 2.0 ml of the Sample Solution to a test tube. Loosely stopper, and place the tube in the water bath to equilibrate for 5 min. At zero time, add 2.0 ml of PNPG Solution, and mix at moderate speed on a vortex mixer. Return the mixture to the water bath. Exactly 10.0 min later, add 3.0 ml of the Sodium Carbonate Solution, mix on the vortex, and remove from the water bath.

For sample blanks, transfer 2.0 ml of the Sample Solution and 3.0 ml of the Sodium Carbonate Solution into a test tube, and mix. Add 2.0 ml of PNPG Solution, and mix. Measure the absorbance of each sample and the blank versus water in a 10-mm cell.

**Note:** *Determine the absorbance of the sample and blank solutions not more than 20 min after adding Sodium Carbonate Solution.*

Calculations

One unit of glucoamylase activity is defined as the amount of glucoamylase that will liberate 0.1 mmol/min of p-nitrophenol from the PNPG Solution under the conditions of the assay.

Calculate the millimolar extinction of the PNP standards using the following equation:

$$\varepsilon_M = A_n/C,$$

in which

$A_n$  is the absorbance of the p-nitrophenol standard, at 400 nm, and  
 $C$  is concentration, in mmol/ml, of p-nitrophenol.

The averaged millimolar extinction coefficient,  $M$ , should be approximately 18.2.

$$\text{Glucoamylase } M = [(AS - AB) \times 7 \times F] / \varepsilon_M \times 10 \times 0.10 \times W \times 2,$$

in which

$AS$  is the sample absorbance;

$AB$  is the blank absorbance;

$F$  is the appropriate dilution factor;

$W$  is the weight of sample, in grams; 7 is the final volume of the test solutions;

10 is the reaction time, in minutes; 0.10 is the amount of PNP liberated, in mmol/min/unit of enzyme;

2 is the sample aliquot, in millilitres; and

$M$  is the millimolar extinction coefficient.

## **$\beta$ -Glucanase Activity**

### Application and Principle

This procedure is used to determine  $\beta$ -glucanase activity of enzyme preparations derived from *Aspergillus niger* var. and *Bacillus subtilis* var. The assay is based on a 15-min hydrolysis of lichenin substrate at 40° and at pH 6.5. The increase in reducing power due to liberated reducing groups is measured by the neocuproine method.

### Reagents and Solutions

*Phosphate Buffer*: Dissolve 13.6 g of monobasic potassium phosphate in about 1900 ml of water, add 70% sodium hydroxide solution until the pH is  $6.5 \pm 0.05$ , then transfer the solution into a 2000-ml volumetric flask, dilute to volume with water, and mix.

*Neocuproine Solution A*: Dissolve 40.0 g of anhydrous sodium carbonate, 16.0 g of glycine, and 450 mg of cupric sulfate pentahydrate in about 600 ml of water. Transfer the solution into a 1000-ml volumetric flask, dilute to volume with water, and mix.

*Neocuproine Solution B*: Dissolve 600 mg of neocuproine hydrochloride in about 400 ml of water, transfer the solution into a 500-ml volumetric flask, dilute to volume with water, and mix. Discard when a yellow colour develops.

*Lichenin Substrate*: Grind 150 mg of lichenin (Sigma Chemical Co., Catalog No. L-6133, or equivalent) to a fine powder in a mortar, and dissolve it in about 50 ml of water at about 85°. After solution is complete (20 to 30 min), add 90 mg of sodium borohydride and continue heating below the boiling point for 1 h. Add 15 g of Amberlite MB-3, or an equivalent ion-exchange resin, and stir continuously for 30 min. Filter with the aid of a vacuum through Whatman No. 1 filter paper, or equivalent, in a Buchner funnel, and wash the paper with about 20 ml of water. Add 680 mg of monobasic potassium phosphate to the filtrate, and re-filter through a 0.22-mm Millipore filter pad, or equivalent. Wash the pad with 10 ml of water, and adjust the pH of the filtrate to  $6.5 \pm 0.05$  with 1 N sodium hydroxide or 1 N hydrochloric acid. Transfer the filtrate into a 100-ml volumetric flask, dilute to volume with water, and mix. Store at 2° to 4° for not more than 3 days.

*Glucose Standard Solution*: Dissolve 36.0 mg of anhydrous dextrose in Phosphate Buffer in a 1000-ml volumetric flask, dilute to volume with water, and mix.

### Test Preparation

Prepare a solution from the enzyme preparation sample so that 1 ml of the final dilution will contain between 0.01 and 0.02  $\beta$ -glucanase units. Weigh the sample, transfer it into a volumetric flask of appropriate size, dilute to volume with Phosphate Buffer, and mix.

### Procedure

Pipet 2 ml of Lichenin Substrate into each of four separate test tubes graduated at 25 ml, and heat the tubes in a water bath at 40° for 10 to 15 min to equilibrate.

After equilibration, add 1 ml of Phosphate Buffer to tube 1 (substrate blank), 1 ml of Glucose Standard Solution to tube 2 (glucose standard), 4 ml of Neocuproine Solution A and 1 ml of the Test Preparation to tube 3 (enzyme blank), and 1 ml of the Test Preparation to tube 4 (sample). Prepare a fifth tube for the buffer blank, and add 3 ml of Phosphate Buffer.

Incubate the five tubes at 40° for exactly 15 min, and then add 4 ml of Neocuproine Solution A to tubes 1, 2, 4, and 5. Add 4 ml of Neocuproine Solution B to all five tubes, and cap each with a suitably sized glass marble.

**Caution:** *Do not use rubber stoppers.*

Heat the tubes in a vigorously boiling water bath for exactly 12 min to develop colour, then cool to room temperature in cold water, and adjust the volume of each to 25 ml with water. Cap the tubes with Parafilm, or other suitable closure, and mix by inverting several times. Determine the absorbance of each solution at 450 nm in 1-cm cells, with a suitable spectrophotometer, against the buffer blank in tube 5.

#### Calculation

One  $\beta$ -glucanase unit (BGU) is defined as that quantity of enzyme that will liberate reducing sugar (as glucose equivalent) at a rate of 1mmol/min under the conditions of the assay.

Calculate the activity of the enzyme preparation taken for analysis as follows:

$$\text{BGU} = [(A4 - A3) \times 36 \times 106] / [(A2 - A1) \times 180 \times 15 \times \text{mg sample}],$$

in which

A4 is the absorbance of the sample (tube 4),

A3 is the absorbance of the enzyme blank (tube 3),

A2 is the absorbance of the glucose standard (tube 2),

A1 is the absorbance of the substrate blank (tube 1), 36 is the micrograms of glucose in the Glucose Standard Solution,

106 is the factor converting micrograms to grams,

180 is the weight of 1  $\mu\text{mol}$  of glucose, and

15 is the reaction time in minutes.

## **Glucose Isomerase Activity**

### Scope

This procedure is designed for the determination of glucose isomerase preparations derived from *Actinoplanes missouriensis*, *Arthrobacter globiformis*, *Bacillus coagulans*, *Streptomyces olivaceus*, *Streptomyces olivochromogenes*, and *Streptomyces rubiginosus*.

### Principle

The assay is based on measurement of the rate of conversion of glucose to fructose in a packed bed reactor.

The procedure as outlined approximates an initial velocity assay method. Specific conditions are: glucose concentration, 45% w/w; pH (inlet) measured at room temperature in the 7.0 to 8.5 range, as specified; temperature, 60.0°; and magnesium concentration,  $4 \times 10^{-3}$  M. The optimum conditions for enzymes from different microbial sources and methods of preparation may vary; therefore, if different pH conditions, buffering systems, or methods of sample preparation are recommended by the manufacturer, such variations in the instructions given herein should be used.

### Reagents and Solutions

**Glucose substrate:** Dissolve 539 g of anhydrous glucose and 1.0 g of magnesium sulfate,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , in 700 ml of water or the manufacturer's recommended buffer, previously heated to 50° to 60°. Cool the solution to room temperature, and adjust the pH as specified by the enzyme manufacturer. Transfer the solution to a 1,000-ml volumetric flask, dilute to volume with water or the specified buffer, and mix. Transfer to a vacuum flask, and de-aerate for 30 min.

*Magnesium sulfate solution:* Dissolve 1.0 g of magnesium sulfate,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , in 700 ml of water. Adjust the pH to 7.5 to 8.0 as specified by the manufacturer, using 1 N sodium hydroxide, dilute to 1,000 ml with water and mix.

**Note:** *Glucose isomerase activity of the commercial enzyme is usually determined on the enzyme that has been immobilized by binding with a polymer matrix or other suitable material. This method is designed for use with such preparations.*

#### Column Assembly and Apparatus

The column assembly is shown in Figure 1 below.

**Note:** *Make all connections with inert tubing, glass or plastic as appropriate.*

Use a 2.5 x 40-cm glass column provided with a coarse sintered glass bottom and a water jacket connected to a constant-temperature water bath, maintained at  $60.0^\circ$  by means of a circulating pump. Connect the top of the column to a variable-speed peristaltic pump having a maximum flow rate of 800 ml per h. The diameter of the tubing with which the peristaltic pump is fitted should permit variation of the pumping volume from 60 to 150 ml per h. Connect the outlet of the column with a collecting vessel.

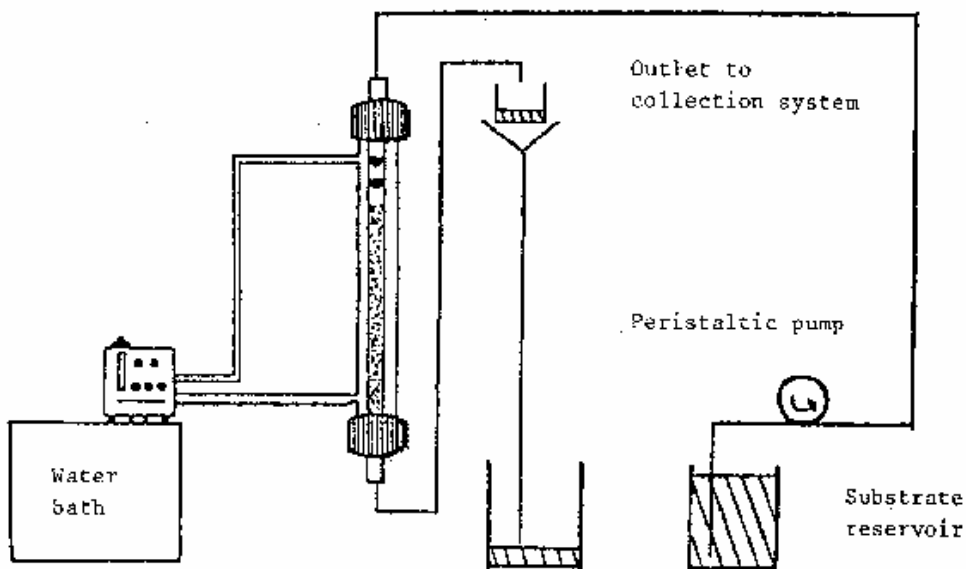


Figure 1. Diagram of a column assembly for assay of Immobilized Glucose Isomerase

#### Sample Preparation

Transfer to a 500-ml vacuum flask an amount of the sample, accurately weighed in g or measured in ml, as appropriate, sufficient to obtain 2,000 to 8,000 glucose isomerase units ( $\text{GI}_c$  U). Add 200 ml of Glucose Substrate, stir gently for 15 sec and repeat the stirring every 5 min for 40 min. De-aerate by vacuum for 30 min.

#### Column Preparation

Quantitatively transfer the Sample Preparation to the column with the aid of Magnesium Sulfate Solution as necessary. Allow the enzyme granules to settle, and then place a porous disk so that it is even with, and in contact with, the top of the enzyme bed. All of the air

should be displaced from the disk. Place a cotton plug about 1 or 2 cm above the disk. (This plug acts as a filter. It ensures proper heating of the solution and traps dissolved gases that may be present in the Glucose Substrate.) Connect the tubing from the peristaltic pump with the top of the column, and seal the connection by suitable means in order to protect the column contents from the atmosphere. Place the inlet tube of the peristaltic pump into the Glucose Substrate solution, and begin a downward flow of the Glucose Substrate into the column at a rate of at least 80 ml per h. Maintain the flow rate for 1 h at room temperature.

#### Procedure

Adjust the flow of the Glucose Substrate to such a rate that a fractional conversion of 0.2 to 0.3 will be produced, based on the estimated activity of the sample. The fractional conversion is calculated from optical rotation values obtained on the starting Glucose Substrate and the sample effluent, as specified in Calculations below. After the correct flow rate has been established, run the column overnight (16 h minimum), then check the pH of the Glucose Substrate, and readjust if necessary to the specified pH. Measure the flow rate, and collect a sample of the column effluent. Cover the effluent sample, allow it to stand for 30 min at room temperature, and then determine the fractional conversion of glucose to fructose (see Calculations below). If the conversion is less than 0.2 or more than 0.3, adjust the flow rate to bring the conversion into this range. If a flow rate adjustment is required, collect an additional effluent sample after allowing the column to re-equilibrate for at least 2 h and then determine the fractional conversion.

Measure the flow rate, and collect an effluent sample. Cover the sample, let it stand at room temperature for 30 min, and determine the fractional conversion.

#### Calculations

##### *Specific rotation*

Measure the optical rotation of the effluent sample and of the starting Glucose Substrate at 25.0°, and calculate their specific rotations by the formula:

$$[\alpha]_{D}^{25} = 100 a/lpd$$

in which

a is the corrected observed rotation, in degrees,

l is the length of the polarimeter tube, in dm,

p is the concentration of the test solution, expressed as g of solute per 100 g of solution, and

d is the specific gravity of the solution at 25°

##### *Fractional conversion*

Calculate the fractional conversion, X, by the formula:

$$X = (\alpha_E - \alpha_S) / (\alpha_F - \alpha_S)$$

in which

$\alpha_E$  is the specific rotation of the column effluent,

$\alpha_S$  is the specific rotation of the Glucose Substrate,

and  $\alpha_F$  is the specific rotation of fructose (which in this case has been calculated to be -94.54).

*Activity*

The enzyme activity is expressed in glucose isomerase units (GI<sub>c</sub>U, the subscript c signifying column process). One GI<sub>c</sub>U is defined as the amount of enzyme that converts glucose to fructose at an initial rate of 1 μmol per min, under the conditions specified.

Calculate the glucose isomerase activity by the formula:

$$\text{GI}_c\text{U per g or ml} = (\text{FS}/\text{W}) [\text{X}_e \ln \text{X}_e / (\text{X}_e - \text{X})]$$

in which

F is the flow rate, in ml per min,

S is the concentration of the Glucose Substrate, in μml per ml,

X<sub>e</sub> is the fractional conversion at equilibrium, or 0.51, and

W is the weight or volume of the sample taken, in g or ml, respectively.

**Glucose Oxidase Activity**Application and Principle

This procedure is used to determine glucose oxidase activity in preparations derived from *Aspergillus niger* var. The assay is based on the titrimetric measurement of gluconic acid produced in the presence of excess substrate and excess air.

Reagents and Solutions

*Chloride–Acetate Buffer Solution*: Dissolve 2.92 g of sodium chloride and 4.10 g of sodium acetate in about 900 ml of water. Adjust the pH to 5.1 with either dilute acetic acid or dilute sodium hydroxide solution and dilute to 1000.0 ml.

*Sodium Hydroxide Solution (0.1 N)*

*Hydrochloric Acid Solution (0.05 N)* Standardized.

*Phenolphthalein Solution (2% w/v)*: Solution in methanol.

*Octadecanol Solution*: Saturated solution in methanol.

*Substrate Solution*: Dissolve 30.00 g of anhydrous glucose in 1000 ml of the Chloride–Acetate Buffer Solution.

Sample Preparation

Dissolve an accurately weighed amount of enzyme preparation in the Chloride–Acetate Buffer Solution, and dilute in the buffer solution to obtain an enzyme activity of 5 to 7 activity units per milliliter.

Procedure

Transfer 25.0 ml of the Substrate Solution to a 32 × 200-mm test tube. To a second 32 × 200 mm test tube transfer 25.0 ml of the Chloride–Acetate Buffer Solution (blank). Equilibrate both tubes in a 35 ± 0.1° water bath for 20 min. Add 3.0 ml of the Sample Preparation to each test tube, mix, and insert a glass sparger into each tube with a pre-adjusted air flow of 700 to 750 ml/min. If excessive foaming occurs, add 3 drops of the Octadecanol Solution to each tube. After exactly 15 min, remove the sparge and rinse any adhering reaction mixture back into the tube with water. Immediately add 10 ml of the Sodium Hydroxide Solution and 3 drops of the Phenolphthalein Solution to each tube. Insert a small magnetic stirrer bar, stir, and titrate to the phenolphthalein endpoint with the standardized 0.05 N hydrochloric Acid Solution.



### Calculation

One Glucose Oxidase Titrimetric unit of activity (GOTu) is the quantity of enzyme that will oxidize 3 mg of glucose to gluconic acid under the conditions of the assay. Determine the enzyme activity using the following equation:

$$\text{GOTu/g} = [(B - T) \times N \times 180 \times F] / [3 \times W],$$

in which

B is the titration volume, in milliliters, of the blank;

T is the titration volume, in milliliters, of the sample;

N is the normality of the titrant; 180 is the molecular weight of glucose;

F is the sample dilution factor;

3 is from the unit definition; and

W is the weight, in grams, of the enzyme preparation contained in each milliliter of the sample solution.

## **Glutaraldehyde Limit Test**

### Scope

This procedure is designed to determine the glutaraldehyde carried over into isomerized syrup during isomerization of glucose syrup by the use of immobilized glucose isomerases crosslinked with glutaraldehyde.

### Principle

The procedure involves sampling the syrup produced during different stages of the enzyme assay "Glucose isomerase activity". Analysis of the sample syrup according to the procedure on page 169 gives the number of mg of glutaraldehyde per kg of syrup. A subsequent calculation gives the amount of glutaraldehyde present per unit of glucose isomerase activity. The enzyme preparation passes the test if the average result is not greater than 0.025.

### Procedure

Samples of syrup during the assay for "Glucose isomerase activity" are taken at steps as prescribed in the following:

*Sample 1:* 25 ml of syrup is taken out at the step called "Sample preparation" (i.e. syrup decanted off, just after the prescribed 40 min soaking period).

*Sample 2:* 25 ml of syrup is taken out at the step called "Procedure" (i.e. isomerized syrup from the column outlet just after the flow rate has been adjusted to the correct level).

*Sample 3:* 25 ml of syrup is taken out at the point of time when samples are taken for determination of the fractional conversion of the glucose to fructose.

As prescribed, this time is at least 16 hours after start-up. In actual practice the time for taking this effluent sample will be in the interval 42-48 hours after start-up.

All three samples (Samples 1, 2, and 3) are subjected to determination for glutaraldehyde as described in "Determination of glutaraldehyde in High Fructose Corn Syrup". As indicated in the text of the assay, it has been determined that the lower detection limit for glutaraldehyde in HFCS (High Fructose Corn Syrup) is 5 mg/kg by this assay.

### Calculation

The relationship between the determination of glutaraldehyde and the determination of activity of the prepared immobilized enzyme can be expressed in the following way:

$$a = (\text{mg GA/kg syrup}) / (\text{GI}_c\text{U/g enzyme})$$

in which

GA is Glutaraldehyde

GI<sub>c</sub>U is the activity unit for glucose isomerase in the column process

#### Interpretation of test results

The enzyme passes test if the average "a" from the three samples tested is not greater than 0.025. (For GA concentrations below the detection limit of 5 mg/kg, the value 5 mg/kg is taken.)

#### *Examples*

- a = 0.025 is equal to an average GA concentration of 5 mg/kg from 200 GI<sub>c</sub>U/g enzyme.
- a = 0.025 is equal to an average GA concentration of 7.5 mg/kg from 300 GI<sub>c</sub>U/g enzyme.

### **Glutaraldehyde Determination in High Fructose Corn Syrup (High Fructose Glucose Syrup)**

#### Scope

This procedure is designed for the determination of Glutaraldehyde in High Fructose Corn Syrup (HFCS).

#### Principle

The assay is based on a measurement using thin layer chromatography.

#### Apparatus

*TLC plates:* Pre-coated TLC plates SIL G-25, available from Macherey-Nagel, Catalog No. 809 013, or equivalent. Activate before use by heating to 100° for at least one h. Use gloves when handling.

#### Reagents

*Solvent system:* Transfer 5.0 ml absolute ethanol to a 100-ml volumetric flask and fill up to the mark with chloroform. Transfer to a 250-ml flask and shake very thoroughly before pouring the mixture into the developing chamber.

*Spray reagents:* (Sufficient for one TLC plate)

I: 1% MBTH: Dissolve 250 mg MBTH (N-methyl-benzothiazolonhydrazon-HCl) in 25 ml water.

II: 2% Ferric chloride: Dissolve 0.5 g ferric chloride (FeCl<sub>3</sub>·6H<sub>2</sub>O) in 25 ml water.

#### *Standard Solutions*

Glutaraldehyde stock solution (1 mg/ml): Transfer 0.4 ml of 25% glutardialdehyde solution (Merck No. 12179) to a 100-ml volumetric flask. Make up to the mark with water.

Glutaraldehyde solution (25 µg/ml): Dilute 250 µl of glutaraldehyde stock solution to 10.0 ml with water. Dilution to be made freshly before use.

Glutaraldehyde solution (3.75 µg/ml): Dilute 1.50 ml of G - 25 µg/ml to 10.0 ml with water. Dilution to be made freshly before use.

#### Assay Solutions

Transfer to 10-ml volumetric flasks:

Assay solution (a): 7.50 g of HFCS sample;

Assay solution (b): 7.50 g of HFCS sample and 1.50 ml of glutaraldehyde solution (25 µg/ml) corresponding to 37.5 µg of glutaraldehyde.

Make both solutions up to volume with water.

#### Procedure

Treat the standard and assay solutions for 30 min in an ultra-sonic bath immediately before use.

Spot the TLC plate as follows:

- Spot 1: 150 µl of glutaraldehyde solution (3.75 µg/ml) equivalent to 0.5625 µg glutaraldehyde.
- Spot 2: 150 µl of assay solution (b) equivalent to 0.5625 µg glutaraldehyde plus 0.1125 g HFCS sample.
- Spot 3: 150 µl of assay solution (a) equivalent to 0.1125 g HFCS sample.

The spots should be placed at least 3 cm from the edges of the plate and 5 cm apart. Allow the spots to dry at room temperature. Run the chromatogram until the solvent front has migrated 15 cm (30-40 min). Allow the plate to dry for at least 30 min at room temperature.

Spray with reagent I using a fine nozzle. Approximately 20 ml are needed.

Wait for 10 min and then spray with reagent II until the spots can be seen. Approximately 25 ml are needed.

#### Estimation

Estimate the glutaraldehyde content of the assay sample (spot 3) by comparison with the standard (spot 1).

If the intensity of assay sample spot 3 is less than the intensity of standard spot 1, then the HFCS sample contains < 5 mg/kg of glutaraldehyde.

Spot 2 is included as proof that the method can detect 5 mg/kg of glutaraldehyde in HFCS.

## **Hemicellulase Activity**

### Scope

This procedure is for the determination of hemicellulase activity of preparations derived from *Aspergillus niger*, var.

### Principle

The test is based on the enzymatic hydrolysis of the interior glucosidic bonds of a defined carob (locust) bean gum substrate at pH 4.5 and 40°. The corresponding reduction in substrate viscosity is determined with a calibrated viscometer.

### Apparatus

*Viscometer:* Use a size 100 calibrated Cannon-Fenske Type Viscometer, or its equivalent. A suitable viscometer is supplied as Catalog No. 2885-100 by Scientific Products, 1210 Waukegan Road, McGraw Park, Ill. 60085.

*Glass Water Bath:* Use a constant-temperature glass water bath maintained at  $40 \pm 0.1^\circ$ . A suitable bath is supplied as Catalog No. W3520 10 by Scientific Products.

### Reagents and Solutions

*Acetate Buffer (pH 4.5):* Add 0.2 N sodium acetate, with continuous agitation, to 400 ml of 0.2 N acetic acid until the pH is  $4.5 \pm 0.05$ , as determined by a pH meter.

*Locust Bean Gum:* Use Powdered Type D-200 locust bean gum, or its equivalent, supplied by Meer Corp., 9500 Railroad Avenue, North Bergen, N.J. 07047. Since the substrate may vary from lot to lot, each lot should be tested in parallel with a previous lot known to be satisfactory. Variations of more than  $\pm 5\%$  viscosity in the average of a series of parallel tests indicate an unsuitable lot.

*Substrate Solution:* Place 12.5 ml of 0.2 N hydrochloric acid and 250 ml of warm water ( $72^\circ$  to  $75^\circ$ ) in the bowl of a power blender (Waring two-speed, or its equivalent, supplied as Catalog No. 58350-1 by Scientific Products), and set the blender on low speed. Slowly disperse 2.0 g of Locust Bean Gum, on a moisture-free basis, into the bowl, taking care not to splash out any of the liquid in the bowl. Wash down the sides of the bowl with warm water, using a rubber policeman, cover the bowl, and blend at high speed for 5 min. Quantitatively transfer the mixture to a 1,000-ml beaker, and cool to room temperature. Using a pH meter, adjust the mixture to pH 6.0 with 0.2 N sodium hydroxide. Quantitatively transfer to a 1,000-ml volumetric flask, dilute to volume with water, and mix. Filter the substrate through gauze before use.

### Sample Preparation

Prepare a solution of the sample in water so that 1 ml of the final dilution will produce a change in relative fluidity between 0.18 and 0.22 in 5 min under the conditions specified in the *Procedure* below. Weigh the enzyme preparation, quantitatively transfer it to a glass mortar, and triturate with water. Quantitatively transfer the mixture to an appropriately sized volumetric flask, dilute to volume with water, and mix. Filter through Whatman No. 1 filter paper, or equivalent, before use.

### Procedure

Scrupulously clean the Viscometer by drawing a large volume of detergent solution, followed by water, through the instrument, and place the viscometer, previously calibrated, in the Glass Water Bath in an exactly vertical position. Pipet 20.0 ml of Substrate Solution and 4.0 ml of Acetate Buffer into a 50-ml Erlenmeyer flask, allowing at least two flasks for each enzyme sample and one flask for a substrate blank. Stopper the flasks, and equilibrate them in the water bath for 15 min. At zero time, pipet 1.0 ml of the Sample Preparation into the equilibrated substrate, start timing with a stopwatch (No. 1), and mix thoroughly. Immediately pipet 10.0 ml of this mixture into the wide arm of the Viscometer. After about 2 min, draw the reaction mixture above the upper mark into the driving fluid head by applying suction with a rubber tube connected to the narrow arm of the instrument. Measure the efflux time by allowing the reaction mixture to flow freely down past the upper mark. As the meniscus falls past the upper mark, start the second stopwatch (No. 2), and at the same time record the reaction time ( $T_R$ ), in min, from stopwatch No. 1. As the meniscus of the reaction mixture falls past the lower mark, record the time ( $T_T$ ), in sec, from stopwatch No. 2.

Immediately re-draw the reaction mixture above the upper mark and into the driving fluid head. As the meniscus falls freely past the upper mark, restart stopwatch No. 2, and at the same time record the reaction time ( $T_R$ ), in min, from stopwatch No. 1. As the meniscus falls past the lower mark, record the time ( $T_T$ ), in sec, from stopwatch No. 2. Repeat the latter operation, beginning with "Immediately re-draw the reaction mixture ..." until a total of four determinations are obtained over a reaction time ( $T_R$ ) of not more than 15 min.

Prepare a substrate blank by pipetting 1.0 ml of water into a mixture of 20.0 ml of Substrate Solution and 4.0 ml of Acetate Buffer, and then immediately pipet 10.0 ml of this mixture into the wide arm of the Viscometer. Determine the time ( $T_S$ ), in sec, required for the meniscus to fall between the two marks. Use an average of five determinations as  $T_S$ .

Prepare a water blank by pipetting 10.0 ml of water, previously equilibrated to  $40 \pm 0.1^\circ$ , into the wide arm of the Viscometer. Determine the time ( $T_w$ ), in sec, required for the meniscus to fall between the two marks. Use an average of five determinations as  $T_w$ .

### Calculation

One hemicellulase unit (HCU) is that activity that will produce a relative fluidity change of 1 over a period of 5 min in a locust bean gum substrate under the conditions specified. Calculate the relative fluidities ( $F_R$ ) and  $T$  values (see definition below) for each of the four efflux times ( $T_T$ ) and reaction times ( $T_R$ ) as follows:

$$F_R = (T_S - T_w)/(T_T - T_w),$$

and

$$T_N = 1/2(T_T/60) + T_R = (T_T/120) + T_R,$$

in which

$F_R$  is the relative fluidity for each reaction time;

$T_S$  is the average efflux time for the substrate blank, in sec;

$T_w$  is the average efflux time for the water blank, in sec;

$T_T$  is the efflux time of the sample reaction mixture, in sec;

$T_R$  is the elapsed time from zero time, in min, i.e., the time from addition of the enzyme solution to the buffered substrate, until the beginning of the measurement of the efflux time ( $T_T$ ); and

$T_N$  is the reaction time ( $T_R$ ), in min, plus one half of the efflux time ( $T_T$ ) converted to min.

Plot the four relative fluidities ( $F_R$ ) as the ordinate against the four reaction times ( $T_N$ ) as the abscissa. A straight line should be obtained. The slope of the line corresponds to the relative fluidity change per min and is proportional to the enzyme concentration. The slope of the best line through a series of experimental points is a better criterion of enzyme activity than is a single relative fluidity value. From the curve determine the  $F_R$  values at 10 and 5 min. They should have a difference in fluidity of not more than 0.22 and not less than 0.18. Calculate the activity of the enzyme sample as follows:

$$\text{HCU/g} = 1,000(F_{R10} - F_{R5})/W,$$

in which

$F_{R10}$  is the relative fluidity at 10 min reaction time;

$F_{R5}$  is the relative fluidity at 5 min reaction time;

1,000 is mg per g; and

$W$  is the weight, in mg, of the enzyme sample contained in the 1.0-ml aliquot of Sample Preparation added to the equilibrated substrate in the Procedure.

## **Invertase Activity**

### Principle

Invertase hydrolyses the non-reducing  $\beta$ -d-fructofuranoside residues of sucrose to yield invert sugar. The invert sugar released is then reacted with 3,5-dinitrosalicylic acid (DNS). The colour change produced is proportional to the amount of invert sugar released, which in turn is proportional to the invertase activity present in the sample. The absorbance is measured at 540 nm and converted into micromoles of reducing sugar produced using a standard curve. One invertase unit is the amount of enzyme which will produce 1 micromole of reducing sugar (expressed as invert sugar) per minute under the conditions specified in this procedure.

### Apparatus

*Spectrophotometer* set at 540 nm

*Water bath* set at  $30 \pm 1.0^\circ$

*Stopwatch*

*Boiling water bath*

*Ice water bath*

*Mixer*

### Reagents and solutions

*0.05 M Sodium acetate buffer, pH 4.7:* Adjust the pH of 200 ml of 0.05 M sodium acetate (4.1 g of sodium acetate anhydrous in 1000 ml of water) to  $\text{pH } 4.7 \pm 0.05$  with 0.05M acetic acid (2.85 ml of glacial acid in 1000 ml of water).

*0.3 M Sucrose:* 5.13 g sucrose in 50.0 ml of water

*20 mM Tris HCl buffer, pH 7.0:* Dissolve 2.42 g of tris (hydroxymethyl) aminomethane in about 800 ml of water. Adjust pH to 7.0 using 5% hydrochloric acid (5 ml of conc. hydrochloric acid in 100.0 ml of water).

*DNS solution:* Weigh 300 g of potassium sodium tartrate tetrahydrate into a one litre conical flask. Add 16 g of sodium hydroxide and 500 ml of water and dissolve by heating gently. When the solution is clear, add slowly 10 g of 3,5-dinitrosalicylic acid (DNS). Keep covered to protect from light until the DNS is totally dissolved. Cool to room temperature and make up to 1 litre with water. Store in a tightly stoppered dark container. Protect from light and carbon dioxide.

*Invert sugar standard (0.01M):* Dry glucose to constant weight at  $105^\circ$  and dry fructose to constant weight at  $70^\circ$  under vacuum. Dissolve 0.9 g of glucose and 0.9 g of fructose in 1000 ml of 0.1% benzoic acid (1 g of benzoic acid in 1000 ml of water).

### Standard curve

Prepare a series of test tubes, in duplicate, according to the table below. The standard curve must include at least four suitable standards

<b>Tube No.</b>	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>Blank</b>
Invert sugar standard (ml)	0.1	0.3	0.5	0.8	1.0	1.2	0.0
Water (ml)	2.4	2.2	2.0	1.7	1.5	1.3	2.5
Acetate buffer (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Content of invert sugar	1.0	3.0	5.0	8.0	10	12	0.0

Reaction and measurement

Mix and incubate for exactly 10 min at  $30 \pm 0.1^\circ$ . Add 2.0 ml of DNS solution to each tube, cover tubes and place all tubes in a boiling water bath for exactly 10 min. Cool rapidly in an ice water bath and add 15 ml of water to each tube. Mix thoroughly. Measure the absorbance at 540 nm of each sample using the blank to zero the spectrophotometer. Plot the absorbance against content of invert sugar.

Sample preparation

Accurately weigh about 1 g of the sample and dissolve in 10 ml of 20 mM Tris HCl buffer. For powder samples it may be necessary to use a magnetic stirrer for up to 10 min. Dilute the sample with 20 mM Tris HCl buffer to obtain a solution for which the measured absorbance will fall within the linear range of 0.14 and 0.30.

Procedure

Into each of a series of 30 ml test tubes, pipette, in quadruplicate, 1.4 ml of water, 0.5 ml of acetate buffer and 0.1 ml of diluted enzyme. Equilibrate the tubes in a  $30^\circ$  water bath. Add 1 ml of 0.3 M sucrose solution to 3 of the 4 tubes. Use the fourth tube as an enzyme blank, adding 2 ml of DNS solution before adding 1.0 ml of 0.3 M sucrose solution. Prepare a reagent blank using 0.1 ml of water in place of diluted enzyme. Continue as described under 'Reaction and measurement'. Read the respective contents of invert sugar from the standard curve.

Calculation

$$\text{Activity for powders (units/minute/g)} = [(C_S - C_B) \times \text{dilution}] / W$$

Where

$C_S$  is Content of invert sugar in sample solution (micromoles)

$C_B$  is Content of invert sugar in enzyme blank solution (micromoles)

W is Weight of sample (g)

$$\text{Activity for liquids (units/minute/ml)} = [(C_S - C_B) \times \text{dilution} \times \text{S.G.}] / W$$

Where

$C_S$  is Content of invert sugar in sample solution (micromoles)

$C_B$  is Content of invert sugar in enzyme blank solution (micromoles)

W is Weight of sample (g)

S.G. is Specific gravity of sample (g/ml)

**Milk Clotting Activity**Scope

This procedure is designed to be applied to enzyme preparations derived from either animal or microbial sources.

Principle

The method is based on a visual flocculation endpoint.

Apparatus

*Bottle-rotating apparatus:* Use a suitable assembly, designed to rotate at a rate of 16 to 18 rpm, such as the Dries-Jacques Associates type model (Available from Dries-Jacques Associates, 1801 East North Avenue, Milwaukee, Wisconsin 53202, USA.) or equivalent

*Sample bottles:* Use 125-ml squat, round, wide-mouth bottles such as those available as Catalog No. 2-903 from Fisher Scientific Co. (Available from Fischer Scientific, 711 Forbes Av., Pittsburgh, PA 15219, USA.), or equivalent.

### Reagents

*Substrate Solution:* Dissolve 60 g of low-heat, non-fat dry milk (such as Peake Grade A (Available from Galloway West, Fond du Lac, Wisc. 54935, USA.)), or equivalent in 500 ml of a solution, adjusted to pH 6.3 if necessary, containing in each ml 2.05 mg of sodium acetate ( $\text{NaC}_2\text{H}_3\text{O}_2$ ) and 1.11 mg of calcium chloride ( $\text{CaCl}_2$ ).

*Standard Preparation:* Use a standard-strength rennet; bovine rennet; milk-clotting enzyme, microbial (*E. parasitica*); or milk-clotting enzyme, microbial (*Mucor* species) as appropriate for the preparation to be assayed. Such standards, which are available from commercial coagulant manufacturers, should be of known activity. Dilute the standard-strength material 1 to 200 with water, and mix. Equilibrate to 30° before use, and prepare no more than 2 h prior to use.

### Sample Preparation

Prepare aqueous solutions or dilutions of the sample to produce a final concentration such that the clotting time, as determined in the Procedure below, will be within 1 min of that of the Standard Preparation. Prepare no more than 1 h prior to use.

### Procedure

Transfer 50.0 ml of the Substrate Solution into each of four 125-ml Sample Bottles. Place the bottles on the Bottle-rotating Apparatus, and suspend the apparatus in a water bath, maintained at  $30^\circ \pm 0.5$ , so that the bottles are at an angle or approximately  $20^\circ$  to  $30^\circ$  to the horizontal. Immerse the bottles so that the water level in the bath is about equal to the substrate level in the bottles. Begin rotating the apparatus at 16-18 rpm, then add 1.0 ml of the Sample Preparation to each of the two bottles, and record the exact time of addition. Add 1.0 ml of the Standard Preparation to each of the other two bottles, recording the exact time. Observe the rotating bottles, and record the exact time of the first evidence of clotting (i.e. when fine granules or flecks adhere to the sides of the bottle). Variations in the response of different lots of the substrate may cause variations in clotting time; therefore, the test samples and standards should be measured simultaneously on the same substrate. Average the clotting time, in sec, of the duplicate samples, recording the time for the Standard Preparation as  $T_s$  and that for the Sample Preparation as  $T_v$ .

### Calculation

Calculate the activity of the enzyme preparation by the formula:

$$\text{Milk-clotting Units/ml} = 100 \times (T_s/T_v) \times (D_s/D_v)$$

in which 100 is the activity assigned to the Standard Preparation,  $D_s$  is the dilution factor for the Standard Preparation, and  $D_v$  is the dilution factor for the Sample Preparation.

**Note:** *The dilution factors should be expressed as fractions; e.g., a dilution of 1 to 200 should be expressed as 1/200.*

## **Protease Activity (Viscometer method)**

### Scope

This procedure is designed for the determination of protease activity at pH 7.



### Principle

This assay is based on the enzymatic hydrolysis of the peptide bonds of a defined gelatin substrate at pH 7.0 and 40°. The corresponding reduction in substrate viscosity is determined with a calibrated viscometer. One Viscometric Protease Unit is defined as that activity which will produce a relative fluidity change of 0.01 per sec in a defined gelatine substrate under the conditions of the assay.

### Special Apparatus

*Calibrated viscometer:* Size 100 Calibrated Cannon-Fenske Type Viscometer, or its equivalent, supplied as Catalog No. P2885-100.

*Constant temperature glass water bath (40 ± 0.1°):* Constant temperature glass water bath, or its equivalent, supplied as Catalog No. W3520-10 (Available from Scientific Products, 1210 Waukegan Rd., McGaw Park, Ill., 60085, USA.).

*Stopwatches:* Stopwatch calibrated in 1/10 min for determining the reaction time ( $T_r$ ) and stopwatch calibrated in 1/5 sec for determining the efflux time ( $T_t$ ).

### Reagents and Solutions

*Disodium monohydrogen phosphate solution (1 N):* Dissolve 47.32 g of anhydrous disodium phosphate in approximately 800 ml of distilled water in a beaker. Quantitatively transfer to a 1,000-ml volumetric flask and dilute to volume with distilled water.

*Monosodium dihydrogen phosphate solution (1 N):* Dissolve 40.00 g of anhydrous monosodium phosphate in approximately 800 ml of distilled water in a beaker. Quantitatively transfer to a 1,000-ml volumetric flask and dilute to volume with distilled water.

*Phosphate buffer (pH 7.0):* Using a standardized pH-meter, add disodium monohydrogen phosphate solution (1 N) with continuous agitation to 800 ml of monosodium dihydrogen phosphate solution (1 N) until the buffer is pH 7.0 ± 0.05.

*Gelatine substrate (4.0% w/v):* With continuous agitation, disperse 20.00 g (moisture-free basis) of gelatin in approximately 400 ml of distilled water in a 1,000-ml Erlenmeyer flask. The dispersion must be free of lumps. Swell the gelatin for 30 min at room temperature with occasional swirling. Place the gelatin solution on a 40 ± 0.1° waterbath. Swirl occasionally until the gelatin is completely solubilized with no particles appearing in solution. Cool to room temperature and quantitatively transfer to a 500-ml volumetric flask and dilute to volume with distilled water.

*Enzyme Preparation:* Prepare an enzyme solution so that 1 ml of the final dilution will produce a relative fluidity change between 0.18 and 0.22 in 5 min under the conditions of the assay. Weigh the enzyme and quantitatively transfer to a glass mortar. Triturate the enzyme with distilled water and quantitatively transfer to an appropriate volumetric flask. Dilute the volume with distilled water and filter the enzyme solution through Whatman No. 1 filter paper, or equivalent, prior to use.

### Procedure

Place the calibrated viscometer in the 40 ± 0.1° water bath in an exactly vertical position. Use only a clean viscometer. Cleaning is readily accomplished by drawing a large volume of detergent solution followed by distilled water through the viscometer. This can be accomplished by using an aspirator with a rubber tube connected to the narrow arm of the viscometer.

Pipet 20 ml of gelatin substrate and 3 ml of phosphate buffer into a 50-ml Erlenmeyer flask. Allow at least two flasks for each enzyme sample and one flask for a substrate blank. Stopper the flasks and equilibrate them in the water bath for 15 min. At zero time pipet 1 ml of the enzyme solution into the equilibrated substrate. Start the stopwatch calibrated in 0.1 min and mix solution thoroughly. Immediately pipet 10 ml of the reaction mixture into the wide arm of the viscometer.

After approximately 2 min apply suction with a rubber tube connected to the narrow arm of the viscometer drawing the reaction mixture above the upper mark into the driving fluid head. Measure the efflux time by allowing the reaction mixture to freely flow down past the upper mark. As the meniscus of the reaction mixture falls past the upper mark, start the other stopwatch. At the same time record the reaction time in min from the first stopwatch ( $T_r$ ). As the meniscus of the reaction mixture falls past the lower mark, record the time in sec from the second stopwatch ( $T_t$ ). Immediately redraw the reaction mixture above the upper mark and into the fluid driving head. As the meniscus of the reaction mixture falls freely past the upper mark, restart the second stopwatch. At the same time, record the reaction time in min from the first stopwatch ( $T_r$ ). As the meniscus of the reaction mixture falls past the lower mark, record the time in sec, from the second stopwatch ( $T_t$ ).

Repeat from redrawing the reaction mixture above the upper mark, until a total of 4 determinations is obtained over a reaction time ( $T_r$ ) of not more than 15 min.

Prepare a substrate blank by pipetting 1 ml of distilled water into 24 ml of buffered substrate. Pipet 10 ml of the reaction mixture into the wide arm of the viscometer. Determine the time ( $T_s$ ) in sec required for the meniscus to fall between the two marks. Use an average of 5 determinations for  $T_s$ .

Prepare a water blank by pipetting 10 ml of equilibrated distilled water into the wide arm of the viscometer. Determine the time ( $T_w$ ) in sec required for the meniscus to fall between the two marks. Use an average of 5 determinations for  $T_w$ .

### Calculation

One Viscometric Protease Unit (VPU) is that activity which will produce a relative fluidity change of 0.01 per sec in a defined gelatin substrate under the conditions of the assay.

Calculate the relative fluidities ( $F_r$ ) and the times ( $T_n$ ) for each of the four (4) efflux times ( $T_t$ ) and reaction times ( $T_r$ ) as follows:

$$F_r = (T_s - T_w)/(T_t - T_w)$$

$$T_n = 1/2 (T_t/60) + T_r = (T_t/120) + T_r$$

where

$F_r$  is relative fluidity for each reaction time,

$T_s$  is average efflux time for the substrate blank in sec,

$T_w$  is average efflux time for the water blank in sec,

$T_t$  is efflux time of the reaction mixture in sec,

$T_r$  is elapsed time in min from zero time, i.e. the time from addition of the enzyme solution to the buffered substrate, until the beginning of the measurement of efflux time ( $T_t$ ),

$T_n$  is reaction time in min ( $T_r$ ), plus one-half of the efflux time ( $T_t$ ) converted to min.

Plot the four relative fluidities ( $F_r$ ) as the ordinate against the four reaction times ( $T_r$ ) as the abscissa. A straight line should be obtained. The slope of this line corresponds to the relative fluidity change per min and is proportional to the enzyme concentration. The slope of the best line through a series of experimental points is a better criterion of enzyme activity than is a

single relative fluidity value. From the graph determine the  $F_r$  values at 10 and 5 min. They should have a difference in fluidity of not more than 0.22 nor less than 0.18. Calculate the activity of the enzyme unknown as follows:

$$\text{VPU/g} = [1,000 (F_{r10} - F_{r5})] / (W \times 300 \times 0.01) = [333 (F_{r10} - F_{r5})] / W$$

where

$F_{r5}$  is relative fluidity at five (5) min of reaction time

$F_{r10}$  is relative fluidity at ten (10) min of reaction time

300 is time of relative fluidity change in sec from  $F_{r10}$  to  $F_{r5}$

1,000 is milligrams per g

W is weight in milligrams of enzyme added to the reaction mixture in a one (1) ml aliquot of enzyme solution

0.01 is change in relative fluidity per sec per VPU.

## **Proteolytic Activity, Bacterial (PC)**

### Scope

This procedure is designed for the determination of protease activity, expressed as PC units.

### Principle

The assay is based on a 30-min proteolytic hydrolysis of casein at 37° and pH 7.0. Unhydrolyzed casein is removed by filtration, and the solubilized casein is determined spectrophotometrically.

### Reagents and Solutions

*Casein: Use Hammarsten-grade casein (Available from Nutritional Biochemical Corp., 21010 Miles Ave., Cleveland, Ohio 44128, USA.) or equivalent.*

*Tris buffer (pH 7.0):* Dissolve 12.1 g of enzyme-grade (or equivalent) tris(hydroxymethyl)aminomethane in 800 ml of water, and titrate with 1 N hydrochloric acid to pH 7.0. Transfer into a 1,000-ml volumetric flask, dilute to volume with water, and mix.

*TCA solution:* Dissolve 18 g of trichloroacetic acid and 19 g of sodium acetate trihydrate in 800 ml of water in a 1,000-ml volumetric flask, add 20 ml of glacial acetic acid, dilute to volume with water, and mix.

*Substrate solution:* Dissolve 6.05 g of tris(hydroxymethyl)aminomethane (enzyme grade) in 500 ml of water, add 8 ml of 1 N hydrochloric acid, and mix. Dissolve 7 g of Casein in this solution, and heat for 30 min in a boiling water bath, stirring occasionally. Cool to room temperature, and adjust to pH 7.0 with 0.2 N hydrochloric acid, adding the acid slowly, with vigorous stirring, to prevent precipitation of the casein. Transfer the mixture into a 1,000-ml volumetric flask, dilute to volume with water, and mix.

### Sample Preparation

Using Tris Buffer, prepare a solution of the sample enzyme preparation so that 2 ml of the final dilution will contain between 10 and 44 PC units.

### Procedure

Pipet 10.0 ml of the Substrate Solution into each of a series of 25 x 150-mm test tubes, allowing one tube for each enzyme test, one tube for each enzyme blank, and one tube for a substrate blank. Equilibrate the tubes for 15 min in a water bath maintained at 37 ± 0.1°. At zero time, rapidly pipet 2.0 ml of the Sample Preparation into the equilibrated substrate,

starting the stopwatch at zero time. Mix, and replace the tubes in the water bath. Add 2 ml of Tris Buffer (instead of the Sample Preparation) to the substrate blank.

After exactly 30 min, add 10 ml of TCA Solution to each enzyme incubation and to the substrate blank to stop the reaction. **Caution:** *Do not use mouth suction for the TCA Solution.* Heat the tubes in the water bath for an additional 30 min to allow the protein to coagulate completely.

At the end of the second heating period, shake each tube vigorously, and filter through 11-cm Whatman No. 42, or equivalent, filter paper, discarding the first 3 ml of filtrate.

**Note:** *The filtrate must be perfectly clear.*

Determine the absorbance of each sample filtrate in a 1-cm cell, at 275 nm, with a suitable spectrophotometer, using the filtrate from the substrate blank to set the instrument at zero. Correct each reading by subtracting the appropriate enzyme blank reading, and record the value so obtained in  $A_u$ .

#### Standard Curve

Transfer 100 mg of L-tyrosine, chromatographic-grade (Available from Calbiochem, La Jolla, Calif. 92037, USA.) or equivalent, previously dried to constant weight, to a 1,000-ml volumetric flask. Dissolve in 60 ml of 0.1 N hydrochloric acid.

When completely dissolved, dilute the solution to volume with water, and mix thoroughly. This solution contains 100  $\mu\text{g}$  of tyrosine in 1.0 ml. Prepare three more dilutions from this stock solution to contain 75.0, 50.0 and 25.0  $\mu\text{g}$  of tyrosine per ml. Determine the absorbance of the four solutions at 275 nm in a 1-cm cell with a suitable spectrophotometer versus 0.006 N hydrochloric acid. Prepare a plot of absorbance versus tyrosine concentration.

#### Calculation

One bacterial protease unit (PC) is defined as that quantity of enzyme that produces the equivalent of 1.5  $\mu\text{g}$  per ml of L-tyrosine per min under the conditions of the assay.

From the Standard Curve, and by interpolation, determine the absorbance of a solution having a tyrosine concentration of 60  $\mu\text{g}$  per ml. A figure close to 0.0115 should be obtained. Divide the interpolated value by 40 to obtain the absorbance equivalent to that of a solution having a tyrosine concentration of 1.5  $\mu\text{g}$  per ml and record the value thus derived as  $A_s$ .

Calculate the activity of the sample enzyme preparation by the formula:

$$\text{PC/g} = (A_u/A_s) \times (22/30W)$$

in which

22 is the final volume, in ml of the reaction mixture,

30 is the time of the reaction, in min, and

W is the weight of the original sample taken, in g.

### **Proteolytic Activity, Fungal (HUT)**

#### Scope

This procedure is for the determination of the proteolytic activity, expressed as haemoglobin units on the tyrosine basis (HUT), of preparations derived from *Aspergillus oryzae*, var., and *Aspergillus niger*, var., and it may be used to determine the activity of other proteases at pH 4.7.

### Principle

The test is based on the 30-min enzymatic hydrolysis of a haemoglobin substrate at pH 4.7 and 40°. Unhydrolyzed substrate is precipitated with trichloroacetic acid and removed by filtration. The quantity of solubilized haemoglobin in the filtrate is determined spectrophotometrically.

### Reagents and Solutions

**Haemoglobin:** Use Haemoglobin Substrate Powder (Sigma Chemicals Co., Catalog No. H 262) or a similar high-grade material that is completely soluble in water.

**Acetate Buffer Solution:** Dissolve 136 g of sodium acetate ( $\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$ ) in sufficient water to make 500 ml. Mix 25.0 ml of this solution with 50.0 ml of 1 M acetic acid, dilute to 1,000 ml with water, and mix. The pH of this solution should be  $4.7 \pm 0.02$ .

**Substrate Solution:** Transfer 4.0 g of the Haemoglobin into a 250-ml beaker, add 100 ml of water, and stir for 10 min to dissolve. Immerse the electrodes of a pH meter in the solution, and adjust the pH to 1.7, stirring continuously, by the addition of 0.3 N hydrochloric acid. After 10 min, adjust the pH to 4.7 by the addition of 0.5 M sodium acetate. Transfer the solution into a 200-ml volumetric flask, dilute to volume with water, and mix. This solution is stable for about 5 days when refrigerated.

**Trichloroacetic Acid Solution:** Dissolve 14.0 g of trichloroacetic acid in about 75 ml of water. Transfer the solution to a 100-ml volumetric flask, dilute to volume with water, and mix thoroughly.

### Sample Preparation

Dissolve an amount of the sample in the Acetate Buffer Solution to produce a solution containing, in each ml, between 9 and 22 HUT. (Such a concentration will produce an absorbance reading, in the procedure below, within the preferred range of 0.2 to 0.5.)

### Procedure

Pipet 10.0 ml of the Substrate Solution into each of a series of 25 x 150-mm test tubes: one for each enzyme test and one for the substrate blank. Heat the tubes in a water bath at 40° for about 5 min. To each tube except the substrate blank add 2.0 ml of the Sample Preparation, and begin timing the reaction at the moment the solution is added; add 2.0 ml of the Acetate Buffer Solution to the substrate blank tube. Close the tubes with No. 4 rubber stoppers, and tap each tube gently for 30 sec against the palm of the hand to mix. Heat each tube in a water bath at 40° for exactly 30 min, and then pipet rapidly 10.0 ml of the Trichloroacetic Acid Solution into each tube. (**Caution:** *Do not use mouth suction on the pipet.*) Shake each tube vigorously against the stopper for about 40 sec, and then allow to cool to room temperature for 1 h, shaking each tube against the stopper at 10 to 12 min intervals during this period. Prepare enzyme blanks as follows: heat, in separate tubes, 10.0 ml of the Trichloroacetic Acid Solution in 10.0 ml of the Substrate Solution, shake well for 40 sec, and to this mixture add 2.0 ml of the preheated Sample Preparation. Shake again, and cool at room temperature for 1 h, shaking at 10 to 12 min intervals.

At the end of 1 h, shake each tube vigorously, and filter through 11-cm Whatman No. 42, or equivalent, filter paper, re-filtering the first half of the filtrate through the same paper. Determine the absorbance of each filtrate in a 1-cm cell, at 275 nm, with a suitable spectrophotometer, using the filtrate from the substrate blank to set the instrument to zero. Correct each reading by subtracting the appropriate enzyme blank reading, and record the value so obtained as  $A_U$ .

**Note:** If a corrected absorbance reading between 0.2 and 0.5 is not obtained, repeat the test using more or less of the enzyme preparation as necessary.

#### Standard Curve

Transfer 100.0 mg of L-tyrosine, chromatographic-grade or equivalent (Aldrich Chemical Co.), previously dried to constant weight, to a 1,000-ml volumetric flask. Dissolve in 60 ml of 0.1 N hydrochloric acid. When completely dissolved, dilute the solution to volume with water, and mix thoroughly. This solution contains 100 µg of tyrosine in 1.0 ml. Prepare three more dilutions from this stock solution to contain 75.0, 50.0, and 25.0 µg of tyrosine per ml. Determine the absorbance of the four solutions at 275 nm in a 1-cm cell on a suitable spectrophotometer versus 0.006 N hydrochloric acid. Prepare a plot of absorbance versus tyrosine concentration. Determine the slope of the curve in terms of absorbance per µg of tyrosine. Multiply this value by 1.10, and record it as  $A_s$ . A value of approximately 0.0084 should be obtained.

#### Calculation

One HUT unit of proteolytic (protease) activity is defined as that amount of enzyme that produces, in 1 min under the specified conditions, a hydrolysate whose absorbance at 275 nm is the same as that of a solution containing 1.10 µg per ml of tyrosine in 0.006 N hydrochloric acid.

Calculate the HUT per g of the original enzyme preparation by the formula,

$$\text{HUT/g} = (A_U/A_s) \times (22/30W),$$

in which

22 is the final volume of the test solution,

30 is the reaction time in min, and

W is the weight of the original sample taken, in g.

**Note:** The value for  $A_s$ , under carefully controlled and standardized conditions, is 0.0084. This value may be used for routine work in lieu of the value obtained from the standard curve, but the exact value calculated from the standard curve should be used for more accurate results and in cases of doubt.

### **Proteolytic Activity, Fungal (SAP)**

#### Scope

This procedure is for the determination of proteolytic activity, expressed in spectrophotometric acid protease units (SAPU), of preparations derived from *Aspergillus niger*, var., and *Aspergillus oryzae*, var.

#### Principle

The test is based on a 30-min enzymatic hydrolysis of a Hammarsten Casein Substrate at pH 3.0 and 37°. Unhydrolyzed substrate is precipitated with trichloroacetic acid and removed by filtration. The quantity of solubilized casein in the filtrate is determined spectrophotometrically.

#### Reagents and Solutions

*Casein:* Use Hammarsten-grade casein, available from Nutritional Biochemical Corp., 21010 Miles Avenue, Cleveland, Ohio 44128.

*Glycine-Hydrochloric Acid Buffer (0.05 M):* Dissolve 3.75 g of glycine in about 800 ml of water. Add 1 N hydrochloric acid until the solution is pH 3.0, determined with a pH meter. Quantitatively transfer the solution to a 1000-ml volumetric flask, dilute to volume with water, and mix.

*TCA Solution:* Dissolve 18.0 g of trichloroacetic acid and 11.45 g of anhydrous sodium acetate in about 800 ml of water, and add 21.0 ml of glacial acetic acid. Quantitatively transfer the solution to a 1000-ml volumetric flask, dilute to volume with water, and mix.

*Substrate Solution:* Pipet 8 ml of 1 N hydrochloric acid into about 500 ml of water, and disperse 7.0 g (moisture-free basis) of Casein into this solution, using continuous agitation. Heat for 30 min in a boiling water bath, stirring occasionally, and cool to room temperature. Dissolve 3.75 g of glycine in the solution, and adjust to pH 3.0 with 0.1 N hydrochloric acid, using a pH meter. Quantitatively transfer the solution to a 1000-ml volumetric flask, dilute to volume with water, and mix.

### Sample Preparation

Using Glycine-Hydrochloric Acid Buffer: Prepare a solution of the sample enzyme preparation so that 2 ml of the final dilution will give a corrected absorbance of enzyme incubation filtrate at 275 nm ( *A*, as defined in the Procedure) between 0.200 and 0.500. Weigh the enzyme preparation, quantitatively transfer it to a glass mortar, and triturate with Glycine-Hydrochloric Acid Buffer. Quantitatively transfer the mixture to an appropriately sized volumetric flask, dilute to volume with Glycine-Hydrochloric Acid Buffer, and mix.

### Procedure

Pipet 10.0 ml of Substrate Solution into each of a series of 25 x 150 mm test tubes, allowing at least two tubes for each sample, one for each enzyme blank, and one for a substrate blank. Stopper the tubes, and equilibrate them for 15 min in a water bath maintained at  $37^{\circ} \pm 0.1^{\circ}$ .

At zero time, start the stopwatch, and rapidly pipet 2.0 ml of the Sample Preparation into the equilibrated substrate. Mix by swirling, and replace the tubes in the water bath. (**Note:** *The tubes must be stoppered during incubation*). Add 2 ml of Glycine-Hydrochloric Acid Buffer (instead of the Sample Preparation) to the substrate blank. After exactly 30 min, add 10 ml of TCA Solution to each enzyme incubation and to the substrate blank to stop the reaction. (**Caution:** *Do not use mouth suction for the TCA Solution*). In the following order, prepare an enzyme blank containing 10 ml of Substrate Solution, 10 ml of TCA Solution, and 2 ml of the Sample Preparation. Heat all tubes in the water bath for 30 min, allowing the precipitated protein to coagulate completely.

At the end of the second heating period, cool the tubes in an ice bath for 5 min, and filter through Whatman No. 42 filter paper, or equivalent. The filtrates must be perfectly clear. Determine the absorbance of each filtrate in a 1-cm cell at 275 nm with a suitable spectrophotometer, against the substrate blank. Correct each absorbance by subtracting the absorbance of the respective enzyme blank.

### Standard Curve

Transfer 181.2 mg of L-tyrosine, chromatographic-grade or equivalent (Calbiochem, La Jolla, Calif. 92037), previously dried to constant weight, to a 1,000-ml volumetric flask. Dissolve in 60 ml of 0.1 N hydrochloric acid. When completely dissolved, dilute the solution to volume with water, and mix thoroughly. This solution contains 1.00  $\mu\text{mol}$  of tyrosine in 1.0 ml. Prepare dilutions from this stock solution to contain 0.10, 0.20, 0.30, 0.40, and 0.50  $\mu\text{mol}$  per ml. Determine the absorbance of each dilution in 1-cm cell at 275 nm, against a water blank. Prepare a plot of absorbance versus  $\mu\text{mol}$  of tyrosine per ml. A straight line must be obtained.

Determine the slope and intercept for use in the Calculation below. A value close to 1.38 should be obtained. The slope and intercept may be calculated by the least squares method as follows:

$$\text{Slope} = [n\Sigma(MA) - \Sigma(M)\Sigma(A)] / [n\Sigma(M^2) - (\Sigma M)^2]$$

$$\text{Intercept} = [\Sigma(A)\Sigma(M^2) - \Sigma(M)\Sigma(MA)] / [n\Sigma(M^2) - (\Sigma M)^2]$$

in which n is the number of points on the standard curve, M is the  $\mu\text{mol}$  of tyrosine per ml for each point on the standard curve, and A is the absorbance of the sample.

#### Calculation

One spectrophotometric acid protease unit is that activity that will liberate 1  $\mu\text{mol}$  of tyrosine per min under the conditions specified. The activity is expressed as follows:

$$\text{SAPU/g} = (A - I) \times 22 / (S \times 30 \times W),$$

in which

A is the corrected absorbance of the enzyme incubation filtrate;

I is the intercept of the Standard Curve;

22 is the final volume of the incubation mixture, in ml;

S is the slope of Standard Curve;

30 is the incubation time, in min; and

W is the weight, in g, of the enzyme sample contained in the 2.0-ml aliquot of Sample Preparation added to the incubation mixture in the Procedure.

### **Proteolytic Activity, Plant**

#### Scope

This procedure is designed for the determination of the proteolytic activity of papain, ficin and bromelain.

#### Principle

The assay is based on a 60 min proteolytic hydrolysis of a casein substrate at pH 6.0 and 40°. Unhydrolyzed substrate is precipitated with trichloroacetic acid and removed by filtration; solubilized casein is then measured spectrophotometrically.

#### Reagents and Solution

*Sodium phosphate solution (0.05 M):* Transfer 7.1 g of anhydrous dibasic sodium phosphate into a 1000-ml volumetric flask, dissolve in about 500 ml of water, dilute to volume with water, and mix. Add 1 drop of toluene as preservative.

*Citric acid solution (0.05 M):* Transfer 10.5 g of citric acid monohydrate into a 1,000-ml volumetric flask, dissolve in about 500 ml of water, dilute to volume with water, and mix. Add 1 drop of toluene as preservative.

*Phosphate-cysteine-EDTA buffer solution:* Dissolve 7.1 g of anhydrous dibasic sodium phosphate in about 800 ml of water, and then dissolve in this solution 14.0 g of disodium EDTA dihydrate and 6.1 g of cysteine hydrochloride monohydrate. Adjust to pH 6.0  $\pm$  0.1 with 1 N hydrochloric acid or 1 N sodium hydroxide, then transfer into a 1,000-ml volumetric flask, dilute to volume with water, and mix.

*Trichloroacetic acid solution:* Dissolve 30 g of trichloroacetic acid in 100 ml of water.



*Casein substrate solution:* Disperse 1 g (moisture-free basis) of Hammarsten casein or equivalent in 50 ml of Sodium Phosphate Solution, and heat for 30 min in a boiling water bath, with occasional shaking. Cool to room temperature, and with rapid and continuous shaking, adjust to pH  $6.0 \pm 0.1$  by the addition of citric acid solution.

**Note:** *Rapid and continuous agitation during the addition prevents casein precipitation.*

Quantitatively transfer the mixture into a 100-ml volumetric flask, dilute to volume with water, and mix.

*Stock standard solution:* Transfer 100.0 mg of USP Papain Reference Standard into a 100-ml volumetric flask, dissolve and dilute to volume with Phosphate-Cysteine-EDTA Buffer Solution, and mix.

*Diluted standard solutions:* Pipet 2, 3, 4, 5, 6 and 7 ml of Stock Standard Solution into a series of 100-ml volumetric flasks, dilute each to volume with Phosphate-Cysteine-EDTA Buffer Solution, and mix by inversion.

*Test solution:* Prepare a solution from the enzyme preparation so that 2 ml of the final dilution will give an absorbance in the Procedure between 0.2 and 0.5. Weigh the sample accurately, transfer it quantitatively to a glass mortar, and triturate with Phosphate-Cysteine-EDTA Buffer Solution. Transfer the mixture quantitatively into a volumetric flask of appropriate size, dilute to volume with Phosphate-Cysteine-EDTA Buffer Solution, and mix.

#### Procedure

Pipet 5 ml of Casein Substrate Solution into each of a series of 25 x 150 mm test tubes, allowing three tubes for the enzyme unknown, six for a papain standard curve, and nine for enzyme blanks. Equilibrate the tubes for 15 min in a water bath maintained at  $40 \pm 0.1^\circ$ . At zero time, rapidly pipet 2 ml of each of the Diluted Standard Solutions, and 2-ml portions of the Test Solution, into the equilibrated substrate, starting the stopwatch at zero time. Mix each by swirling, stopper and place the tubes back in the water bath. After 60.0 min. add 3 ml of Trichloroacetic Acid Solution to each tube. (**Caution:** *Do not use mouth suction*). Mix each tube immediately by swirling.

Prepare enzyme blanks containing 5.0 ml of Casein Substrate Solution, 3.0 ml of Trichloroacetic Acid Solution, and 2.0 ml of one of the appropriate Diluted Standard Solutions or the Test Solution.

Return all tubes to the water bath, and heat for 30.0 min allowing the precipitated protein to coagulate completely. Filter each mixture through Whatman No. 42, or equivalent, filter paper, discarding the first 3 ml of filtrate. The subsequent filtrate must be perfectly clear. Determine the absorbance of each filtrate in a 1-cm cell at 280 nm with a suitable spectrophotometer, against its respective blank.

#### Calculation

One papain unit (PU) is defined in this assay as that quantity of enzyme that liberates the equivalent of 1  $\mu\text{g}$  of tyrosine per h, under the conditions of the assay. Prepare a standard curve by plotting the absorbances of filtrates from the Diluted Standard Solutions against the corresponding enzyme concentrations, in mg/ml. By interpolation from the standard curve, obtain the equivalent concentration of the filtrate from the Test Solution. Calculate the activity of the enzyme preparation taken for analysis as follows:

$$\text{PU/mg} = (A \times C \times 10)/W$$

in which

A is the activity of USP Papain Reference Standard, in PU per mg,

C is the concentration, in mg per ml, of Reference Standard from the standard curve, equivalent to the enzyme unknown,

10 is the total volume, in ml, of the final incubation mixture, and

W is the weight, in mg, of original enzyme preparation in the 2-ml aliquot of Test Solution added to the incubation mixture.

## **Pullulanase Activity**

### Scope

This procedure is designed for the determination of the pullulanase activity. (Pullulan is produced by deep fermentation of food grade hydrolysed starch by *Aureobasidium pullulans*.)

### Principle

Pullulanase hydrolyses  $\alpha$  1-6 glycosidic links in branched poly-saccharides and breaks down pullulan to yield maltotriose only. After the reaction is complete, the reducing sugars formed are estimated by the reaction with dinitrosalicylic acid. Thus one unit of Pullulanase is the activity which will produce reducing sugars equivalent to 1 mg of anhydrous maltose after one min, under the conditions of the assay. (*Maltose is used as the standard of comparison, because maltotriose is expensive and not of the highest purity. The method measures the reducing end groups of maltotriose and higher sugars using maltose as a reference.*)

### Reagents

*Pullulan solution:* Add 1 g of standard pullulan to 70 ml of distilled water. Boil for 5 min, cool and add 10 ml of molar acetate buffer pH 5.0 then dilute to 100 ml. Filter if necessary. This solution can be stored up to two weeks in a refrigerator.

*3,5-Dinitrosalicylic acid reagent (DNS):* Add 1 g of DNS to 16 ml of 10% w/v sodium hydroxide solution. Add 30 g of Rochelle salt (potassium sodium tartrate tetrahydrate) and 50 ml of distilled water and then warm until dissolved. Dilute this solution to 100 ml. It may be kept for 5 days at 5°.

### Procedure

Pipet 1 ml of substrate pullulan solution into a 17 x 1.5 cm test tube and place in a water bath at 50° for 5 min. Add 1 ml of enzyme solution and allow reaction to proceed for exactly 10 min. Stop reaction by adding 2 ml of DNS reagent.

Prepare a blank by adding 2 ml of DNS reagent to substrate before the enzyme is added.

Place the two tubes in a boiling water bath for exactly 5 min and then cool rapidly and add 10 ml of distilled water. Mix solutions well by shaking.

Measure the absorbance of the test solution against the blank using 2-cm glass cells at a wavelength of 540 nm.

### Standardization

The reducing value measured is compared with that of a standard maltose solution. A standard maltose graph is not necessary as, for accurate results, the absorbance produced in the test should be between 0.2 - 0.5. As 1 mg of maltose will give an absorbance of 0.82, for the purpose of the calculation the definition is adjusted to read "0.4 units of activity will produce 0.4 mg of anhydrous maltose equivalent...". Therefore a standard maltose solution is made so that 1 ml contains 0.4 mg of anhydrous maltose and this solution is used for the test

in place of the 1 ml of enzyme solution. The absorbance is read as before and should be 0.325. This reading is so constant that, if any difference is found, the wavelength calibration on the spectrophotometer should be checked. This is critical since very small errors in the wavelength can have large effects on the absorbance.

#### Calculation

For an unknown sample several dilutions are made up and tested. A graph of absorbance against enzyme concentration is plotted (see Figure 2) and the concentration of enzyme which will give an absorbance of 0.325 is found. Then, by definition this concentration of enzyme contains 0.4 Pullulanase units. Thus the activity of Pullulanase preparation is found by:

$$\text{Pullulanase activity/mg} = 1,000 / \text{mg of enzyme in test} \times (0.4 / 10)$$

Enzyme concentration	mg in test	Absorbance
0.002%	0.02	0.170
0.003%	0.03	0.245
0.004%	0.04	0.325
0.005%	0.05	0.390
0.006%	0.06	0.465
0.008%	0.08	0.595
0.010%	0.10	0.720

From the graph, an absorbance of 0.325 is given by 0.004% w/v enzyme solution. Therefore the activity equals

$$(1,000 / 0.04) \times (0.4 / 10) = 1,000 \text{ units per g}$$

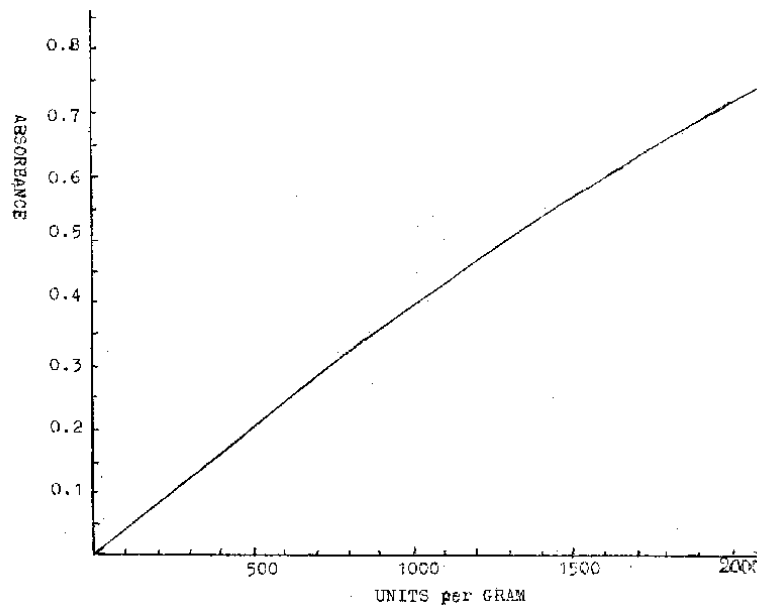


Figure 2. Pullulanase Assay for 50 mg/kg solution

This can now be used to construct a standard graph of absorbance against Pullulanase units for a fixed enzyme concentration. This graph can be used for all further samples. If the 0.005% solution is taken as standard, then its absorbance of 0.39 must give 1,000 units/g (as above). From this, a graph can be constructed for any sample at a concentration of 0.005%

Enzyme concentration	Absorbance	Units/g
0.002%	0.170	400
0.003%	0.245	600
0.004%	0.325	800
0.005%	0.390	1,000
0.006%	0.465	1,200
0.008%	0.595	1,600
0.010%	0.720	2,000

A graph is drawn on absorbance against units/g for a 0.005% enzyme solution.

#### Example

For an enzyme made up to concentration of 0.0025%, 0.005% and 0.0075%, the absorbances would be:

Concentration	Absorbance	Units g
0.0025%	0,200	480
0.005%	0.375	950
0.0075%	0.553	1,470

Thus the activity is found as follows:

$$0.0025\% \ 480 \times 0.005 / 0.0025 = 960 \text{ u/g}$$

$$0.005\% \ 950 \times 0.005 / 0.005 = 950 \text{ u/g}$$

$$0.0075\% \ 1,470 \times 0.005 / 0.0075 = 980 \text{ u/g}$$

Average = 953 units/g

### **Xylanase activity (Method 1)**

#### Principle

Xylanase samples are incubated with a remazol-stained wheat arabinoxylan substrate. Unconverted substrate is precipitated with ethanol. The intensity of blue colouring of the supernatant due to unprecipitated remazol-stained substrate degradation products is proportional to the endoxylanase activity. Xylanase activity is measured relative to an enzyme standard and calculated in Farvet Xylanase Units (FXU). The colour profile may vary from enzyme to enzyme.

Apparatus

*Spectrophotometer*  
*Thermostatic water bath*  
*Centrifuge*  
*10-ml plastic test tubes*  
*Stopwatch*

Reagents and substrates

*Phosphate buffer stock solution, 1.0 M:* Dissolve 1210 g sodium dihydrogen phosphate monohydrate and 218.9 g disodium hydrogen phosphate dihydrate in demineralised water. Add 40 ml 4 N NaOH and make up to 10 l with water.

*Phosphate buffer, 0.1 M, pH 6.00 ± 0.05:* Take 1000 ml phosphate buffer stock solution and adjust the pH to 6.0 ± 0.05 using either 4 N NaOH or 2 N HCl. Make up to 10 L with demineralised water.

*Azo-wheat arabinoxylan substrate (Megazyme Ltd., Bray, Ireland) 0.5% w/v pH 6.00 ± 0.05:* Weigh 0.500 g Azo-wheat arabinoxylan into a 150-ml beaker. Add about 90 ml of 0.1 M phosphate buffer, and heat to approximately 50°, while stirring. Continue stirring at 50° for a further 20 min. Cool the substrate solution and adjust to pH 6.00 ± 0.05 before transferring to a 100-ml graduated flask. Fill to the mark with phosphate buffer.

*Stop reagent:* Pipette 6.65 ml 2 N HCl into a 100 ml graduated flask. Fill up to the mark with 99.9% ethanol.

*Standard solutions:* Reference enzyme stock and working solutions: Accurately weigh approximately 1g FXU standard into a suitable graduated flask, add 0.1 M phosphate buffer to volume and dissolve the standard by stirring for approximately 15 min. Use this stock solution to prepare at least 6 FXU standard working solutions to give a range of activities between 0.2 and 1.4 FXU/ml for the construction of the standard curve. Prepare additional samples of known activity for inclusion at the beginning and the end of each analysis series or at least every 20 samples.

*Samples:* Samples are diluted on the basis of their anticipated activity so that the activity of the final dilution is between 0.4-1.4 FXU/ml. Results outside the working range may be used to assess the activity of the sample for the next run. Weigh dry or liquid samples directly into the flask. Granulated products may take a considerable time to dissolve.

Procedure

Pipette 0.100 ml working standard or sample solution into 10-ml test tubes, add 0.900 ml of the substrate and mix. Incubate the tubes in a 50° water bath for 30 min. Add 5 ml stop reagent and mix for 10-20 sec.

Leave the tubes to stand at room temperature, for 15-60 min and centrifuge at 4000 rpm for 15 min. Measure the absorbance of the supernatant at 585 nm within 20 min.

Calculation

Use the measurements for the enzyme standards to plot a standard curve. The data may be fitted to a third order polynomial. Determine the corresponding enzyme activity values from the standard curve for the samples. The activity of each sample is calculated as follows:

$$\text{Sample activity (in FXU/g)} = \frac{C \times F \times D}{W}$$

Where:

- C is enzyme activity read from the standard curve (FXU/ml)  
 F is volume of sample (ml)  
 D is further dilution of sample (e.g. second or third dilution)  
 W is weight of sample (g)

## Xylanase activity (Method 2)

### Principle

Xylanase samples are incubated with azurine-crosslinked wheat arabinoxylan substrate. Xylanase hydrolyses the substrate to water-soluble fragments with the concomitant change in colour. The reaction is terminated after a designated time and the optical density (OD) of the reaction mixture is measured at 590 nm (OD<sub>590</sub>). Xylanase activity is calculated based on the rate of release of the azurine dye. One xylanase unit (XU) is defined as the amount of enzyme that increases the OD<sub>590</sub> at a rate of one OD per 10 minutes under standard conditions (pH 5.00; 40°).

### Apparatus

*Spectrophotometer*  
*Magnetic stirrer*  
*Thermostatic water bath*  
*Whatman No. 1 filter paper*  
*Test tubes (15 ml)*

### Reagents

*Citric acid monohydrate*  
*Disodium hydrogen phosphate dihydrate*  
*TRIS (tris (hydroxyl methyl) amino methane)*  
*Sodium hydroxide*  
*Substrate (azurine-crosslinked wheat arabinoxylan: Xylazyme tablets from Megazyme, Ireland)*

**Note:** *a new batch of the substrate should be compared with a previous batch by analyzing the same enzyme preparation using both substrates. If a difference in enzymatic activity is noted, an appropriate correction factor should be calculated and applied to the results obtained with the new batch of the substrate.*

*Reaction buffer (McIlvaine buffer, pH 5.00):* Dissolve 10.19 g of citric acid monohydrate and 18.33 g disodium hydrogen phosphate dihydrate in 850 ml distilled water in a 1000-ml volumetric flask. Adjust the pH to 5.00 using either 0.1 M citric acid monohydrate or 0.2 M disodium hydrogen phosphate dihydrate. Add water to 1000 ml. The buffer can be stored for up to 6 months at 2-5°.

*Stop solution (2% w/v TRIS, pH 12.0):* Dissolve 20 g of TRIS in 850 ml distilled water in a 1000-ml volumetric flask. Adjust the pH to 12.0 with 5 M NaOH. The solution can be stored for up to six months at 2-5°.

*Test sample solutions:* Accurately weigh a quantity of the enzyme preparation that would give an OD increase within the range of 0.3 – 1.2 in a 100 ml volumetric flask. Add 60 ml of the reaction buffer. Stir the solution using a magnetic stirrer for 10 minutes. Remove the magnet and add the reaction buffer to volume. Transfer the enzyme solution to a glass beaker and let it stand for 5 minutes or until the precipitate settles. Use clear solution for analysis.

*Blank:* Pre-heat 1.0 ml reaction buffer at 40.0° for 5 min. Add one Xylazyme tablet. After exactly 10 min at 40.0°, add 10.0 ml stop solution and filter the sample through Whatman No.1 filter.

#### Procedure

Prepare 3 test tubes for each test sample. Pipette 1 ml of the reaction buffer to each tube and add 50, 75, and 100 microliters of the test sample solution.

- Pre-heat all test sample solutions at 40.0° for 5 min.
- Add one Xylazyme tablet to each tube. Do not stir.
- After 10 minutes ( $\pm 1$  sec), terminate the reaction by adding 10 ml stop solution.
- Filter all solutions through Whatman No. 1 filter paper.
- Measure OD of each test sample solution against the blank at 590 nm.

#### Calculations

Perform linear regression on  $OD_{590}$  as a function of test sample volumes (in ml) used in the analysis. Calculate the activity of the enzyme preparation in xylanase units (XU) per gram (g) using the following equation:

$$\frac{XU}{g} = S \frac{V}{W}$$

Where:

S is the slope obtained from linear regression of the  $OD_{590}$  as a function of sample volume in ml

V is the volume of the volumetric flask used to prepare the test sample solution in ml (multiplied by further dilutions, if applicable)

W is the weight of the enzyme preparation in g

# GLYCEROL ESTERS OF ROSINS

## Ring and ball softening point method

The ring-and-ball softening point is defined as the temperature at which a disk of the sample held within a horizontal ring is forced downward a distance of 1 in. (25.4 mm) under the weight of a steel ball as the sample is heated at a prescribed rate in a water or glycerol bath.

### Apparatus

The apparatus illustrated in Figures 1 and 2 consists of the components described in the following paragraphs.

### Ring

A brass-shouldered ring conforming to the dimensions shown in Figure 1a should be used. If desired, the ring may be attached by brazing or other convenient manner to a brass wire of about 13 B & S gauge (0.06 to 0.08 in., or 1.52 to 2.03 mm, in diameter) as shown in Figure 2a.

### Ball

A steel ball, 3/8 in. (9.53 mm) in diameter, weighing between 3.45 and 3.55 g, should be used.

### Ball-Centering Guide

A guide for centering the ball, constructed of brass and having the general shape and dimensions, as illustrated in Figure 1c may be used if desired.

### Container

Use a heat-resistant glass vessel, such as an 800-ml low-form Griffin beaker, not less than 3.34 in. (8.5 cm) in diameter and not less than 5 in. (12.7 cm) in depth from the bottom of the flare.

### Support for Ring and thermometer

Any convenient device for supporting the ring and thermometer may be used, provided that it meets the following requirements: (1) the ring shall be supported in a substantially horizontal position; (2) when using the apparatus shown in Figure 1d, the bottom of the ring shall be 1.0 in. (25.4 mm) above the horizontal plate below it, the bottom surface of the horizontal plate shall be at least 0.5 in. (13 mm) and not more than 0.75 in. (18 mm) above the bottom of the container, and the depth of the liquid in the container shall be not less than 4.0 in. (10.2 cm); (3) when using the apparatus shown in Figure 1e, the bottom of the ring shall be 1.0 in. (25.4 mm) above the bottom of the container, with the bottom end of the rod resting on the bottom of the container, and the depth of the liquid in the container shall be not less than 4.0 in. (10.2 cm), as shown in Figure 1 a, b and c; and (4) in both assemblies, the thermometer shall be suspended so that the bottom of the bulb is level with the bottom of the ring and within 0.5 in. (13 mm) but not touching the ring.

### Thermometers (mercury-in-glass)

Depending upon the expected softening point of the sample, use either an ASTM 15C low-softening-point thermometer (-2° to 80°) or an ASTM 16C high-softening-point thermometer



(30° to 200°).

#### Stirrer

Use a suitable mechanical stirrer rotating between 500 and 700 rpm. To ensure uniform heat distribution in the heating medium, the direction of the shaft rotation should move the liquid upward. (See Figure 2d for recommended dimensions.)

#### Sample Preparation

Select a representative sample of the material under test consisting of freshly broken lumps free of oxidized surfaces. Scrape off the surface layer of samples received as lumps immediately before use, avoiding inclusion of finely divided material or dust. The amount of sample taken should be at least twice that necessary to fill the desired number of rings, but in no case less than 40 g. Immediately melt the sample in a clean container, using an oven, hot plate, or sand or oil bath to prevent local overheating. Avoid incorporating air bubbles in the melting sample, which must not be heated above the temperature necessary to pour the material readily without inclusion of air bubbles. The time from the beginning of heating to the pouring of the sample shall not exceed 15 min. Immediately before filling the rings; preheat them to approximately the same temperature at which the sample is to be poured. While being filled, the rings should rest on an amalgamated brass plate. Pour the sample into the rings so as to leave an excess on cooling. Cool for at least 30 min, and then cut off the excess material cleanly with a slightly heated knife or spatula. Use a clean container and a fresh sample if the test is repeated.

#### Procedure

*Materials having softening points above 80°:* Fill the glass vessel with glycerol to a depth of not less than 4.0 in. (10.2 cm) and not more than 4.25 in. (10.8 cm). The starting temperature of the bath shall be 32°. For resins (including rosin), the glycerol should be cooled to not less than 45° below the anticipated softening point, but in no case lower than 35°. Position the axis of the stirrer shaft near the back wall of the container, with the blades clearing the wall and with the bottom of the blades 0.75 in. (18 mm) above the top of the ring. Unless the ball-centering guide is used, make a slight indentation in the center of the sample by pressing the ball or a rounded rod, slightly heated for hard materials, into the sample at this point. Suspend the ring containing the sample in the glycerol bath so that the lower surface of the filled ring is 1.0 in. (25.4 mm) above the surface of the lower horizontal plate (see Figure 1d), which is at least 0.5 in. (13 mm) and not more than 0.75 in. (18 mm) above the bottom of the glass vessel, or 1.0 in. (25.4 mm) above the bottom of the container (see Figure 2e). Place the ball in the glycerol but not on the test specimen. Suspend an ASTM high-softening-point thermometer (16C) in the glycerol so that the bottom of its bulb is level with the bottom of the ring and within 0.5 in. (13 mm) but not touching the ring. Maintain the initial temperature of the glycerol for 15 min, and then, using suitable forceps, place the ball in the center of the upper surface of the sample in the ring. Begin stirring, and continue stirring at 500 to 700 rpm until completion of the determination. Apply heat at such a rate that the temperature of the glycerol is raised 5° per min, avoiding the effects of drafts by using shields if necessary.

[Note: The rate of rise of the temperature shall be uniform and shall not be averaged over the test period. Reject all tests in which the rate of rise exceeds  $\pm 0.5^\circ$  for any minute period after the first three.]

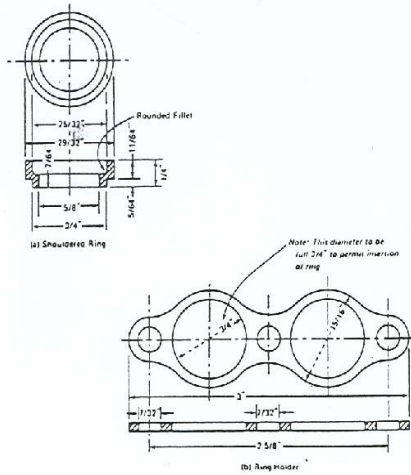
Record as the softening point the temperature of the thermometer at the instant the sample

touches the lower horizontal plate (see Figure 1d) or the bottom of the container (see Figure 2e). Make no correction for the emergent stem of the thermometer.

**Materials having softening points of 80° or below:** Follow the above procedure, except use an ASTM low-softening-point thermometer (15C) and use freshly boiled water cooled to 5° as the heating medium. For resins (including rosins), use water cooled to not less than 45° below the anticipated softening point, but in no case lower than 5°.

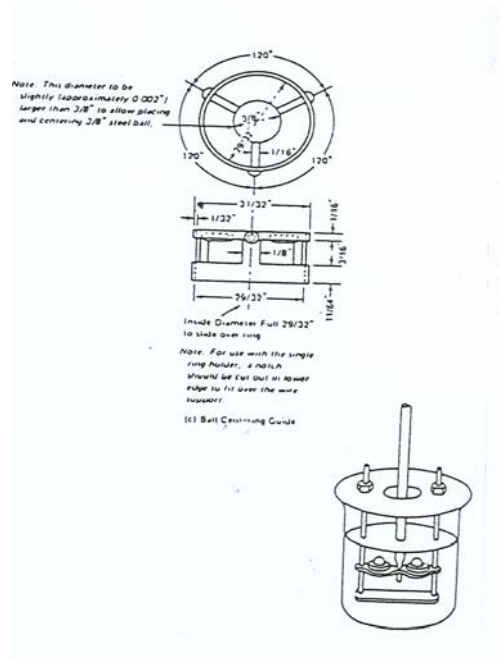
**Apparatus - Ring and Ball Softening Point**

(a) Shouldered Ring



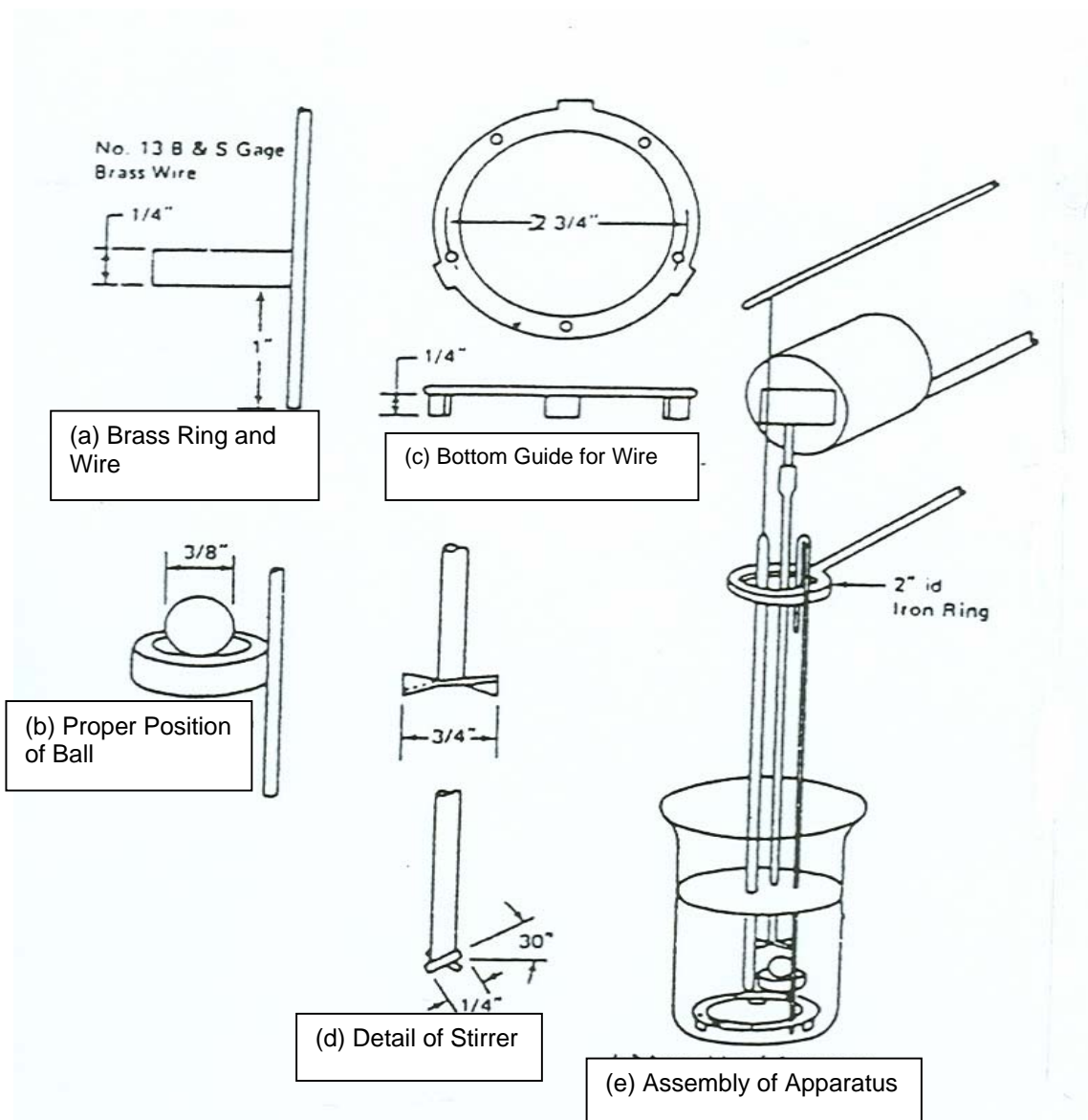
(b) Ring Holder

(c) Bell Centering Guide



(d) Assembly Apparatus with Two Rings

**Figure 1.** Shouldered Ring, Ring Holder Ball-Centering Guide, and Assembly of Apparatus Showing Two Rings



**Figure 2.** Assembly of Apparatus Showing Stirrer and Single Shouldered Ring.

Figures 1 and 2 are referenced from the Food Chemicals Codex, 6<sup>th</sup> Edition, 2008, p. 1161 (figures 40 and 41).

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## FATS, OILS AND HYDROCARBONS

### Acid Value

Acid value is defined as the number of mg of potassium hydroxide required to neutralize the acids in 1 g of fatty material.

Unless otherwise directed, weigh accurately about 5 g of sample into a 500-ml Erlenmeyer flask, and add 75-100 ml of hot neutral ethanol. Agitation and further heating may be necessary to effect complete solution of the sample. For some samples, it may be necessary to use as the solvent a 1:1 mixture of neutralized diethyl ether/ethanol or petroleum spirit/ethanol. Add 0.5 ml of phenolphthalein TS and titrate immediately, while shaking, with 0.5 N KOH until the pink colour persists for at least 30 sec. (For acidity less than 2% by weight, 0.1 N KOH should be used for the titration; for acidity less than 0.2% by weight, it is necessary, in addition, to first neutralize the carbon dioxide in the reaction vessel.)

$$\text{Acid value} = (56.1 \times T \times N) / W$$

where

T is the titre (ml);

N is the normality of potassium hydroxide solution; and

W is the weight of sample (g).

### Aromatic Hydrocarbons Determination

Determine by Gas Chromatography using the following conditions or equivalent that will elute n-decane before benzene:

#### Apparatus

Liquid phase:	Tetracyanoethylated Pentaerythritol (TCEPE)
Length:	30 m
i.d.:	0.25 mm
Temperatures:	
Inlet:	275°
Detector:	250°
Column:	95°
Carrier gas:	N <sub>2</sub>
Flow rate:	3 cm <sup>3</sup> /min
Detector	Flame ionization
Split	100 – 1

#### Reagents

*Isooctane*: 99 mole percent minimum containing less than 0.05 mole percent aromatic material.

*Benzene*: 99.5 mole percent minimum.

*Internal Standard*: n-Decane and either n-undecane or n-dodecane according to the requirement of the System Suitability Test.

*Reference Solution A*: Prepare a standard solution containing 0.5% by weight each of the Internal Standard and of benzene in isooctane.

*Reference Solution B:* Prepare a standard solution containing about 0.5% by weight each of n-decane, of Internal Standard, and of benzene in isooctane.

### Calibration

Select the instrument conditions necessary to give the desired sensitivity. Inject a known volume of Reference Solution A, and change the attenuation, if necessary, so that the benzene peak is measured with a chart deflection of not less than 25% or more than 95% of full scale. When choosing the attenuation, consider all unresolved peaks to represent a single compound. There may be tailing of the non-aromatic peak, but do not use any conditions that lead to a depth of the valley ahead of the benzene peak (A) less than 50% of the weight of the benzene peak (B) as depicted in Figure 1

If there is tailing of the non-aromatic material, construct a baseline by drawing a line from the bottom of the valley ahead of the benzene peak to the point of tangency after the peak (see Figure 2). Determine the areas of the benzene peak and the internal standard peak by use of an electronic integrator. Do not use integrators on peaks without a constant baseline, unless the integrator has provision for making baseline corrections with accuracy at least as good as that of manual methods.

Calculate a response factor for benzene (R<sub>b</sub>) relative to the Internal Standard by the formula

$$A_i/W_i \times B_v/A_b,$$

in which

A<sub>i</sub> is the area of the Internal Standard peak in arbitrary units corrected for attenuation;

W<sub>i</sub> is the weight percent of Internal Standard in Reference Solution A;

A<sub>b</sub> is the area of the benzene peak in arbitrary units corrected for attenuation; and

W<sub>b</sub> is the weight percent of benzene in Reference Solution A.

### System Suitability Test

Inject the same volume of Reference Solution B as in the Calibration and record the chromatogram. n-Decane must be eluted before benzene, and the ratio of A to B (Figure 1) must be at least 0.5 where A is equal to the depth of the valley between the n-decane and benzene peaks and B is equal to the height of the benzene peak.

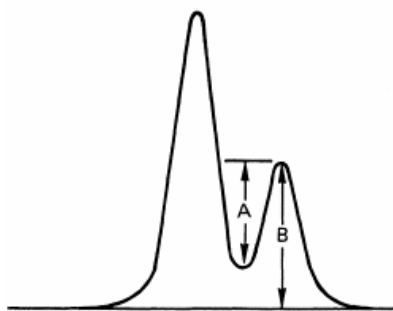


Figure 1. Illustration of A/B Ratio.

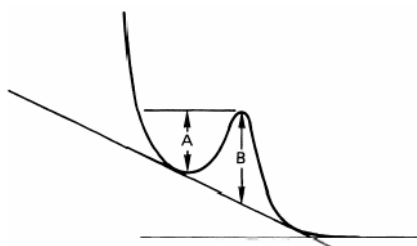


Figure 2. Illustration of A/B Ratio for a Small Component Peak on the Tail of a Large Peak.

### Procedure

Place approximately 0.1 ml of Internal Standard into a tared 25-ml volumetric flask, weigh on an analytical balance, dissolve in and dilute to volume with the sample to be analyzed.

Using the exact instrumental conditions that were used in the calibration, inject the same volume of sample containing the Internal Standard. Before measuring the area of the Internal Standard and benzene peaks, change the attenuation to ensure at least 25% chart deflection.

Measure the area of the Internal Standard and benzene peaks in the same manner as was used for the calibration. Calculate the weight percent of benzene in the sample ( $W_B$ ) by the formula

$$(A_b \times R_b \times W_i \times 100)/(A_i \times S),$$

in which

$A_b$  is the area of the benzene peak corrected for attenuation;

$R_b$  is the relative response factor for benzene;

$W_i$  is the weight, in grams, of Internal Standard added;

$A_i$  is the area of the Internal Standard peak corrected for attenuation; and

$S$  is the weight, in grams of the sample taken.

Retention Times in Minutes for Selected Hydrocarbons Under the given Conditions are:

- Benzene 6.1
- Toluene 7.0
- Ethylbenzene 8.0
- p- and m-Xylenes 8.5
- o-Xylene 10.0
- n-Dodecane 6.5

### **Average Molecular Weight**

(ASTM D 2502 See Test for Viscosity for Copyright permission)

Determine the kinematic viscosity of the sample at 37.8 and 98.9° as described in the method for Viscosity, 100°. Read the value of H that corresponds to the measured viscosity at 37.8° by the use of Table 1; linear interpolation between adjacent columns may be required. Read a viscosity -molecular weight chart for H and 98.9° viscosity (the chart is available from the American Society for Testing and Materials (ASTM)). A simplified version is shown in Figure 3 for illustration purposes only. Interpolate where necessary between adjacent lines of 98.89° viscosity. After locating the point corresponding to the value of H (ordinate) and the 98.89° viscosity (superimposed lines), read the molecular weight along the abscissa.

Kinematic viscosity, mm <sup>2</sup> / at 37.8°	Table1 - Tabulation of H Function				
	0	0.2	0.4	0.6	0.8
2	-176	-151	-126	-104	-85
3	-67	-52	-30	-25	-13
4	-1	9	19	28	36
5	44	52	59	66	73
6	79	85	90	96	101
7	106	111	116	120	124
8	128	132	136	140	144
9	147	151	154	157	160
10	163	166	169	172	175
11	178	180	183	185	188
12	190	192	195	197	199
13	201	203	206	208	210
14	211	213	215	217	219
15	212	222	224	226	227
16	229	231	232	234	235
17	237	238	240	241	243
18	244	245	247	248	249
19	251	252	253	255	256
20	257	258	259	261	262
21	263	264	265	266	267
22	269	270	271	272	273
23	274	275	276	277	278
24	279	280	281	281	282
25	283	284	285	286	287
26	288	289	289	290	291
27	292	293	294	294	295
28	295	297	298	298	299
29	300	301	301	302	303
30	304	304	305	306	306
31	307	308	308	309	310
32	310	311	312	312	313
33	314	314	315	316	316
34	317	317	318	319	319
35	320	320	321	322	322
36	323	323	324	325	325
37	325	326	327	327	328
38	328	329	329	330	331
39	331	332	332	333	333

	H									
	0	1	2	3	4	5	6	7	8	9
40	334	336	339	341	343	345	347	349	352	354
50	355	357	359	361	363	364	366	368	369	371
60	371	374	375	377	378	380	381	382	384	385
70	386	387	388	390	391	392	393	394	395	397
80	398	399	400	401	402	403	404	405	406	407
90	409	409	410	410	411	412	413	414	415	416
100	416	417	418	419	420	420	421	422	423	423
110	424	425	423	426	427	428	428	429	430	430
120	431	432	432	433	433	434	435	435	436	437
130	437	438	438	439	439	440	441	441	442	442
140	443	443	444	444	445	446	446	447	447	448
150	448	449	449	450	450	450	451	451	452	452
160	453	453	454	454	455	455	456	456	456	457
170	457	458	458	459	459	460	460	460	461	461
180	461	462	462	463	463	463	464	464	465	465
190	465	466	466	466	467	467	468	468	468	469
	H									
	0	10	20	30	40	50	60	70	80	90
200	469	473	476	479	482	485	487	490	492	493
300	497	499	501	503	505	507	509	511	512	514
400	515	517	518	520	521	523	524	525	527	528
500	529	530	531	533	534	535	536	537	538	539
600	540	541	542	543	544	545	546	547	547	548
700	549	550	551	551	552	553	554	554	555	556
800	557	557	558	559	559	560	561	562	562	563
900	563	564	565	565	566	566	567	567	568	569
1000	569	574	578	583	587	591	594	597	600	603
2000	605	608	610	614	616	618	620	621	623	625
3000	625	626	628	629	631	632	633	634	636	637
4000	638	639	640	641	642	643	644	645	646	647
5000	648	649	650	651	652	652	653	654	655	656
6000	656	657	658	658	659	660	660	661	662	662
7000	663	664	664	665	665	666	666	667	667	668
8000	668	669	670	670	671	671	671	672	672	673
9000	673	674	674	675	675	676	676	677	677	677
10000	673	681	684	688	691	694	696	699	701	703
20000	705	707	709	711	712	715	715	717	718	719
30000	720	722	723	724	725	726	727	728	729	730
40000	731	732	732	733	734	735	736	736	737	738
50000	739	739	740	741	741	742	743	743	744	744
60000	745	746	746	747	747	748	748	749	749	750
70000	750	751	751	752	752	753	753	753	754	754
80000	755	755	756	756	756	757	757	758	758	758
90000	759	759	759	760	760	760	761	761	761	762
100000	762	762	763	763	763	764	764	764	764	765



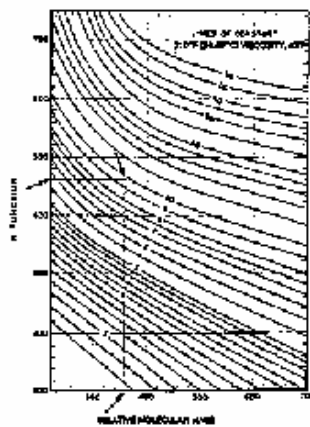


Figure 3. Lines of Constant Viscosity ( $\text{mm}^2/\text{g}$ ) at  $98.89^\circ$

### Carbon Number at 5% Distillation Point

(ASTM D 2887 See Test for Viscosity for Copyright permission.)

"Carbon number" is number of carbon atoms in a molecule. Determine the boiling point distribution of the sample by gas chromatography using the following conditions:

The system must have the following performance characteristics:

- sensitivity: 1% dodecane must be detected with a peak height of at least 10% of full scale under the conditions prescribed below.
- stability: when operated at the required sensitivity level, the baseline drift is not more than 1% of full scale per hour
- repeatability of retention times: 6 sec for each component of the calibration mixture.
- resolution (R): determined for a solution of 1% of each of hexadecane and octadecane in n-octane is not less than three and not more than eight, using the following formula:

$$R = 2d / (W_1 + W_2)$$

where

d is the distance in mm between the peak maxima of hexadecane and octadecane

W1 is the peak width in mm at the baseline of hexadecane

W2 is the peak width in mm at the baseline of octadecane

Typical conditions which may be used are:

- Column: packed with: 5% SE-30
- Column temperature:  $10 - 350^\circ$  rate:  $6.5^\circ / \text{min}$ .
- Carrier gas: helium
- Detector: FID; temperature:  $370^\circ$
- Injection block temperature:  $370^\circ$

### Calibration mixture:

Prepare a mixture of hydrocarbons of known boiling points covering the range of the sample. At least one compound must have a boiling point lower than the initial boiling point of the sample.

### Calibration

Cool the column to the selected starting temperature (the retention time for the initial boiling point must be at least 1 min) and inject the calibration mixture. Record the retention time of each peak maximum and the peak areas for each component. Plot the retention time of each peak versus the corresponding normal boiling point of that component in degrees Celsius to obtain a calibration curve.

### Sample analysis

Using the exact conditions used in the calibration run, inject the sample. Record the area of each time segment at fixed time intervals not greater than 1% of the retention time equivalent to a boiling point of 538° obtained from the calibration curve.

### Calculation

Sum the area segments to obtain the cumulative area at each time interval during the run. At the point of the chromatogram, where the baseline at the end first becomes steady, observe the cumulative area counts. Move back along the record until a cumulative area equal to 99.5% of the total at the steady point appears. Mark this point as the final boiling point. Observe the area counts at the start of the run until the point is reached, where the cumulative area count is equal to 0.5% of the total area. Mark this point as the initial boiling point of the sample. Divide the cumulative area at each interval between the initial and final boiling points by the total cumulative area and multiply by 100. This will give the cumulative percent of the sample recovered at each time interval. Tabulate the cumulative percent recovered at each interval and the retention time at the end of the interval. Using linear interpolation, if necessary, determine the retention time associated with 5% and read the corresponding boiling temperature from the calibration curve.

## **Congealing Range**

Melt in a glass tube (25 mm in diameter and 100 mm in length, the glass being 1 mm in thickness) about 5 g of the sample by heating gently to 15-20° above the expected congealing range. By means of a perforated stopper, fasten the tube in a wide-mouthed bottle of clear glass, approximately 70 mm in diameter and 150 mm in height. Suspend a standard thermometer in the melted sample so that it will serve as a stirrer, cool if necessary, and stir the mass slowly until the mercury remains stationary for 30 sec. Discontinue stirring and allow the thermometer to hang, with the bulb in the centre of the sample, and observe the rise of the mercury column. The highest point to which it rises is the congealing temperature.

## **Free Fatty Acids**

(Based on AOCS Method Ca 5a-40)

Unless otherwise directed in the specification monograph, weigh accurately the appropriate amount of the sample, indicated in the table below, into a 250-ml Erlenmeyer flask or other suitable container. Add 2 ml of phenolphthalein TS to the specified amount of hot alcohol, neutralize with alkali to the first faint but permanent pink colour, and then add the hot neutralized alcohol to the sample container. Titrate with the appropriate normality of sodium hydroxide, shaking vigorously, to the first permanent pink colour of the same intensity as that

of the neutralized alcohol. The colour must persist for at least 30 sec. Calculate the percentage of free fatty acids (FFA) in the sample by the formula  $VN/W$ , in which  $V$  is the volume and  $N$  is the normality, respectively, of the sodium hydroxide used,  $W$  is the weight of the sample, in g, and  $e$  is the equivalence factor given in the monograph.

FFA Range (%)	g of sample	ml of alcohol	Strength of NaOH
0.00-0.2	$56.4 \pm 0.2$	50	0.1
0.2-1.0	$28.2 \pm 0.2$	50	0.1
1.0-30.0	$7.05 \pm 0.05$	75	0.25
30.0-50.0	$7.05 \pm 0.05$	100	0.25-1.0
50.0-100	$3.525 \pm 0.001$	100	1.0

## Hydroxyl Value

(Based on AOCS Method Cd 13-60)

**Note:** *This method involves use of pyridine which should be used with appropriate caution.*

Hydroxyl value is defined as the number of mg of potassium hydroxide required to neutralize the amount of acetic acid capable of combining by acetylation with 1 g of sample.

Weigh accurately the appropriate amount of sample according to the expected hydroxyl value and transfer it into a 250-ml glass-stoppered Erlenmeyer flask.

Hydroxyl value	Sample weight (g)
0 to 20	10
20 to 50	5
50 to 100	3
100 to 200	2

Pipet 5.0 ml of pyridine/acetic anhydride TS into the flask. (For samples having a 0-20 hydroxyl value, add an additional 5 ml of pyridine/acetic anhydride TS to the flask.) Thoroughly mix the contents by gently swirling. Pipet 5.0 ml of pyridine/acetic anhydride TS into an empty flask for the reagent blank. (If 10.0 ml of the reagent were used for the acetylation, use a 10.0-ml blank.) Place the flasks on a steam bath, under reflux condensers, and heat for 1 h. To hydrolyze excess acetic anhydride, add sufficient water (not exceeding about 10 ml) through the condensers to the flasks. If the solution separates into two layers, add sufficient pyridine to obtain a homogeneous solution. Heat on a steam bath for 10 min with reflux condensers attached. Add 25 ml of neutralized n-butanol, about half of it through the condensers and the remainder to wash down the sides of the flasks after removal of the condensers. Add 1 ml of phenolphthalein TS and titrate to a faint pink endpoint with 0.5 N ethanolic KOH solution. To correct for free acid, mix about 10 g of the sample, accurately weighed, with 10 ml of pyridine (neutralized to phenolphthalein), add 1 ml of

phenolphthalein TS and titrate to a faint pink endpoint with 0.5 N ethanolic potassium hydroxide.

Calculate the hydroxyl value by the formula:

$$\text{Hydroxyl value} = [(B + (W_A / C) - S) \times N \times 56.1] / W$$

where

A is ml of KOH solution required for the free acid determination;

B is ml of KOH solution required for the reagent blank;

C is the weight of sample used for the free acid determination;

S is ml of KOH solution required for titration of the acetylated sample;

W is weight of sample used for acetylation; and

N is normality of the ethanolic KOH solution.

## Identification Tests for Functional Groups

### Ester Hydrolysis

Reflux 1 g of sample with 15 ml of 0.5 N ethanolic potassium hydroxide for 1 h. Add 15 ml of water, acidify with dilute hydrochloric acid TS (about 6 ml). Oily drops or a white to yellowish-white solid is produced which is soluble in 5 ml of hexane.

Remove the hexane layer, extract again with 5 ml of hexane and again remove the hexane layer. Collect all the hexane extracts together. The fatty acids thus extracted may be identified by gas-liquid chromatography (see Test A). Carry out the whole of the procedure in a fume cupboard. The aqueous layer is used for Tests B through H.

### Test A: Methyl esters of fatty acids

(Based on AOCS Methods Ce 1-62, Ce 1f-96, Ce 1h-05)

#### *Apparatus*

Use a suitable gas chromatograph equipped with a flame ionization detector (FID) and containing a 50-m × 0.25-mm id capillary fused silica column, or equivalent, containing a suitable highly polar stationary phase (0.20 μm) film, such as CP™-Sil 88, SP-2650, SP-2340, BPX-70, or SP2560.

**Note:** For accurate determination of all fatty acids present in non-ruminant animal and vegetable oils and fats, a 100m SP2560 or CP-Sil 88 column is recommended.

#### *Operating Conditions*

The operating conditions may vary with the instrument used, but a suitable chromatogram may be obtained isothermally at temperatures between 170° and 198°, depending on the column stationary phase. Inlet temperature (injector), 250°; detector, 250°; and a suitable hydrogen or helium carrier gas flow.

#### *Standard Solutions*

Column performance is checked using a suitable mixture of fatty acid methyl esters covering the range of fatty acids under investigation. Fatty acid methyl esters with a wide range of carbon numbers and double-bond configurations can be purchased. A mixture containing C12:0; 9c-18:1; 11c-18:1; 9c,12c,15c-18:3; 11c-20:1; and an Internal Standard (C21:0) using each carrier gas and column combination. Since commercial GC designs are different, to achieve optimal separation small changes in the sample size, sample concentration or oven

temperature may be required. If so, adjust the sample size, sample concentration or oven temperature until the best separation results are obtained. Baseline separation of the various components in both the standard and the sample preparations is desirable.

*Sample Preparation (for fats and oils) (Based on AOCS Method Ce 2-66)*

Introduce 100 to 1000 mg of the fat into a 50- or 125-mL reaction flask. Add 4 to 10 ml of 0.5 N methanolic sodium hydroxide, and add a boiling chip. Attach a condenser, and heat the mixture on a steam bath until the fat globules go into solution. This step should take 5 to 10 min. Add 5 to 12 ml of 12.5% boron fluoride–methanol reagent (this reagent contains 125 g/l of boron fluoride in methanol and is available commercially) through the condenser, and boil for 2 min. Add 2 to 5 ml of heptane through the condenser, and boil for 1 min longer. Remove from heat, remove condenser, and add about 15 ml of saturated sodium chloride solution. Stopper the flask, and shake vigorously for 15 sec. Transfer about 1 ml of the heptane solution into a test tube and add a small amount of anhydrous sodium sulfate. The dry heptane solution may then be injected directly into a gas chromatograph.

The methyl esters should be analyzed as soon as possible. They may be kept in an atmosphere of nitrogen in a screwcap vial at 2° for 24 h. For longer storage, they should be sealed in a glass ampoule, subjected first to a vacuum and then backfilled with nitrogen and stored at -20° (freezer).

*Procedure*

Inject an appropriate volume (1 µl) of sample into the chromatograph. If an automated system is used, follow the manufacturer's instructions; if calculations are to be done manually, proceed as follows:

Calculate the area percent of each component (CN) by the equation

$$CN = [AN/TS] \times 100,$$

in which AN is the area of the peak corresponding to component CN and TS is the total area for all detected components [TS = ΣAN].

*Calculated Iodine Value (Based on AOCS Method Cd 1c-85)*

Using the fatty acid composition determined above, calculate the Iodine value (IV) as follows:

- Triglycerides, iodine value = (% hexadecenoic acid × 0.950) + (% octadecenoic acid × 0.860) + (% octadecadienoic acid × 1.732) + (% octadecatrienoic acid × 2.616) + (% eicosenoic acid × 0.785) + (% docosenoic acid × 0.723)
- Free fatty acids, iodine value = (% hexadecenoic acid × 0.9976) + (% octadecenoic acid × 0.8986) + (% octadecadienoic acid × 1.810) + (% octadecatrienoic acid × 2.735) + (% eicosenoic acid × 0.8175) + (% docosenoic acid × 0.7497).

**Note:** This procedure is not intended to be a rapid method, but rather gives two results from one analysis. For oils with an unsaponifiable content greater than 0.5% (e.g., fish oils), and for materials with low iodine value the calculation tends to be low. Calculated IV based on GC fatty acid composition of non-triglyceride lipid materials such as partial esters of glycerol, partial esters of sorbitol/sorbitan/isosorbide esters, partial esters of polyoxyethylene sorbitol/sorbitan/isosorbide or glycerol, etc. will provide the calculated IV of only the fatty acids used to prepare the partial esters. To obtain the actual IV of partial esters with nonfatty acid polyol diluents, the chlorinated Wijs Reagent IV method should be used. IV

*values of partial esters via the Wijs method are lower than those obtained by GC because of the dilution effect of the polyol material.*

**Test B: Acetic Acid**

Transfer about 5 ml of the aqueous layer resulting from the hydrolysis into a dish, add excess calcium carbonate and evaporate until dry. Transfer the major part of the residue into a glass tube. Place a filter paper, moistened with Reagent for acetone (a saturated solution of o-nitrobenzaldehyde in sodium hydroxide TS, freshly prepared) on top of the tube. Heat over a micro flame. The yellow colour of the paper changes into greenish blue by reaction of the Reagent for acetone, with the calcium acetate formed.

**Test C: Succinic Acid**

Transfer one drop of the aqueous layer resulting from the hydrolysis and a drop of a 0.5% solution of ammonium chloride and several mg of zinc powder into a micro test tube.

The mouth of the tube is covered with a disk of filter paper moistened with a solution in benzene of 5% p-dimethylamino-benzaldehyde and 20% trichloroacetic acid. The bottom of the test tube is heated vigorously with a micro flame for about 1 min. Depending on the amount of succinic acid or succinimide, a red-violet or pink stain appears on the paper.

**Test D: Fumaric Acid**

Transfer 1 ml of the aqueous layer resulting from the hydrolysis with 1 ml of 2 N sodium carbonate into a test tube. Add 2 or 3 drops of 0.1 N potassium permanganate. The solution is promptly discoloured.

**Test E: Tartaric Acid**

Evaporate about 5 ml of the aqueous layer resulting from the hydrolysis in a porcelain dish until dry. Add 2 ml of concentrated sulfuric acid containing 0.5% of pyrogallol and heat on a steam bath. An intense violet colour is produced.

**Test F: Citric Acid**

To 3 ml of the aqueous layer resulting from the hydrolysis add a few drops of 1% potassium permanganate and warm until the colour has disappeared. Then add an excess of bromine TS. A white precipitate (pentabromoacetone) is formed immediately or on cooling.

Evaporate 1 ml of the aqueous layer resulting from the hydrolysis in a porcelain dish, add 1 ml of a mixture of 1 vol acetic anhydride and 5 vol of pyridine into the warm dish. A violet colour is produced. (Tartaric acid produces a green colour.)

**Test G: Lactic Acid**

Transfer 0.2 ml of the aqueous layer resulting from the hydrolysis and 2 ml of concentrated sulfuric acid into a test tube and place for 2 min in boiling water. Cool and add 1 or 2 drops of a 5% guaiacol solution in ethanol. A red colour is immediately produced.

If tartaric acid is present according to Test E, it must be removed as follows: transfer 3 ml of the aqueous layer resulting from the Hydrolysis and an excess of calcium hydroxide as a powder into a test tube, place in boiling water for 5 min, shaking several times, cool and filter.

**Test H: Glycerol**

Transfer 5 ml of the aqueous layer resulting from the hydrolysis into a test tube. Add excess calcium hydroxide as a powder, place in boiling water for 5 min, shaking several times, cool and filter.

Transfer one drop of the filtrate into a tube and add about 50 mg of potassium hydrogen sulfate. Place a filter paper, moistened with Reagent for acrolein (a 5% solution of disodium pentacyanonitrosylferrate in water and a 20% piperidine solution in water; mix the solutions 1:1 immediately before use) on the top of the tube. Heat over a micro flame. A blue coloured filter paper indicates the presence of glycerol. The colour changes to light red after addition of sodium hydroxide TS.

The test cannot be employed in the presence of ethylene glycol or lactic acid, since they decompose under the prescribed conditions yielding acetaldehyde which reacts with the reagents in the same manner as acrolein.

**Iodine Value (Modified Wijs Method)**

(Based on AOCS Method Cd 1d-92)

The iodine value (IV) is a measure of unsaturation and is expressed as the number of g of iodine absorbed, under the prescribed conditions, by 100 g of the test substance. For fats and oils, the Iodine Value may be calculated from the results of gas chromatographic quantification of methyl ester (see Methyl Esters of Fatty Acids, above)

**Reagents**

Cyclohexane

Glacial acetic acid

Wijs Solution: this reagent should be purchased commercially.

Potassium iodide TS

N sodium thiosulfate

**Procedure**

The appropriate weight of the sample, in g, is calculated by dividing the number 25 by the expected iodine value. Melt the sample, if necessary, and filter it through a dry filter paper. Transfer the accurately weighed quantity of sample into a clean, dry, 500-ml glass-stoppered bottle or flask containing 20 ml of glacial acetic acid/cyclohexane, 1:1, v/v, and pipet 25.0 ml of Wijs Solution into the flask. The excess of iodine should be between 50% and 60% of the quantity added, that is, between 100% and 150% of the quantity absorbed. Swirl, and let stand in the dark for 1.0 h where the iodine value is <150 and for 2.0 h where the iodine value is  $\geq 150$ . Add 20 ml of potassium iodide TS and 100 ml of recently boiled and cooled water, and titrate the excess iodine with 0.1 N sodium thiosulfate, adding the titrant gradually and shaking constantly until the yellow colour of the solution almost disappears. Add starch TS, and continue the titration until the blue colour disappears entirely. Toward the end of the titration, stopper the container and shake it violently so that any iodine remaining in solution in the glacial acetic acid/cyclohexane layer may be taken up by the potassium iodide solution. Concomitantly, conduct two determinations on blanks in the same manner and at the same temperature.

**Calculation**

Calculate the iodine value by the formula

$$(B - S) \times 12.69N/W$$

in which

B – S is the difference between the volumes of sodium thiosulfate required for the blank and for the sample, respectively;

N is the normality of the sodium thiosulfate;

W is the weight, in g, of the sample.

## 1-Monoglyceride and Free Glycerol Contents

### Preparation of Samples

*Solid Samples in Flake Form:* Mix without melting and take a portion for analysis.

*Solid Samples not in Flake Form:* Melt at not more than 10° above melting point, mix thoroughly and take a portion for analysis. Do not attempt to test samples which contain so much free glycerol that it separates when the sample solidifies.

*Semi-solid and Liquid Samples:* Liquefy by heating at not more than 10° above melting point, mix thoroughly, and take a portion for analysis. Do not attempt to test samples which contain so much free glycerol that it separates from the sample when cooled to room temperature.

**Caution:** *The sample must not be subjected to a temperature in excess of that required to melt it, as this may reduce the monoglyceride content if any soap is present.*

### Procedure for 1-Monoglyceride

Weigh to the nearest mg duplicate samples of 1 g into a 100-ml glass-stoppered volumetric flask. Dissolve in 50 ml of chloroform. Add 25 ml of water and shake vigorously for 30-60 sec. Transfer the aqueous layer to a glass-stoppered 100-ml volumetric flask, using a glass siphon. If an emulsion forms due to the presence of soap in the sample, add 3 or 4 ml of glacial acetic acid to break the emulsion. Extract 3 more times using 25, 25 and 20 ml of distilled water. Add chloroform to the flask until the level of the chloroform coincides with the 100-ml mark. Using the glass siphon, transfer as much as possible of the aqueous layer above the chloroform layer to the flask containing the aqueous extracts. The aqueous extracts in the volumetric flask are saved for the determination of free glycerol.

Pipet 50 ml of acetic periodic acid TS into each of a series of 500-ml glass-stoppered Erlenmeyer flasks. Prepare 3 for blanks, adding 50 ml of chloroform to two and 50 ml of water to the third. The titrations of the water and chloroform blanks are used as a check (within 0.5 ml) on the chloroform. Pipet 50 ml of chloroform sample solution into one the flasks containing 50 ml of acetic periodic acid TS and shake gently to effect thorough mixing. Allow to stand for at least 30 min but not longer than 1.5 h. To each flask add 20 ml of potassium iodide TS. Mix by gentle shaking, allow to stand at least 1 min but not more than 5 min before titrating. Do not allow to stand in strong sunlight. Add 100 ml of distilled water and titrate with 0.1 N sodium thiosulfate. Use a variable speed magnetic stirrer to keep the solution thoroughly mixed. Continue the titration to the disappearance of the brown iodine colour from the aqueous layer. Add 2 ml of starch TS and continue the titration to the disappearance of iodine from the chloroform layer and the disappearance of the blue iodo-starch colour from the aqueous layer.

Calculation of 1-monoglyceride content as pure monostearate:

$$\% \text{ 1-monoglyceride} = [(B - S) \times N \times 17.927] / W$$



where

B is the sodium thiosulfate consumed in the titration of blank containing 50 ml of chloroform;

S is the sodium thiosulfate consumed in the titration of sample;

N is the exact normality of 0.1 N sodium thiosulfate; and

W is the weight of sample, represented by aliquot pipetted for test.

The 1-monoglyceride content may be calculated in terms of a monoester other than the monostearate by dividing the molecular weight of the monoglyceride by 20 and substituting this value for 17.927 in the formula above.

#### Procedure for free Glycerol

Add distilled water to the combined aqueous extracts from the monoglyceride determination until the volume is 100 ml and mix thoroughly. Pipet 50 ml of acetic periodic acid TS into each of a series of 500-ml glass-stoppered Erlenmeyer flasks. Pipet 50 ml of aqueous sample solution into one of the flasks containing 50 ml of acetic periodic acid TS and shake gently to effect thorough mixing. Continue as described under the procedure for monoglyceride, second paragraph commencing "Allow to stand for at least 30 min...".

Calculation of glycerol content:

$$\% \text{ free glycerol} = [(B - S) \times N \times 2.30] / W$$

where

B is the sodium thiosulfate consumed in the titration of blank containing 50 ml of water;

S is the sodium thiosulfate consumed in the titration of sample;

N is the exact normality of 0.1 N thiosulfate; and

W is the weight of sample represented by aliquot pipetted for test.

## **Polycyclic Aromatic Hydrocarbons**

### General Instructions

Because of the sensitivity of the test, the possibility of errors arising from contamination is great. It is of the greatest importance, therefore, that all glassware be scrupulously cleaned to remove all organic matter such as oil, grease, detergent residues, etc. Examine all glassware, including stoppers and stopcocks, under ultraviolet light to detect any residual fluorescent contamination. As a precautionary measure it is a recommended practice to rinse all glassware with purified isooctane immediately before use. No grease is to be used on stopcocks or joints. Great care to avoid contamination of wax samples in handling and to assure absence of any extraneous material arising from inadequate packaging is essential. Because some of the polynuclear hydrocarbons sought in this test are very susceptible to photo-oxidation, the entire procedure is to be carried out under subdued light.

### Apparatus

*Separatory funnels:* 250-ml, 500-ml, 1,000-ml, and preferably 2,000-ml capacity, equipped with tetrafluoroethylene polymer stopcocks.

*Reservoir:* 500 ml capacity, equipped with a 24/40 standard taper male fitting at the bottom and a suitable balljoint at the top for connecting to the nitrogen supply. The male fitting should be equipped with glass hooks.

*Chromatographic tube:* 180 mm in length, inside diameter to be  $15.7 \pm 0.1$  mm, equipped with a coarse, fritted-glass disc, a tetrafluoroethylene polymer stopcock, and a female 24/40

standard tapered fitting at the opposite end. (Overall length of the column with the female joint is 235 mm).

*Disc:* Tetrafluoroethylene polymer 2-inch diameter disc approximately 3/16-inch thick with a hole bored in the center to closely fit the stem of the chromatographic tube.

*Heating jacket:* Conical, for 500-ml separatory funnel. (Used with variable transformer heat control).

*Suction flask:* 250-ml or 500-ml filter flask.

*Condenser:* 24/40 joints, fitted with a drying tube, length optional.

*Evaporation flask (optional):* 250-ml or 500-ml capacity all-glass flask equipped with standard taper stopper having inlet and outlet tubes permit passage of nitrogen across the surface of the liquid to be evaporated.

*Vacuum distillation assembly:* All glass (for purification of dimethyl sulfoxide); 2-l distillation flask with heating mantle; Vigreux vacuum-jacketed condenser (or equivalent) about 45 cm in length and distilling head with separable cold finger condenser. Use of tetrafluoroethylene polymer sleeves on the glass joints will prevent freezing. Do not use grease on stopcocks or joints.

*Spectrophotometric cells:* Fused quartz cells, optical path length in the range of  $5.000 \pm 0.005$  cm; also for checking spectrophotometer performance only, optical path length in the range  $1.000 \pm 0.005$  cm. With distilled water in the cells, determine any absorbance differences.

*Spectrophotometer:* Spectral range 250 - 400 nm with spectral slit width of 2 nm or less, under instrument operating conditions for these absorbance measurements, the spectrophotometer shall, also meet the following performance requirements:

- Absorbance repeatability,  $\pm 0.01$  at 0.4 absorbance.
- Absorbance accuracy,  $\pm 0.05$  at 0.4 absorbance.
- Wavelength repeatability,  $\pm 0.2$  nm.
- Wavelength accuracy,  $\pm 1.0$  nm.

*Nitrogen cylinder:* Water-pumped or equivalent purity nitrogen in cylinder equipped with regulator and valve to control flow at 5 p.s.i.g.

#### Reagents and materials

##### *Organic solvents:*

All solvents used throughout the procedure shall meet the specifications and tests described below. The isooctane, benzene, acetone, and methyl alcohol designated in the list following this paragraph shall pass the following test:

To the specified quantity of solvent in a 250-ml Erlenmeyer flask, add 1 ml of purified n-hexadecane and evaporate on the steam bath under a stream of nitrogen (a loose aluminium foil jacket around the flask will speed evaporation). Discontinue evaporation when not over 1 ml of residue remains. (to the residue from benzene add a 10-ml portion of purified isooctane, re-evaporate, and repeat once to insure complete removal of benzene).

Alternatively, the evaporation time can be reduced by using the optional evaporation flask. In this case the solvent and n-hexadecane are placed in the flask on the steam bath, the tube assembly is inserted, and a stream of nitrogen is fed through the inlet tube while the outlet tube is connected to a solvent trap and vacuum line in such a way as to prevent any flow-back of condensate into the flask.

Dissolve the 1 ml of hexadecane residue in isooctane and make to 25 ml volume. Determine the absorbance in the 5 cm path length cells compared to isooctane as reference. The absorbance of the solution of the solvent residue (except for methyl alcohol) shall not exceed 0.01 per cm path length between 280 and 400 nm. For methyl alcohol this absorbance value shall be 0.00.

*Isooctane (2,2,4-trimethylpentane):* Use 180 ml for the test described in the preceding paragraph. Purify, if necessary, by passage through a column of activated silica gel (Grade 12 or equivalent) about 90 cm in length and 5 cm to 8 cm in diameter.

*Benzene, reagent grade:* Use 150 ml for the test. Purify, if necessary, by distillation or otherwise.

*Acetone, reagent grade:* Use 200 ml for the test. Purify, if necessary, by distillation.

*Eluting mixtures:*

- 10% benzene in isooctane: Pipet 50 ml of benzene into a 500-ml glass-stoppered volumetric flask and adjust to volume with isooctane, with mixing.
- 20% benzene in isooctane: Pipet 50 ml of benzene into a 250-ml glass-stoppered volumetric flask, and adjust to volume with isooctane, with mixing.
- Acetone-benzene-water mixture: Add 20 ml of water to 380 ml of acetone and 200 ml of benzene, and mix.

*n-Hexadecane, 99% olefin-free:* Dilute 1.0 ml of n-hexadecane to 25 ml with isooctane and determine the absorbance in a 5-cm cell compared to isooctane as reference point between 280-400 nm. The absorbance per centimeter path length shall not exceed 0.00 in this range. Purify, if necessary, by percolation through activated silica gel or by distillation.

*Methyl alcohol, reagent grade:* Use 10.0 ml of methyl alcohol. Purify, if necessary, by distillation.

*Dimethyl sulfoxide:* Pure grade, clear, water-white, m.p. 18° minimum. Dilute 120 ml of dimethyl sulfoxide with 240 ml of distilled water in a 500-ml separatory funnel, mix and allow to cool for 5-10 min. Add 40 ml of isooctane to the solution and extract by shaking the funnel vigorously for 2 min. Draw off the lower aqueous layer into a second 500 ml separatory funnel and repeat the extraction with 40 ml of isooctane. Draw off and discard the aqueous layer. Wash each of the 40 ml isooctane portions three times with 50 ml portions of distilled water. Shaking time for each wash is 1 min. Discard the aqueous layers. Filter the first isooctane portion through anhydrous sodium sulfate prewashed with isooctane (see sodium sulfate below for preparation of filter), into a 250-ml Erlenmeyer flask, or optionally into the evaporating flask. Wash the first separatory funnel with the second 40 ml isooctane portion, and pass through the sodium sulfate into the flask. Then wash the second and first separatory funnels successively with a 10 ml portion of isooctane, and pass the solvent through the sodium sulfate into the flask. Add 1 ml of n-hexadecane and evaporate the isooctane on the steam bath under nitrogen. Discontinue evaporation when not over 1 ml of residue remains. To the residue, add a 10 ml portion of isooctane and re-evaporate to 1 ml of hexadecane. Again, add 10 ml of isooctane to the residue and evaporate to 1 ml of hexadecane to insure complete removal of all volatile materials. Dissolve the 1 ml of hexadecane in isooctane and make to 25 ml volume. Determine the absorbance in 5 cm path length cells compared to isooctane as reference. The absorbance of the solution should not exceed 0.02 per cm path length in the 280-400 nm range.

**Note:** *Difficulty in meeting this absorbance specification may be due to organic impurities in the distilled water. Repetition of the test omitting the dimethyl sulfoxide will disclose their presence. If necessary to meet the specification, purify the water by re-distillation, passage through an ion-exchange resin, or otherwise.*

Purify, if necessary, by the following procedure: To 1,500 ml of dimethyl sulfoxide in a 2 l glass-stoppered flask, add 6.0 ml of phosphoric acid and 50 g of Norit A (decolorizing carbon, alkaline) or equivalent. Stopper the flask, and with the use of a magnetic stirrer (tetrafluoro-ethylene polymer coated bar) stir the solvent for 15 min. Filter the dimethyl sulfoxide through four thicknesses of fluted paper (18.5 cm, Schleicher & Schuell, No. 597, or equivalent). If the initial filtrate contains carbon fines, refilter through the same filter until a clear filtrate is obtained. Protect the sulfoxide from air and moisture during this operation by covering the solvent in the funnel and collection flask with a layer of isooctane. Transfer the filtrate to a 2-l separatory funnel and draw off the dimethyl sulfoxide into the 2-l distillation flask of the vacuum distillation assembly and distil at approximately 3 mm Hg pressure or less. Discard the first 200 ml fraction of the distillate and replace the distillate collection flask with a clean one. Continue the distillation until approximately 1 l of the sulfoxide has been collected.

At completion of the distillation, the reagent should be stored in glass-stoppered bottles since it is very hygroscopic and will react with some metal containers in the presence of air.

*Phosphoric acid, 85% reagent grade*

*Sodium borohydride, 98%*

*Magnesium oxide* (Sea Sorb 43, Food Machinery Company, Westvaco Division, distributed by chemical supply firms, or equivalent): Place 100 g of the magnesium oxide in a large beaker, add 700 ml of distilled water to make a thin slurry, and heat on a steam bath for 30 min with intermittent stirring. Stir well initially to insure that all the absorbent is completely wetted. Using a Buchner funnel and a filter paper (Schleicher & Schuell No. 597, or equivalent) of suitable diameter, filter with suction. Continue suction until water no longer drips from the funnel. Transfer the absorbent to a glass trough lined with aluminium foil (free from rolling oil). Break up the magnesia with a clean spatula and spread out the absorbent on the aluminium foil in a layer about 1-2 cm thick. Dry for 24 h at  $160 \pm 1^\circ$ . Pulverize the magnesia with mortar and pestle. Sieve the pulverized absorbent between 60-180 mesh. Use the magnesia retained on the 180-mesh sieve.

*Celite 545:* Johns-Manville Company, diatomaceous earth, or equivalent.

*Magnesium oxide-Celite 545 mixture:* Place the magnesium oxide (60-180 mesh) and the Celite 545 in 2 to 1 proportions, respectively, by weight in a glass-stoppered flask large enough for adequate mixing. Shake vigorously for 10 min. Transfer the mixture to a glass trough lined with aluminium foil (free from rolling oil) and spread it out on a layer about 1 to 2 cm thick. Reheat the mixture at  $160 \pm 1^\circ$  for 2 h, and store in a tightly closed flask.

*Sodium sulfate, anhydrous, reagent grade, preferably in granular form:* For each bottle of sodium sulfate reagent used, establish as follows the necessary sodium sulfate prewash to provide such filters required in the method: Place approximately 35 g of anhydrous sodium sulfate in a 30 ml coarse, fritted-glass funnel or in a 65 ml filter funnel with glass wool plug; wash with successive 15 ml portions of the indicated solvent until a 15 ml portion of the wash shows 0.00 absorbance per cm path length between 280 nm and 400 nm when tested as prescribed under Organic solvents above. Usually three portions of wash solvent are sufficient.

### Procedure

Before proceeding with the analysis of a sample, determine the absorbance in a 5 cm path cell between 250 nm and 400 nm for the reagent blank by carrying out the procedure, without a wax sample, at room temperature, recording the spectra after the extraction stage and after the complete procedure as prescribed. The absorbance per cm path length following the extraction stage should not exceed 0.040 in the wavelength range from 250 to 400 nm; the absorbance per cm path length following the complete procedure should not exceed 0.070 in the wavelength range from 250 to 299 nm, inclusive, or 0.045 in the wavelength range from 300 nm to 400 nm. If in either spectrum the characteristic benzene peaks in the 250-260 nm region are present, remove the benzene by the procedure under Organic solvents, above, and record absorbance again.

Place 300 ml of dimethyl sulfoxide in a 1-l separatory funnel and add 75 ml of phosphoric acid. Mix the contents of the funnel and allow to stand for 10 min. (The reaction between the sulfoxide and the acid is exothermic. Release pressure after mixing, then keep funnel stoppered). Add 150 ml of isooctane and shake to pre-equilibrate the solvents. Draw off the individual layers and store in glass-stoppered flasks.

Place a representative 1 kg sample of wax, or if this amount is not available, the entire sample, in a beaker of a capacity about three times the volume of the sample and heat with occasional stirring on a steam bath until the wax is completely melted and homogenous. Weigh four  $25 \pm 0.2$  g portions of the melted wax in separate 100 ml beakers. Reserve three of the portions for later replicate analyses as necessary. Pour one weighed portion immediately after re-melting (on the steam bath) into a 500 ml separatory funnel containing 100 ml of the pre-equilibrated sulfoxide-phosphoric acid mixture that has been heated in the heating jacket at a temperature just high enough to keep the wax melted. (**Note:** *In preheating the sulfoxide-acid mixture, remove the stopper of the separatory funnel at intervals to release the pressure*).

Promptly complete the transfer of the sample to the funnel in the jacket with portions of the pre-equilibrated isooctane, warming the beaker, if necessary, and using a total volume of just 50 ml of the solvent. If the wax comes out of solution during these operations, let the stoppered funnel remain in the jacket until the wax re-dissolves. (Remove stopper from the funnel at intervals to release pressure).

When the sample is in solution, remove the funnel from the jacket and shake it vigorously for 2 min. Set up three 250 ml separatory funnels with each containing 30 ml of pre-equilibrated isooctane. After separation of the liquid phases, allow to cool until the main portion of the sample-isooctane solution begins to show a precipitate. Gently swirl the funnel when precipitation first occurs on the inside surface of the funnel to accelerate this process. Carefully draw off the lower layer, filter it slowly through a thin layer of glass wool fitted loosely in a filter funnel into the first 250 ml separatory funnel, and wash in tandem with the 30 ml portions of isooctane contained in the 250 ml separatory funnels. Shaking time for each wash is 1 min. Repeat the extraction operation with two additional portions of the sulfoxide-acid mixture, replacing the funnel in the jacket after each extraction to keep the sample in solution and washing each extractive in tandem through the same three portions of isooctane.

Collect the successive extractives (300 ml total) in a separatory funnel (preferably 2-liter), containing 480 ml of distilled water, mix, and allow to cool for a few min after the last extractive has been added. Add 80 ml of isooctane to the solution and extract by shaking the funnel vigorously for 2 min. Draw off the lower aqueous layer into a second separatory funnel (preferably 2-l) and repeat the extraction with 80 ml of isooctane. Draw off and

discard the aqueous layer. Wash each of the 80 ml extractives three times with 100 ml portions of distilled water. Shaking time for each wash is 1 min. Discard the aqueous layers. Filter the first extractive through anhydrous sodium sulfate prewashed with isooctane (see Sodium Sulfate above for preparation of filter) into a 250-ml Erlenmeyer flask (or optionally into the evaporation flask). Wash the first separatory funnel with the second 80 ml isooctane extractive and pass through the sodium sulfate. Then wash the second and first separatory funnels successively with a 20 ml portion of isooctane and pass the solvent through the sodium sulfate into the flask. Add 1 ml of n-hexadecane and evaporate the isooctane on the steam bath under nitrogen. Discontinue evaporation when not over 1 ml of residue remains. To the residue, add a 10 ml portion of isooctane, re-evaporate to 1 ml of hexadecane, and repeat this operation once more.

Quantitatively transfer the residue with isooctane to a 25 ml volumetric flask, make to volume, and mix. Determine the absorbance of the solution in the 5 cm path length cells compared to isooctane as reference between 280 nm and 400 nm (take care to lose none of the solution in filling the sample cell). Correct the absorbance values for any absorbance derived from reagents as determined by carrying out the procedure without the sample. If the corrected absorbance does not exceed the limits prescribed in the Characteristics, the sample meets the ultraviolet absorbance specifications. If the corrected absorbance per cm path length exceeds the limits prescribed in the Characteristics, proceed as follows:

Quantitatively transfer the isooctane solution to a 125 ml flask equipped with 24/40 joint and evaporate the isooctane on the steam bath under a stream of nitrogen to a volume of 1 ml of hexadecane. Add 10 ml of methyl alcohol and approximately 0.3 g of sodium borohydride (Minimize exposure of the borohydride to the atmosphere. A measuring dipper may be used). Immediately fit a water-cooled condenser equipped with a 24/40 joint and with a drying tube into the flask, mix until the borohydride is dissolved, and allow to stand for 30 min at room temperature, with intermittent swirling. At the end of this period, disconnect the flask and evaporate the methyl alcohol on the steam bath under nitrogen until the sodium borohydride begins to come out of the solution. Then add 10 ml of isooctane and evaporate to a volume of about 2-3 ml. Again, add 10 ml of isooctane and concentrate to a volume of approximately 5 ml. Swirl the flask repeatedly to assure adequate washing of the sodium borohydride residues.

Fit the tetrafluoroethylene polymer disc on the upper part of the stem of the chromatographic tube, then place the tube with the disc on the suction flask and apply the vacuum (approximately 135 mm Hg). Weigh out 14 g of the 2:1 magnesium oxide-Celite 545 mixture and pour the adsorbent mixture into the chromatographic tube in approximately 3 cm layers. After the addition of each layer, level off the top of the adsorbent with a flat glass rod or metal plunger by pressing down firmly until the adsorbent is well packed. Loosen the topmost few mm of each adsorbent layer with the end of a metal rod before the addition of the next layer. Continue packing in this manner until all the 14 g of the adsorbent is added to the tube. Level off the top of the adsorbent by pressing down firmly with a flat glass rod or metal plunger to make the depth of the adsorbent bed approximately 12.5 cm in depth. Turn off the vacuum and remove the suction flask. Fit the 500 ml reservoir onto the top of the chromatographic column and pre-wet the column by passing 100 ml of isooctane through the column. Adjust the nitrogen pressure so that the rate of descent of the isooctane coming off of the column is between 2-3 ml per min. Discontinue pressure just before the last of the isooctane reaches the level of the adsorbent. (**Caution:** *Do not allow the liquid level to recede below the adsorbent level at any time*). Remove the reservoir and decant the 5 ml isooctane concentrate solution onto the column and with slight pressure again allow the liquid level to recede to barely above the adsorbent level. Rapidly complete the transfer similarly with two 5

ml portions of isooctane, swirling the flask repeatedly each time to assure adequate washing of the residue. Just before the final 5 ml wash reaches the top of the adsorbent, add 100 ml of isooctane to the reservoir and continue the percolation at the 2-3 ml per min rate. Just before the last of the isooctane reaches the adsorbent level, add 100 ml of 10% benzene in isooctane to the reservoir and continue the percolation at the aforementioned rate. Just before the solvent mixture reaches adsorbent level, add 25 ml of 20% benzene in isooctane to the reservoir and continue the percolation at 2-3 ml per min until all this solvent mixture has been removed from the column. Discard all the elution solvents collected up to this point.

Add 300 ml of the acetone-benzene-water mixture to the reservoir and percolate through the column to elute the polynuclear compounds. Collect the eluate in a clean 1-l separatory funnel. Allow the column to drain until most of the solvent mixture is removed. Wash the eluate three times with 300 ml portions of distilled water, shaking well for each wash. (The addition of small amounts of sodium chloride facilitates separation). Discard the aqueous layer after each wash. After the final separation, filter the residual benzene through anhydrous sodium sulfate prewashed with benzene (see Sodium sulfate under "Reagents and Materials" for preparation of filter) into a 250-ml Erlenmeyer flask (or optionally into the evaporation flask). Wash the separatory funnel with two additional 20 ml portions of benzene which are also filtered through the sodium sulfate. Add 1 ml of n-hexadecane and completely remove the benzene by evaporation under nitrogen, using the special procedure to eliminate benzene as previously described under Organic Solvents. Quantitatively transfer the residue with isooctane to a 25 ml volumetric flask and adjust the volume. Determine the absorbance of the solution in the 5 cm path length cells compared to isooctane as reference between 250 and 400 nm. Correct for any absorbance derived from the reagents as determined by carrying out the procedure without a wash sample. If either spectrum shows the characteristic benzene peaks in the 250 - 260 nm region, evaporate the solution to remove benzene by the procedure under Organic Solvents. Dissolve the residue, transfer quantitatively, and adjust to volume in isooctane in a 25 ml volumetric flask. Record the absorbance again. If the corrected absorbance does not exceed the limits prescribed in the Characteristics the sample meets the ultraviolet absorbance specifications.

## **Polyglycerol Determination in Polyglycerol Esters**

### Principle

Polyglycerol esters are saponified with alcoholic potassium hydroxide solution and the fatty acids removed by extraction. The polyols are converted to trimethylsilyl (TMS) derivatives and analyzed by gas liquid chromatography.

### Procedure

#### *Preparation of the polyol sample*

Weigh about 0.5 g of sample and reflux with 20 ml of ethanolic potassium hydroxide solution (1 N) for 2 h. Reduce the volume of ethanol by evaporation at 45-50° in a stream of nitrogen. Add 10 ml of water and convert the soaps to free fatty acids by acidifying with concentrated hydrochloric acid. Extract the fatty acids from the aqueous phase with successive 20 ml portions of light petroleum (boiling range 40-60°). Wash the combined petroleum extracts with water (20 ml) and combine the wash with the aqueous phase.

Adjust the aqueous polyol solution to pH 7.0 with aqueous potassium hydroxide solution with the aid of a pH-meter. Evaporate to a small volume (2-3 ml) under reduced pressure and extract three times with 30 ml of boiling ethanol. Filter off any residue and evaporate the ethanol under reduced pressure to yield a viscous liquid sample of polyols.

Dissolve a 0.1 g sample of polyol in 0.5 ml of warm pyridine (previously dried over potassium hydroxide) in a 10-ml capped vial. Add 0.2 ml hexamethyl disilazane, shake, add 0.2 ml trimethylchlorosilane and shake again. Place on a warm plate (about 80°) for 3-5 min. Check that white fumes are present indicating an excess of reagent.

#### *Gas-liquid chromatography*

Any suitable gas chromatograph equipped as follows:

Stationary phase: 3% OV-1

Carrier gas: Nitrogen

Temperature of injection port: 275°

Column temperature: 90° to 330° at 4-6°/min

Detector type: FID, temperature: 350°

Inject a 2.0 µl sample of TMS derivatives of polyols. The following sequence of peaks are recorded on the resultant chromatogram :

<b>Elution sequence of peaks</b>	<b>Identity</b>	<b>Description</b>
1	Solvent	Out of scale
2	Glycerol	Single peak
3	Cyclic diglycerols	Single peak
4	Diglycerols	Single peak
5	Cyclic triglycerols	Single peak
6	Triglycerols	Single peak
7	Cyclic tetraglycerols	Single peak
8	Tetraglycerols	Multiple peaks
9	Pentaglycerols	Single peak
10	Hexaglycerols	Single peak
11	Heptaglycerols	Single peak
12	Octaglycerols	Single peak
13	Nonaglycerols	Barely discernible in the tail of peak 12

#### Calculation

Measure each peak area by a suitable method.

$$\% \text{ di-, tri- and tetraglycerols} = \frac{\text{Sum of corrected areas of peaks 3 to 8} \times 100}{\text{Sum of corrected areas of peaks 3 to 13}}$$

$$\% \text{ polyglycerols equal to or greater than heptaglycerol} = \frac{\text{Sum of corrected areas of peaks 11 to 13} \times 100}{\text{Sum of corrected areas of peaks 3 to 13}}$$

### **Polyols**

#### Principle

The sample is hydrolysed. Fatty acids are removed by ion exchange in combination with hexane extraction. The components of the filtrate are separated by thin layer chromatography.



Procedure

Reflux 1 g of sample with 15 ml of 0.5 N ethanolic potassium hydroxide for 1 h. Add 25 g of strong cation ion exchange resin (such as Amberlite I R 120, H-form), 50 ml of hexane and 25 ml of water. Stir the mixture for about 1 h. Filter off the resin and, after allowing the layers of the filtrate to separate, take the aqueous layer for TLC.

Spot 2 to 5  $\mu$ l portions of the aqueous layer onto a silica gel G plate and also 2  $\mu$ l of 5% solutions of glycerol, ethylene glycol and 1,2-propylene glycol.

Develop the chromatogram using chloroform:acetone:5 N ammonia (10:80:10) as the solvent system. After development, dry the plate in a stream of air until the water and ammonia have been removed.

Spray the plate with a solution of lead acetate (1% w/v in toluene) and heat the plate for 5 min at 110°. 1,2-Diols are revealed as white spots on a brown background.

The following are examples of  $R_f$  values that may be obtained:

Polyol	$R_f$
Glycerol	0.35
Ethylene glycol	0.70
1,2-Propylene glycol	0.85

**Propylene Glycol Dimer and Trimer Determination**Principle

Propylene glycol esters of fatty acids are saponified with alcoholic potassium hydroxide and the fatty acids are removed by extraction. The aqueous polyol fraction is analyzed by gas-liquid chromatography for di- and tripropylene glycol.

Reagents

Potassium hydroxide, ethanolic solution (56.1 g/l ethanol)

Sodium hydroxide solution (50% w/v in water)

Hydrochloric acid (1 + 1 by volume)

Hexane

*Standards:* propylene glycol (1,2-propanediol) dipropylene glycol (1,1-oxydi-2-propanol) tripropylene glycol triethylene glycol

Procedure*Preparation of polyols*

Weigh about 50 g of sample, to the nearest 0.01 g, together with about 2 g to nearest 0.001 g, of triethylene glycol (as internal standard) into a 1-l saponification flask. Add 350 ml of ethanolic potassium hydroxide solution and reflux under an air condenser for 2 h with stirring. Transfer the contents of the flask quantitatively to an 800-ml beaker. Wash the flask and condenser with 200 ml of hot distilled water and evaporate the combined sample solution and washings to about 200 ml on a steam bath. Acidify the hot residue to pH 2 by the dropwise addition, with agitation, of hydrochloric acid (1 + 1). Transfer the hot mixture quantitatively to a 2-l separatory funnel with 200 ml of hexane and shake. Allow the layers to

separate. Transfer the lower aqueous layer to a 500-ml separatory funnel and add 200 ml of fresh hexane. Shake, then allow the layers to separate. Draw off the lower aqueous layer into a 600-ml beaker and add the hexane phase to the original 2-l separatory funnel. Wash the 500-ml separator with two further 200-ml portions of hexane and add these to the 2-l separator. Wash the original 800-ml beaker with 100 ml of water and add to the hexane solution. Mix thoroughly and allow the layers to separate. Draw off the aqueous layer into the 600-ml beaker containing the aqueous fractions. Wash the 800-ml beaker once more with 100 ml of water and add the drained aqueous layer to the combined aqueous fractions. Adjust the pH of the combined aqueous solution to pH 7.0-7.05 (using pH-meter) with sodium hydroxide solution and evaporate to about 150 ml on a steam bath. Transfer quantitatively to a 250 ml round bottom flask and concentrate further to about 50 ml by distilling through a vertical Vigreux column to prevent the loss of low boiling glycols. Decant the concentrated polyols from the precipitated salts, through a filter funnel containing Whatman No. 1 paper, into a 100-ml volumetric flask. Wash the salts and flask twice with 20 ml of water and add to the volumetric flask via the filter funnel. Dilute to the mark with water, mix well and use for the GLC polyol analysis. If salts reprecipitate in the volumetric flask refilter before sampling.

#### *Gas-liquid chromatography*

Any suitable gas chromatograph equipped with:

Stationary phase: 15% Carbowax 20 M

Carrier gas: Helium

Temperature of injection port: 290°

Column temperature: 150° to 230° at 2°/min

Detector type: FID; temperature: 250°.

Prepare a reference solution of glycols in water by weighing the glycol standards, to the nearest 0.1 mg, into a 100 ml volumetric flask as follows:

- Propylene glycol: 1 g
- Dipropylene glycol: 0.01 g
- Tripropylene glycol: 0.005 g
- Triethylene glycol: 0.01 g

Make up to the mark with water and mix.

Inject aliquots of this reference standard solution and establish the sensitivity setting to yield measurable peaks. Similarly inject the prepared sample solution.

#### Calculation

Measure each peak area by a suitable method, such as multiplying the peak height by the peak width at half the peak height, and calculate the % dimer and trimer in the sample as follows:

$$\% \text{ dimer} = (A_{DS} \times W_{DR}) / A_{DR} \times (W_{IS} \times A_{IR}) / (A_{IS} \times W_{IR}) \times 100 / W$$

$$\% \text{ trimer} = (A_{TS} \times W_{TR}) / A_{TR} \times (W_{IS} \times A_{IR}) / (A_{IS} \times W_{IR}) \times 100 / W$$

where

$A_{DS}$  is the peak area of dipropylene glycol (sample solution);

$A_{DR}$  is the peak area of dipropylene glycol (reference solution);

$A_{TS}$  is the peak area of tripropylene glycol (sample solution);

$A_{TR}$  is the peak area of tripropylene glycol (reference solution);

$A_{IS}$  is the peak area of triethylene glycol (sample solution);

$A_{IR}$  is the peak area of triethylene glycol (reference solution);  
 $W$  is the weight (g) of sample of propylene glycol esters of fatty acids;  
 $W_{DR}$  is the <sup>1</sup>weight (g) of dipropylene glycol in the reference solution;  
 $W_{TR}$  is the <sup>1</sup>weight (g) of tripropylene glycol in the reference solution;  
 $W_{IS}$  is the <sup>1</sup>weight (g) of triethylene glycol added to the sample solution; and  
 $W_{IR}$  is the <sup>1</sup>weight (g) of triethylene glycol in the reference solution.

<sup>1</sup> Corrected for the actual content of polyol (e.g.  $W_{DR}$  is the weight of dipropylene glycol taken x % of assay).

## Saponification

(AOCS Methods Tl 1a-64 and Cd 3-25)

Weigh accurately about 20 g of the sample and subject to alkaline hydrolysis by refluxing for 2 h with ethanolic potassium hydroxide TS containing a quantity of potassium hydroxide 100% in excess of the calculated amount required to saponify the sample completely. After hydrolysis, convert the ethanolic soap solution to an aqueous solution by the addition of water and evaporation of the alcohol on a steam bath. Acidify the hot aqueous soap solution with sulfuric acid to liberate the fatty acid. Extract the acid solution with 3 portions of petroleum ether to remove the fatty acid. Evaporate the petroleum ether extracts on a steam bath and dry the residue to constant weight under vacuum at 75° to recover the fatty acid. Multiply the weight of recovered fatty acid by 100/W to obtain the yield of fatty acid from a 100-g sample (where W is the exact weight of sample taken). The fatty acid can be identified by determination of the physical and chemical constants, e.g. the solidification temperature, or by gas-liquid chromatography.

Neutralize the aqueous polyol solution to pH 7 with potassium hydroxide. Evaporate the polyol solution to a moist residue on a steam bath and extract the polyol from the salts with 3 portions of hot absolute ethanol. Evaporate off the alcohol on a steam bath and dry the residue to constant weight under vacuum at 75° to yield the polyol moiety of the sample. Multiply the weight of recovered polyol by 100/W to obtain the yield of polyols from a 100-g sample (where W is the exact weight of sample taken).

## Saponification Value

### Definition

Saponification value is defined as the number of mg of potassium hydroxide required to neutralize the free acids and saponify the esters in 1 g of test substance.

### Procedure

Melt the sample, if necessary, and filter it through a dry filter paper to remove any traces of moisture. Unless otherwise directed, weigh accurately into a 250-ml flask a sample of such size (usually about 4-5 g) that the titration of the sample solution after saponification will require between 45 and 55% of the volume of 0.5 N hydrochloric acid required for the blank. Add 50.0 ml of ethanolic potassium hydroxide TS from a pipet and allow the pipet to drain for a definite period of time. Prepare and conduct blank determinations simultaneously with the sample and similar in all respects. Connect an air condenser to each flask and boil gently but steadily, with occasional mixing, until the sample is completely saponified. (This usually requires about 1 h for normal samples). After the flasks and condensers have cooled somewhat but not sufficiently for the contents to gel, wash down the inside of the condensers with a few ml of distilled water. Disconnect the condensers, add about 1 ml of

phenolphthalein TS to reach flask, and titrate with 0.5 N hydrochloric acid until the pink colour has just disappeared.

$$\text{Saponification value} = [56.1 \times N (A - B)] / W$$

Where

A is ml of HCl required for the titration of the blank;  
 B is ml of HCl required for the titration of the sample;  
 W is the weight of sample in g; and  
 N is normality of the HCl.

## **Sorbitan Ester Content**

### Principle

Sorbitan esters may be assayed by alkaline saponification followed by recovery of the polyol and determination of the isosorbide content by gas-liquid chromatography.

### Procedure

#### *Saponification and recovery of the polyol*

Weigh accurately about 25 g of the sample into a 500-ml round-bottomed boiling flask. Add 250 ml of ethanol and a quantity of potassium hydroxide 100% in excess of the calculated amount required for saponification (approximately 7.5 g). Boil the mixture for 2 h under reflux. Transfer the saponification mixture to an 800-ml beaker. Rinse the flask with about 100 ml of water and add to the mixture. Place the beaker on a steam bath to evaporate the alcohol. Add water occasionally to replace the ethanol. When the odour of ethanol can no longer be detected, adjust the volume of the soap solution to approximately 250 ml with hot water.

Acidify the hot soap solution with stirring using sufficient 1:1 sulfuric acid to provide a 10% excess. Heat and stir the mixture until the fatty acid layer separates. Transfer the hot mixture to a 500-ml separating funnel using hot water to rinse the beaker. Cool the contents of the funnel and extract three times with 100-ml portions of petroleum ether. Combine the petroleum ether extracts in a second funnel and wash once with 100 ml of water. Combine the water wash with the aqueous phase in an 800-ml beaker.

Neutralize the polyol solution with 10% aqueous potassium hydroxide solution to pH 7 using a pH meter. Place the beaker in a steam bath and evaporate the solvent to incipient dryness. Extract the residue four times with 150-ml portions of boiling absolute ethanol. Filter the combined extracts into a 1-l suction flask through a 10-cm Buchner funnel containing a 1-3 cm bed of silicagel. Wash the funnel with absolute ethanol. Transfer the filtrate and washings to a 1,000-ml volumetric flask. Cool to room temperature and dilute to volume with ethanol. Use this as the sample solution for gas-liquid chromatography.

#### *Gas-liquid chromatography*

The experimental operating conditions for the isosorbide analyses are not critical, suitable conditions are listed below. Minor fluctuations in temperature and gas flow rate do not affect resolution or analytical results.

Stationary phase: 15% Carbowax 20 M

Carrier gas: Argon

Temperature of injection port: 295°

Column temperature: 195°

Detector type: FID; 250°

### Calculation

The isosorbide content of an aliquot of the recovered polyol solution is estimated directly from a calibration curve prepared from a standard sorbitan ester or by multiplying the observed peak area by the slope of the curve ( $\mu\text{g}$  of isosorbide per unit area).

$$\% \text{ Sorbitan ester} = (I \times 20) / (f \times W)$$

where

I is  $\mu\text{g}$  of isosorbide found in the aliquot of recovered polyol solution by gas chromatography; W is g of sorbitan ester taken for analysis; and f is fractional isosorbide yield from standard sorbitan esters (see **Note** below).

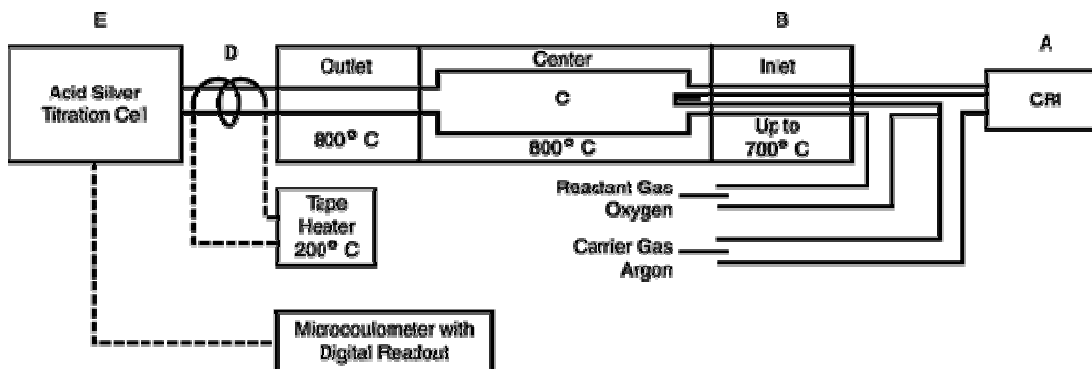
**Note:** A known sample of sorbitan ester is treated as described under Saponification and recovery of the polyol above. Suitable aliquots of the solution are subjected to the gas chromatographic procedure. The fractional yield of isosorbide is calculated from the weight of sample corrected to a dry, fatty-acid-free basis. The procedure is estimated to have an accuracy of 5%.

### Sulfur

**Note:** All reagents used in this test should be reagent grade: water should be of high purity, and gases must be high-purity grade.

### Apparatus

The Dohrmann Microcoulometric Titrating System (MCTS-30), or its equivalent as shown in the figure below should be used. It consists of a constant rate injector (A), a pyrolysis furnace (B), a quartz pyrolysis tube (C), a granular tin scrubber (D), a titration cell (E), and a microcoulometer with a digital readout (F).



**Granular-Tin Scrubber:** Place 5 g of 20/30 mesh granular reagent grade tin between quartz-wool plugs in an elongated 18/8-12/5 standard-taper adaptor which connects the pyrolysis tube and the titration cell.

**Microcoulometer:** Must have variable attenuation, gain control, and be capable of measuring the potential of the sensing-reference electrode pair, and comparing this potential with a bias potential, amplifying the potential difference, and applying the amplified difference to the working-auxiliary electrode pair so as to generate a titrant. Also the microcoulometer output voltage signal must be proportional to the generating current.

*Pyrolysis Furnace:* The sample should be pyrolyzed in an electric furnace having at least two separate and independently controlled temperature zones, the first being an inlet section that can maintain a temperature sufficient to volatilize the entire organic sample. The second zone shall be a pyrolysis section that can maintain a temperature sufficient to pyrolyze the organic matrix and oxidize all the organically bound sulfur. A third outlet temperature zone is optional.

*Pyrolysis Tube:* Must be fabricated from quartz and constructed in such a way that a sample, which is vaporized completely in the inlet section, is swept into the pyrolysis zone by an inert gas where it mixes with oxygen and is burned. The inlet end of the tube shall hold a septum for syringe entry of the sample and side arms for the introduction of oxygen and inert gases. The center, or pyrolysis section, should be of sufficient volume to ensure complete pyrolysis of the sample.

*Sampling Syringe:* A microliter syringe of 10- $\mu$ l capacity capable of accurately delivering 1 to 10  $\mu$ l of sample into the pyrolysis tube. Three-inch x 24-gauge needles are recommended to reach the inlet zone of the pyrolysis furnace.

*Titration Cell:* Must contain a sensor-reference pair of electrodes to detect changes in triiodide ion concentration, a generator anode-cathode pair of electrodes to maintain constant triiodide ion concentration, and an inlet for a gaseous sample from the pyrolysis tube. The sensor electrode shall be platinum foil and the reference electrode platinum wire in saturated triiodide half cell. The generator anode and cathode half-cell shall also be placed on a magnetic stirrer.

#### Preparation of Apparatus

Carefully insert the quartz pyrolysis tube into the furnace, attach the tin scrubber, and connect the reactant and carrier-gas lines. Add the Cell Electrolyte Solution (see below) to the titration cell and flush the cell several times. Maintain an electrolyte level of 3.8 cm (1.5 in.) above the platinum electrodes. Place the titration cell on a magnetic stirrer and connect the cell inlet to the tin scrubber outlet. Position the platinum foil electrodes (mounted on the movable cell head) so that the gas-inlet flow is parallel to the electrodes with the generator anode adjacent to the generator cathode. Assemble and connect the coulometer in accordance with the manufacturer's instructions. Double-wrap the adaptor containing the tin scrubber with heating tape and turn the heating tape on. Adjust the flow of the gases, the pyrolysis furnace temperature, the titration cell, and the coulometer to the desired operating conditions. Typical operating conditions are as follows:

Reagent gas flow (oxygen): 200 cm<sup>3</sup>/min

Carrier-gas flow (Ar, He): 400 cm<sup>3</sup>/min

Furnace temperatures

Inlet zone: 700° (maximum)

Pyrolysis zone: 800 - 1000°

Outlet zone: 800° (maximum)

Tin-Scrubber flow rate: 200 cm<sup>3</sup>/min

Titration cell: Stirrer speed set to produce slight vortex

Coulometer

Bias voltage: 160 mV

Gain: 50

Constant Rate Injector: 0.25  $\mu$ l/sec

The tin scrubber must be conditioned to sulfur, nitrogen, and chlorine before quantitative analysis can be achieved. A solution containing 10 mg/kg butyl sulfide, 100 mg/kg pyridine,

and 200 mg/kg chlorobenzene in isooctane has proven an effective conditioning agent. With a fresh scrubber installed and heated, two 30- $\mu$ l samples of this conditioning agent injected at a flow rate of 0.5  $\mu$ l/sec produces a steady increasing response, with final conditioning indicated by a constant reading from the offset during the second injection.

### Reagents

- Argon or Helium, (Argon preferred) High-purity grade: two-stage regulators must be used.
- Cell Electrolyte Solution: dissolve 0.5 g of potassium iodide and 0.6 g of sodium azide in 500 ml of high-purity water, add 5 ml of glacial acetic acid and dilute to 1 L. Store in a dark bottle or in a dark place and prepare fresh at least every 3 months.
- Oxygen: high-purity grade.
- Iodine: resublimed, 20 mesh or less.
- Sulfur Standard (approximately 100 mg/kg): weigh accurately 0.1569 g of n-butyl sulfide, into a tared 500-ml volumetric flask. Dilute to the mark with isooctane and reweigh. Calculate the sulfur concentration (S), in percent, by the formula:

$$S = \frac{W_s}{W_c} \times 2.192 \times 10^5$$

where

Ws = weight of n-butyl sulfide, and

Wc = weight of the solution.

### Calibration

Prepare a calibration standard (approximately 5 mg/kg) by pipetting 5 ml of Sulfur Standard into a 10-ml volumetric flask and diluting to volume with isooctane. Fill and clamp the syringe onto the constant rate injector, push the sliding carriage forward to penetrate the septum with the needle, and zero the meter in case of long-term drift in the automatic baseline zero circuitry. Switch S<sub>1</sub> automatically starts the stepper-motor syringe drive and initiates the analysis cycle. At 2.5 min (after setting switch S<sub>1</sub>) set the digital meter with the scan potentiometer to correspond to the sulfur content of the known standard to the nearest 0.01 mg/kg. At the 3-min point, the number displayed on the meter stops, the plunger drive block is retracted to its original position, as preset by switch S<sub>2</sub> and a baseline re-equilibration period equal to the injection period must be allowed before a new sample may be injected. Repeat the Calibration step a total of at least four times.

### Procedure

Rinse the syringe several times with sample: then fill it, clamp it onto the constant-rate injector push the sliding carriage forward to penetrate the septum with the needle, and zero the meter. Turn on switch S<sub>1</sub> to start the stepper-motor syringe drive automatically and initiate the analysis cycle. After the 3-min hold point, the number displayed on the meter corresponds to the sulfur content of the injected sample.

### **Viscosity, 100°**

(ASTM D 445 Adapted, with permission, from the Annual Book of ASTM Standards, copyright American Society for Testing and Materials, 100 Harbor Drive, West Conshohocken, PA 19428. Copies of the complete ASTM standard may be purchased direct from ASTM)

Use a viscometer of the glass capillary type, calibrated and capable of measuring kinematic viscosity with a repeatability exceeding 0.35 % only in one case in twenty. Immerse the viscometer in a liquid bath at the temperature required for the test  $\pm 0.1^\circ$  ensuring that at no time of the measurement will any portion of the sample in the viscometer be less than 20 mm below the surface of the bath liquid or less than 20 mm above the bottom of the bath. Charge the viscometer with sample in the manner dictated by the design of the instrument. Allow the sample to remain in the bath for about 30 min. Where the design of the viscometer requires it, adjust the volume of sample to the mark. Use pressure to adjust the head level of the sample to a position in the capillary arm of the instrument about 5 mm ahead of the first mark. With the sample flowing freely, measure, in seconds ( $\pm 0.2$  s), the time required for the meniscus to pass from the first to the second timing mark. If the time is less than 200 s, select a viscometer with a capillary of smaller diameter and repeat the operation. Make a second measurement of the flow time. If two measurements agree within 0.2 %, use the average for calculating the kinematic viscosity. If the measurements do not agree, repeat the determination after thorough cleaning and drying the viscometer.

$$\text{Viscosity, } 100^\circ \text{ (mm}^2\text{/s)} = C \times t$$

where

C is the calibration constant of the viscometer ( $\text{mm}^2\text{/s}^2$ ), and  
t is the flow time (s)





## FLAVOURING AGENTS

### Acid Value

Dissolve about 10 g of sample, accurately weighed, in 50 ml of ethanol, previously neutralized to phenolphthalein TS with 0.1 N sodium hydroxide. Add 1 ml of phenolphthalein TS and titrate with 0.1 N sodium hydroxide until the solution remains faintly pink after shaking for 10 sec, unless otherwise directed. Calculate the Acid Value (AV) by the formula:

$$AV = (5.61 \times S) / W$$

in which

S is the number of ml of 0.1 N sodium hydroxide consumed in the titration of the sample, and W is the weight of the sample in g.

### Assay

For Gas Chromatographic analysis procedures see Gas Chromatography in the Section on Analytical Techniques.

### Boiling Point

See the Section on Appearance and Physical Properties, under General Methods.

### Ester Determination

Transfer an accurately weighed quantity of the sample specified in the monograph into a 125-ml Erlenmeyer flask containing a few boiling stones. Add to this flask, and, simultaneously, to a similar flask for a blank test, 25.0 ml of 0.5 N ethanolic potassium hydroxide. Connect each flask to a reflux condenser, and heat the mixtures on a steam bath for exactly 1 h, unless otherwise directed in the monograph. Allow the mixtures to cool, add 10 drops of phenolphthalein TS, to each flask, and titrate the excess alkali in each flask with 0.5 N hydrochloric acid. Calculate the percentage of Ester (E) in the sample by the formula:

$$E = [(b - S)(100e)] / W$$

in which

b is the number of ml of 0.5 N hydrochloric acid consumed in the titration of the blank;  
S is the number of ml of 0.5 N hydrochloric acid consumed in the titration of the sample; and  
e is the equivalence factor given in the monograph, and W = the weight of the sample in mg.

### Melting Point (Melting Range)

See the Section on Appearance and Physical Properties, under General Methods.

### Refractive Index

See the Section on Appearance and Physical Properties, under General Methods.

### Solubility in Ethanol

Unless otherwise stated in the specification, transfer a 1 ml sample into a calibrated 10-ml glass-stoppered cylinder graduated in 0.1-ml subdivisions, and add slowly, in small portions,

ethanol, the concentration and quantity of which are specified in the monograph. Maintain the temperature at 20°. A clear solution free from foreign matter should be obtained.

**Solubility in General**

See the Section on Appearance and Physical Properties, under General Methods.

**Specific Gravity**

See the Section on Appearance and Physical Properties, under General Methods.

## HPLC Method for certain flavourings

**3-methyl-2-oxobutanoic acid (631)**

**Sodium 3-methyl-2-oxobutanoate (631.1)**

**3-methyl-2-oxopentanoic acid (632)**

**Sodium 3-methyl-2-oxopentanoate (632.1)**

**4-methyl-2-oxopentanoic acid (633)**

**Sodium 4-methyl-2-oxopentanoate (633.1)**

**2-oxo-3-phenylpropionic acid (1478)**

**Sodium 2-oxo-3-phenylpropionate (1479)**

Determine by HPLC using the following:

Note: All solutions should be prepared with ultra high quality (UHQ) deionized water

### Apparatus:

HPLC system with a suitable pump, injector, and a data station

Column: Stainless steel; 300 x 7.6 mm  
Stationary phase: Bio-Rad Aminex<sup>®</sup> HPX-87H or equivalent  
Detector: UV

### HPLC conditions:

Column temperature: 35°  
Mobile phase: 0.004 M Sulfuric acid  
Flow rate: 0.6 ml/min  
Injection volume: 50 µl  
Detection: 210 nm  
Run time: 30 min

Note: *The retention times of the compounds are as follows:*

3-methyl-2-oxobutanoic acid is 13.0 min  
3-methyl-2-oxopentanoic acid is 14.8 min  
4-methyl-2-oxopentanoic acid is 16.7 min  
2-oxo-3-phenylpropionic acid is 24.7 min

### Procedure:

Weigh about 100 mg of the sample, dissolve in a minimum amount of 0.2 M sodium hydroxide solution, and make up to 100 ml in a volumetric flask. Set up and condition the HPLC using the mobile phase. Inject the sample solution and determine the purity of the sample by the area normalization method.

Note: This method is also available from the on-line database on specifications for flavouring agents in the link to Analytical methods (Volume 4).

## FOOD COLOURS

### Chloride as Sodium Chloride

**Note:** *This determination is done in connection with Water Content (Loss on Drying) for food colours and the result is included in that calculation.*

#### Apparatus

- Potentiometric titration apparatus
- Silver indicator electrode
- Glass body calomel reference electrode or calomel reference electrode with potassium sulfate bridge

#### Reagents

- Nitric acid, 1.5 N, reagent grade
- Silver nitrate, 0.1 N, standard solution

#### Procedure

Accurately weigh 0.5 - 1.0 g of the colour sample ( $W_s$ ), dissolve in 100 ml of water, and acidify with 5 ml of 1.5 N nitric acid. Place the silver and glass body calomel electrodes in the colour solution. If only a standard calomel reference electrode is available, connect it to the solution by means of the saturated potassium sulfate bridge. (Use of a glass body electrode as the reference electrode eliminates the need for the potassium sulfate bridge; this simplifies the apparatus considerably, and the glass body electrode is sufficiently constant to be used as a reference for this type of titration.)

Determine the chloride content of the solution by titration with the 0.1 N silver nitrate. (Each ml of 0.1 N silver nitrate is equivalent to 0.00585 g of sodium chloride.)

#### Calculation

Calculate the chloride content of the sample as percent sodium chloride using the following equation:

$$\% \text{ sodium chloride} = 100 \times (\text{ml titrant} \times 0.00585 \text{ g})/W_s.$$

### Chloroform-Insoluble Matter

#### Apparatus

- Oven, 0 - 200° range
- Hot plate
- Crucible, fitted with glass fiber disk
- Vacuum flask
- Source of vacuum
- Desiccator

#### Reagents

- Chloroform, reagent grade

#### Procedure

Accurately weigh the quantity of sample indicated in each specification monograph ( $W_1$ ) into a 250-ml beaker. Mix with 100 ml of chloroform (b.p. 61.1°). Stir and heat to boiling on the hot plate in a fume hood. Filter the hot solution through a weighed crucible ( $W_2$ ). Transfer

the residue in the beaker to the crucible with chloroform. Wash the residue in the crucible with 10-ml portions of chloroform until the washings are colourless. Place the crucible in the oven at 100 - 150° for 3 h; cool the crucible in the desiccator. Weigh the cooled crucible ( $W_3$ ).

#### Calculation

The percent chloroform-insoluble matter in the sample is  $100 \times (W_3 - W_2) / W_1$ .

## **Colouring Matters**

### Identification

Many of the colours used by food manufacturers are mixtures of colouring matters of the type described in the specification monographs, and some of the mixtures contain added diluents. A simple test to establish whether a powdered sample of colouring matter is a single colouring matter or a mixture of colouring matters is to sprinkle a very small quantity of the powder into each of two beakers, one containing water and the other containing concentrated sulfuric acid. Under these conditions, the specks of individual colouring matters can easily be seen as they dissolve; the test is surprisingly sensitive.

The positive identification of individual food colours is often quite difficult. A large number are the sodium salts of sulfonic acids, which have no precise melting point. In addition, synthesized colours usually contain subsidiary colouring matters, while colouring matters extracted from natural sources generally are mixtures of colours themselves. Identification, therefore, is best achieved by comparison of the observed properties with the properties of authentic commercial samples. The principle techniques in use are chromatography and spectrophotometry. Frequently, both are required, because the presence of subsidiary colouring matters might affect the observed spectra so that positive identification of the principal colour component cannot be made. For this reason, separation of the individual colouring matters by column, paper, or thin layer chromatography is advisable before attempting additional identification by spectrophotometry.

Subsidiary colouring matters are defined as those colouring matters that are produced during the manufacturing process in addition to the principal named colouring matter(s). Paper chromatography has been used for many years for identifying subsidiary colouring matters in water-soluble food colours. The assumption is generally made that spectrophotometric absorbances of subsidiary colouring matters are similar to that of the main colouring matter. Accordingly, standards of individual subsidiary colour matters are not required. The presence of colouring matters other than the principal and subsidiary colouring matters is usually detected on the chromatograms used to determine subsidiary colouring matters. Interpretation of the chromatograms for these colour impurities usually requires additional information.

High-performance liquid chromatography (HPLC) has been used successfully to separate, identify, and quantitate the subsidiary colouring matter contents of various food colours. Standards for individual subsidiary colouring matters are needed for this method. However, the specification limits in the monographs are, unless otherwise stated, linked to the paper chromatographic method and the conditions are provided under "Tests" in the specification.

### Identification by chromatography

Paper and thin layer chromatography are often useful in identification of colouring matters and do not require expensive equipment. But it must be kept in mind that the  $R_f$ -value of a substance is generally an unreliable quantity because many factors, most of which are beyond the analyst's control, can have a major influence on the  $R_f$ -values. These factors

include: composition and age of the solvent mixture, concentration of solvent vapour in the atmosphere, quality of the chromatography paper, machine direction of commercially made paper, kind and quality of subsidiary colouring matters, concentration, pH-value of the solution, and temperature. For this reason, comparative chromatography using reference colours should always be used. By simultaneously running several colouring matters of similar concentration a number of these factors are eliminated.

Coincidence of migration distances with a single solvent system should be looked upon only as one criterion of identity and further tests should be made to confirm the finding.

The following table contains examples of the  $R_f$ -values that may be expected when 1% aqueous solutions of various colouring matters are subjected to thin layer chromatography on Silica Gel G in the ten solvent systems listed below. The compositions of the solvent systems, all of which must be freshly prepared, are:

Solvent System Number

1. iso-Propanol:ammonia (sp.gr. 0.880):water (7:2:1)
2. iso-Butanol:ethanol:water:ammonia (sp.gr. 0.880) (10:20:10:1)
3. Saturated aqueous potassium nitrate solution
4. Phenol:water (4:1, w/v)
5. Hydrochloric acid (sp.gr. 1.18):water (23:77)
6. Trisodium citrate:ammonia (sp.gr. 0.880):water (2 g:15 ml:85 ml)
7. Acetone:2-butanone :ammonia (sp.gr. 0.880):water (60:140:1:60)
8. n-Butanol:ethanol:pyridine:water (2:1:1:2)
9. iso-Propanol:ammonia (sp.gr. 0.880) (4:1)
10. n-Butanol:acetic acid (glacial):water (10:5:6)

**R<sub>f</sub> Values of Some Water-Soluble Colours** (This Table does not indicate the acceptability of the listed colours for food use.)  
**Note:** Numbers 1 to 10 refer to solvent systems (see above).

<b>REDS</b>	C.I. No.	INS No.	1	2	3	4	5	6	7	8	9	10
Ponceau 4R or Cochineal Red A	16255	124	0.66 (0.85)	0.75	0.88	0.03	0.95	1.00	0.60	0.90	0.11	0.52 0.00-0.57
Carmosine or Azorubine	14720	122	0.65 (0.77)	0.81	0.00-0.42	0.16	0.00 (0.00-0.32)	1.00	0.65	0.88	0.34 (0.46)	0.63 (0.11-0.70)
Amaranth	16185	123	0.62 (0.48, 0.76)	0.75 (0.83)	1.00 (0.00- 1.00)	0.04 (0.16)	1.00	1.00	0.40 (0.64, 0.66)	0.90	0.10 (0.41)	0.39, 0.67
Erythrosine RS	45430	127	0.85 (0.68, 0.79)	0.91 (0.86, 0.74, 0.81)	0.00, 0.10	0.00-0.90 (0.41)	0.00	0.00-0.95	0.64, 0.66 (0.58)	0.89	0.66 (0.57, 0.43)	1.00
Red 2G	18050	--	0.68	0.80	0.37	0.12	0.00-0.71	1.00	0.64	0.90	0.36	0.68
<b>ORANGES</b>												
Orange G	16230	--	0.71 (0.67, 0.88)	0.80 (0.75)	0.64 (1.00, 0.35)	0.23, 0.15, 0.04	0.73	1.00	0.64 (0.62, 0.50, 0.67)	0.91	0.36 (0.32, 0.17)	0.69 (0.46, 0.82)
Orange RN	15970	--	0.83 (0.62)	0.88 (0.78)	0.00 (0.00- 0.42)	0.42 (0.13)	0.13 (0.38)	0.76 (1.00)	0.68 (0.65)	0.92	0.64 (0.29)	0.82, 0.71
Sunset Yellow FCF or Orange Yellow S	15985	110	0.75 (0.68)	0.82 (0.74)	1.00 (0.00- 1.00)	0.17, 0.03	1.00	1.00	0.65 (0.48)	0.90	0.34 (0.10, 0.22)	0.67 (0.46)
<b>YELLOWS</b>												
Tartrazine	19140	102	0.66	0.77	0.46-1.00	0.08	1.00	1.00	0.52	0.93	0.14	0.50
Yellow 2G	18965	--	0.63	0.80	0.77	0.21	0.74	1.00	0.62	0.92	0.21	0.75
Quinoline Yellow	47005	104	0.83, 0.88	0.88 (0.82)	0.00-1.00	0.65 (0.21)	0.26-1.00, 0.00-0.38	0.95 (0.35)	0.54 (0.68)	0.88	0.00- 0.31, 0.64	0.11-0.75 (0.83)
Fast Yellow AB	13015	--	0.77	0.81	1.00	0.14	0.97	1.00	0.56	0.93	0.36	0.66
<b>GREENS, BLUES, AND VIOLETS</b>												
Green S or Acid Brilliant-Green BS or Lissamine Green	44090	142	0.44 (0.52, 0.68, 0.74)	0.61 (0.67, 0.75, 0.81, 0.84)	0.49 (0.24)	0.53 (0.05, 0.36, 1.00)	0.29 (0.43)	1.00	0.46 (0.56, 0.71)	0.75 (0.89, 0.92)	0.07	0.55
Indigo Carmine or Indigotin	73015	132	0.56 (0.70)	0.50-0.76 (0.78)	0.00 (0.05,	0.09, 0.18 (0.52)	0.92	0.94	0.66 (0.71,	0.89, 0.84	0.37 (0.00-	0.00-0.63





Assessment of the colour shade should be made while the chromatograms are still moist with solvent and then again after drying. The shade should be assessed in both incident and transmitted daylight as well as under ultraviolet (UV) light, in which many colours show characteristic colour changes. Furthermore, UV light can often be used to identify the presence of colourless fluorescent impurities. If possible, use two UV emitters which yield different wave lengths; one lamp should emit around 250 nm.

Tests with acids, alkalis and other suitable reagents, in order to confirm the results, should be made. All tests may be carried out with fine capillary pipettes on each colour spot.

The following requirements should be met when identifying the colours in colouring matters by comparing to reference colours:

- equal migration distances in several solvent systems;
- equal shade in daylight and ultraviolet light; and
- equal colour changes with reagents.

#### Identification by spectrophotometry

Spectrophotometric methods of examination are among the most useful means of identification of colours. The UV, visible, and infrared regions of the electromagnetic spectrum are all employed.

The visible region of the spectrum is ordinarily examined as the first step in attempting to identify an unknown colour. Many colours show characteristic absorption bands in the visible region. Spectra in the UV region may also be of use and should be obtained together with the visible spectrum, if possible.

In the application of UV-visible spectrophotometry, spectra should always be obtained in more than one solvent, or if in a single solvent, under various conditions. Spectra of aqueous solutions should be obtained under neutral (buffered with ammonium acetate), acid (0.1 N hydrochloric acid), and alkaline (0.1 N sodium hydroxide) conditions.

A UV-visible absorption spectrum is ordinarily displayed as a plot of absorbance vs. wavelength. In addition to the wavelength maximum, the most characteristic and useful features of the absorption spectrum can be the "shoulders" or inflection points on the spectral curve. These features often make it possible to distinguish between two or more colouring matters that have absorption maxima at the same wavelength. Many colours can be definitively characterized by observing the extent to which the absorption maxima and other features of the absorption curve are changed by variation in pH or by other changes in the solvent.

Infrared absorption spectra offer another useful means of identification of compounds. An example of their use is in distinguishing Sunset Yellow and Orange GGN. Whereas the UV-visible absorption spectra of these colours are nearly identical, their infrared spectra are quite different in the region of the spectrum in which the sulfonic acid groups absorb strongly.

Infrared spectra of substances can be obtained using various sample preparations; the more commonly used are:

- solutions of the material in suitable solvents;
- suspensions of the material in a suitable liquid;
- potassium bromide pellets (in this technique, a small amount of the colouring matter, usually from 1 to 3 mg, is thoroughly mixed with pure, dry potassium bromide and the mixture is transferred to a suitable die and pressed into a thin pellet by exerting a pressure of 700 to 1,400 kg/cm<sup>2</sup>).

Spectra are ordinarily displayed as % transmittance vs. wavenumber ( $\text{cm}^{-1}$ ). The salient features of the spectra are the intensities of the absorption peaks, and their shapes.

Detailed discussion of the infrared absorption technique and interpretation of infrared spectra are beyond the scope of this volume. It must be pointed out, however, that some difficulties exist in the practical use of this technique for identifying colours.

The crystal structure or other physical state of the sample may affect the spectra obtained from suspensions or potassium bromide pellets. It is necessary to make certain that the unknown material has been treated in exactly the same manner as was the standard or known sample.

Care must be taken to ensure that absorption bands due to contaminants are identified. All materials to be tested should be free from water or other solvent before an infrared spectrum is obtained because water and all organic solvents absorb infrared radiation. Water-soluble colouring matters can often be prepared for analysis by dissolving samples in water, adding a little acetic acid, evaporating to apparent dryness, and then drying at about  $100^\circ$  to remove the residual water. Infrared spectra should be obtained of the dried solids, as well as blanks.

#### Other identification techniques

Sometimes chromatographic and spectrophotometric techniques will fail to provide positive identification of colouring matters. In such cases, the problem can often be solved by reducing the colouring matter or otherwise degrading it and identifying the resulting products. This technique is particularly applicable to identifying azo colours. The amine compounds resulting from the reduction can frequently be readily identified by chromatographic and spectrophotometric techniques.

Many other techniques have been applied to the identification of colouring matters. For example, many pigments have a well defined crystalline structure and can be identified by their X-ray diffraction patterns or by X-ray crystallography. Some colouring matters can be converted to crystalline derivatives and similarly identified. The descriptions of these and other available techniques are beyond the scope of this volume.

#### Total colouring matters content

Two general methods are used for determination of total colouring matters: 'Colouring Matters Content by Spectrophotometry' and 'Colouring Matters Content by Titration with Titanous Chloride.'

When using the spectrophotometric method, the analyst should take into account the accuracy and precision of the spectrophotometer used for the analysis. All colours present in the sample that absorb in the same region as that of the main colour will contribute to the absorbance figure used to calculate the results; subsidiary colouring matters of markedly different hue will not be accounted for by this method. This method uses accepted absorptivity figures obtained from purified standard colours for calculating the total colouring matters content.

The titanous chloride reduction method assumes that isomers and subsidiary colouring matters have the same titanous chloride equivalent as the main colouring matter.

#### Colouring Matters Content by Spectrophotometry

Three experimental procedures are described. Procedure 1 is used for water-soluble colouring matters. Procedure 2 is used for organic solvent-soluble colouring matters, especially the synthetic carotenoids. (The solutions prepared in Procedure 2 are used in the identification tests for the carotenoids.) Procedure 3 is used for lakes.

Principle

The absorbance of a solution of the colouring matter is determined at its wavelength of maximum absorption and the total colouring matters content is calculated using a standard absorptivity value quoted in the specification monograph.

Apparatus

- UV-visible range spectrophotometer capable of accurate ( $\pm 1\%$  or better) measurement of absorbance in the region of 350 - 700 nm with an effective slit width of 10 nm or less
- Spectrophotometer cells, 1 cm path length

Procedure 1 – Colouring matters content of water-soluble colouring matters

Accurately weigh 0.25 g ( $\pm 0.02$  g) of the sample (W). Transfer to a 1-liter volumetric flask. Add freshly distilled water or the solvent prescribed in the specification monograph and swirl to dissolve. Make up to volume and mix. Dilute to a solution of suitable strength according to the details given in the specification monograph. Measure the absorbance (A) at the wavelength of maximum absorption in a 1 cm cell, using water or the prescribed solvent as the blank.

Calculation

Calculate the total colouring matters content of the sample using either of the following equations:

$$\% \text{ total colouring matters} = 100 \times (A \times 1 \text{ liter} \times F) / (a \times 1 \text{ cm} \times W)$$

or

$$\% \text{ total colouring matters} = 100 \times (A/A^{1\%}_{1\text{cm}}) \times (F/W)$$

where

A is the absorbance of the sample solution at the wavelength of maximum absorption;  
 $A^{1\%}_{1\text{cm}}$  is the specific absorbance of the standard indicated in the specification monograph;  
 a is the absorptivity of the standard in liter/(g·cm); and  
 F is the dilution factor (Volume diluted / Volume measured).

Procedure 2 – Colouring matters content of organic solvent-soluble colouring mattersReagents

- Chloroform, reagent grade, acid free
- Cyclohexane, reagent grade

Accurately weigh 0.08 g ( $\pm 0.01$  g) of the sample (W) into a 100-ml volumetric flask ( $V_1$ ). Add 20 ml of chloroform and dissolve by swirling briefly. Make sure that the solution is clear. Make up to volume with cyclohexane and mix. Pipet 5.0 ml of the solution ( $v_1$ ) into a second 100-ml volumetric flask ( $V_2$ ) and make up to volume with cyclohexane. Pipet 5.0 ml of this diluted solution ( $v_2$ ) into the final 100-ml volumetric flask ( $V_3$ ) and make up to volume with cyclohexane. Measure the absorbance (A) of the twice-diluted solution at the wavelength of maximum absorption in a 1 cm cell, using cyclohexane as the blank.

Perform this procedure promptly, avoiding exposure to air insofar as possible and undertaking all operations in the absence of direct sunlight.

Calculation

Calculate the total colouring matters content of the sample using either of the following equations:

$$\% \text{ total colouring matters} = 100 \times (A \times V_1 \times V_2 \times V_3) / (a \times 10^{-3} \times v_1 \times v_2 \times W)$$

or

$$\% \text{ total colouring matters} = (A \times V_1 \times V_2 \times V_3) / (v_1 \times v_2 \times W \times A^{1\%}_{1 \text{ cm}})$$

where

A is absorbance of the sample solution at the wavelength of maximum absorption;  
 $A^{1\%}_{1 \text{ cm}}$  is the specific absorbance of the standard indicated in the specification monograph;  
 a is the absorptivity of the standard in liter/(g·cm)  
 $V_1$ ,  $V_2$ , and  $V_3$  are the volumes of the three volumetric flasks (each 100 ml);  
 $v_1$  and  $v_2$  are the volumes of the two pipets (each 5 ml);  
 a is absorptivity of the standard in liter/(g·cm); and  
 $10^{-3}$  is the correction factor for a in ml/liter.

Procedure 3 – Colouring matters content of lakesReagents

- Potassium dihydrogen phosphate, reagent grade
- Sodium hydroxide, reagent grade
- Phosphoric acid, reagent grade
- Hydrochloric acid, reagent grade

Prepare pH 7 phosphate buffer as follows: Weigh 13.61 g of potassium dihydrogen phosphate into a 2000-ml beaker, dissolve in 200 ml of water, and dilute to 1,000 ml. Add about 90 ml of 1 N sodium hydroxide. Determine the pH using a pH-meter and adjust the pH to 7.0 using 0.1 N sodium hydroxide or diluted phosphoric acid.

Accurately weigh a quantity of lake which will give an absorbance approximately equal to that of the parent colour when the latter is tested according to Procedure 1, above. Transfer to a 250- ml beaker containing 10 ml hydrochloric acid previously diluted with water to approximately 50 ml. Heat with stirring to dissolve the lake, then cool to ambient temperature. Transfer to a 1-liter volumetric flask, make up to volume with pH 7 phosphate buffer, and mix. Proceed as detailed in Procedure 1, above, and in the specification monograph, using pH 7 phosphate buffer as the spectrophotometric blank.

Colouring Matters Content by Titration with Titanous ChloridePrinciple

Titanous chloride (titanium trichloride,  $\text{TiCl}_3$ ) reduces the colouring matter to yield titratable reduction products. The method assumes that isomers and subsidiary colouring matters have the same titanium trichloride equivalent as the main colouring matter.

Apparatus

- Titration apparatus (See Figures 1 and 2 below):
  - Bottle (borosilicate glass) for titrant (may be up to 5 liter volume, as needed), with 29/42 ground glass center neck (for burette), side arm for inlet gas sparge, side arm stopcock (for gas outlet), and side arm with glass stopper for refilling bottle (**Note:** *bottle may need to be custom-made*)

- Digital burette, 25 ml – Brinkmann Digital Burette II™ or equivalent
  - 500-ml conical flasks, sealable with No. 10 rubber stoppers
  - Overhead stirrer
  - Stopper assembly – No. 10 rubber stopper with five holes for accommodating the stirrer rod, burette delivery tip, argon source, gas outlet tubing, and 10 ml pipet
  - Glass rod stopper for the pipet inlet in the stopper assembly
  - Tubing, glass and flexible plastic, for connections
- Hot plate

### Reagents

- Titanium trichloride (20% in HCl), reagent grade
- Hydrochloric acid, reagent grade
- Ferrous ammonium sulfate  $[\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}]$ , reagent grade
- Sulfuric acid, reagent grade
- Potassium dichromate, 0.100 N standard solution
- Ammonium thiocyanate, reagent grade
- Sodium citrate, reagent grade
- Sodium hydrogen tartrate, reagent grade
- Boiling chips
- Argon, UHP compressed gas (Carbon dioxide from a Kipp apparatus may be also be used, but is much less convenient; compressed nitrogen gas may be used provided residual oxygen is removed.)

### Procedure for Colouring Matters

**Note:** *A water bubbler should be placed in line between the argon source and the titration apparatus.*

#### Preparation of 0.1 N titanium trichloride

Measure 800 ml of water for each liter of solution required into a beaker of appropriate size. On a hot plate, boil the water vigorously for 1 min, cover with a watch glass, and allow to cool to room temperature. In a fume cupboard, using graduated cylinders, add 90 ml of hydrochloric acid, stir, and add 100 ml of 20% titanium trichloride solution, for each liter of solution required. (Avoid transferring any white precipitate from the titanium trichloride reagent bottle.) Mix the solution and transfer to the titrant bottle. Attach the burette and connect the argon. Pass argon through the solution for 1-2 h with the sidearm stopcock on the bottle open to maintain ambient pressure. While maintaining a slow flow of gas, draw up titrant into the burette. Drain the burette, discard the titrant, and refill. Drain and refill the burette two more times. Stop the gas flow, close the sidearm stopcock, and store the solution for at least 72 h before use.



Figure 1



Figure 2

### Apparatus for titanium chloride titrations

#### Standardization of 0.1 N titanium trichloride

Drain and refill the burette with 0.1 N titanium trichloride. Use within 1 h. Weigh 3.0 g ( $\pm 0.2$  g) of ferrous ammonium sulfate into a 500-ml conical flask. Add 200 ml of water. Using a graduated cylinder, add 25 ml of 10 N sulfuric acid. Pipet 20 ml of standard 0.100 N potassium dichromate into the flask. Swirl to mix. Connect the flask securely to the stopper assembly (rubber stopper fitted with stirrer, gas inlet and outlet tubes, burette tip, and glass rod stopper). Gently bubble argon into the flask. Turn on the stirrer and slowly increase the speed until the solution is stirring vigorously without splashing. Wait 1 min before beginning the titration, and continue stirring throughout the procedure.

After adding 15-17 ml of 0.1 N titanium trichloride drop-wise within about 2 min, stop the flow of titrant and reduce the argon flow. Remove the solid glass rod from the stopper assembly, and pipet 10 ml of 50% ammonium thiocyanate (indicator solution) into the flask. The colour of the solution will become brownish-red. Remove the pipet, re-insert the glass rod, and restore the argon flow. Add 0.1 N titanium trichloride dropwise, with 2-3 sec pauses between drops, until a sharp colour change from brownish-red to light green is observed. The endpoint (20-21 ml) is reached when the solution returns to the original light green colour and remains that colour for 20 sec. Stop the argon flow and gradually turn off the stirrer. Record the volume (V) of 0.1 N titanium trichloride used to the nearest 0.05 ml. Perform the titration procedure in triplicate.

Determine the indicator blank by repeating the above procedure without the 0.100 N potassium dichromate. The blank determination should require less than 0.5 ml of 0.1 N titanium trichloride. Record the volume used to the nearest 0.05 ml.

For each titration, the concentration of the titanium trichloride solution is  $(N \times 20)/(V - V_B)$ ,

where

N is the concentration of the standard potassium dichromate solution;

20 is the aliquot (ml) of the potassium dichromate solution;

V is the volume (ml) of titanium trichloride solution required to titrate the aliquot of standard potassium dichromate solution; and

$V_B$  is the volume (ml) of titanium trichloride solution used to determine the blank.

Calculate the concentration of the standard titanium trichloride solution by averaging the three titration results. (Restandardize the solution weekly by performing one titration of 0.100 N potassium dichromate and determining one indicator blank.)

#### Determination of total colouring matters content of sample

Accurately weigh the quantity of sample indicated in each specification monograph ( $W_S$ , in mg) into a 500-ml conical flask. Add 10 g of sodium citrate or 15 g of sodium hydrogen tartrate, as specified in each monograph, a few boiling chips, and 150 ml of water. Wash down the walls of the flask with water, cover with a watch glass, and gently swirl to dissolve. In a fume hood, heat the solution to boiling on a hot plate. Boil vigorously for at least 10 sec to remove dissolved oxygen. (Avoid sample decomposition by boiling the solution for no more than 2 min.) Using gloves, remove the flask from the hot plate. Within 2-4 min of removing the flask from the hot plate, remove the watch glass, and connect the flask securely to the stopper assembly (flask might still be hot). Gently bubble argon into the flask. Turn on the stirrer and slowly increase the speed until the solution is stirring vigorously without splashing. Wait 1 min before beginning the titration, and continue stirring throughout the procedure. The colour will act as its own indicator unless otherwise stated in the appropriate monograph.

Rapidly add standardized 0.1 N titanium trichloride dropwise until the colour of the solution begins to change, then stop for 15-20 sec. Continue adding the titrant dropwise, with 1-2 sec pauses between drops. When the solution is close to the final colour, stop again for 20 sec. Continue adding 0.1 N titrant dropwise, with 5-10 sec pauses between drops, until the final colour is observed. The endpoint is reached when the final colour is stable for 20 sec. Stop the argon flow and gradually turn off the stirrer. Record the volume of titrant used to the nearest 0.05 ml

#### Calculation

The percent total colouring matters content of the sample is  $100 \times (V \times F \times N) / (W_S)$ ,

where

V is the ml of standardized titanium trichloride solution required;

F is  $D/(1.00 \text{ ml} \times 0.1 \text{ meq/ml})$ , where D is the weight (mg) of colouring matters equivalent to 1.00 ml of 0.1 N titanium trichloride, quoted in the specification monograph); and

N is the concentration of standardized titanium trichloride solution (in meq/ml).

#### Procedure for Lakes

Add 150 ml of water to a 500-ml conical flask and dissolve in it the buffer compound specified in the monograph for the parent colour. Accurately weigh a quantity of lake equivalent to 35-40 ml of 0.1 N titanium trichloride and transfer it to the flask. Add a few boiling chips, wash down the walls of the flask with water, and cover with a watch glass. In a fume hood, heat the mixture to boiling or until the lake has completely dissolved. Using gloves, remove the flask from the hot plate. Titrate with standardized 0.1 N titanium



trichloride in the manner described under Determination of total colouring matters content of sample, above.

## **Subsidiary Colouring Matter Content**

### Principle

In this method, the subsidiary colouring matters are separated from the main colouring matter by ascending paper chromatography and are extracted separately from the paper. The absorbance of each extract is measured at its wavelength of maximum absorbance by visible spectrophotometry.

Because it is impractical to identify each subsidiary colouring matter and because the subsidiary colouring matters are usually minor components of food colours, the method assumes that the specific absorbance of each subsidiary colouring matter is the same as that of the total colouring matters. The subsidiary colouring matters content is calculated by adding together the absorbances of the extracts in conjunction with the total colouring matters content of the sample.

### Apparatus

Chromatography tank and ancillary equipment (Figures 3 and 4 or equivalent) comprising:

- Glass tank (A) and cover (B)
- Supporting frame (C) for the chromatography paper
- Solvent tray (D)
- Secondary frame (E) for supporting "drapes" of the filter paper
- Whatman No. 1 chromatography grade paper or equivalent, 20 cm x 20 cm sheets
- Microsyringe, capable of delivering 0.1 ml with a tolerance of  $\pm 0.002$  ml
- Visible range spectrophotometer
- Spectrophotometer cells, closed, 40 mm path length
- Test tubes
- Filter paper, 9 cm, coarse porosity

### Chromatography solvents (all reagent grade)

- Water:ammonia (sp.gr. 0.880):trisodium citrate (95 ml:5 ml:2 g)
- n-Butanol:water:ethanol:ammonia (sp.gr. 0.880) (600:264:135:6)
- 2-Butanone:acetone:water (7:3:3)
- 2-Butanone:acetone:water:ammonia (sp.gr. 0.880) (700:300:300:2)
- 2-Butanone:acetone:water:ammonia (sp.gr. 0.880) (700:160:300:2)
- n-Butanol:glacial acetic acid:water (4:1:5)

Shake for 2 min, allow layers to separate. Use the upper layer as the chromatography solvent.

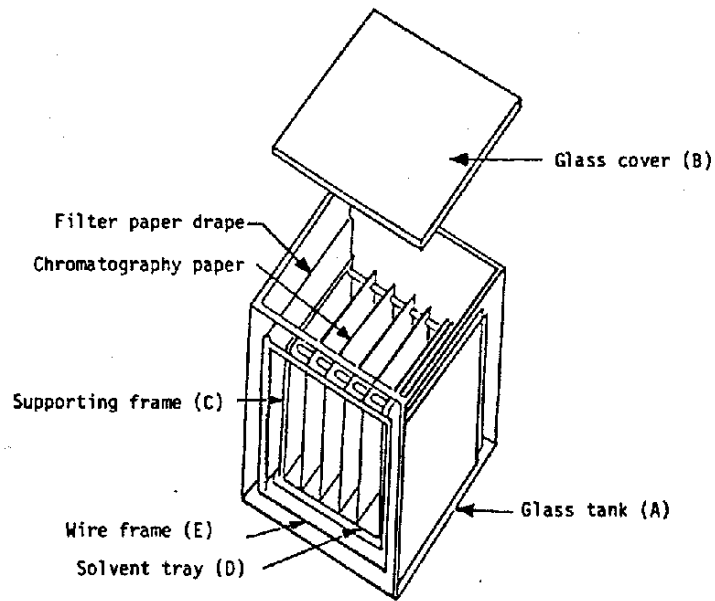


Figure 3. Assembly of Chromatography Apparatus

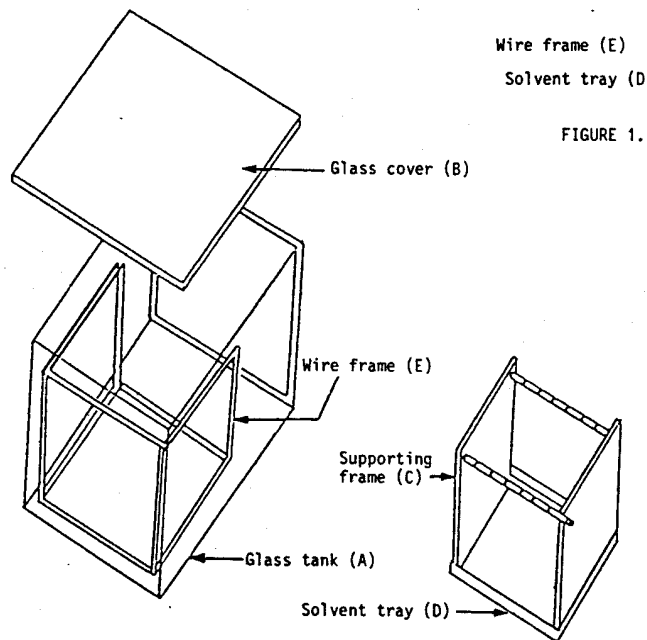


Figure 4. Components of Chromatography Apparatus

#### Other reagents

- Acetone, reagent grade
- Sodium hydrogen carbonate, reagent grade

#### Procedure

Not less than 2 h before carrying out the determination, arrange the filter-paper drapes in the glass tank and pour over the drapes and into the bottom of the tank sufficient chromatography

solvent to cover the bottom of the tank to a depth of approximately 1 cm. Place the solvent tray in position and fit the cover to the tank.

Prepare a 1.0% aqueous solution of the sample. Mark out a sheet of chromatography paper as shown in Figure 5. Apply 0.10 ml of the sample solution as uniformly as possible within the confines of the 18 cm x 7 mm rectangle, holding the nozzle of the microsyringe steadily in contact with the paper. Allow the paper to dry at room temperature for 1 - 2 h or at 50° in a drying cabinet for 5 min, followed by 15 min at room temperature. Mount the dried sheet, together with a plain sheet to act as a blank on the supporting frame. (If required, several dried sheets may be developed simultaneously.)

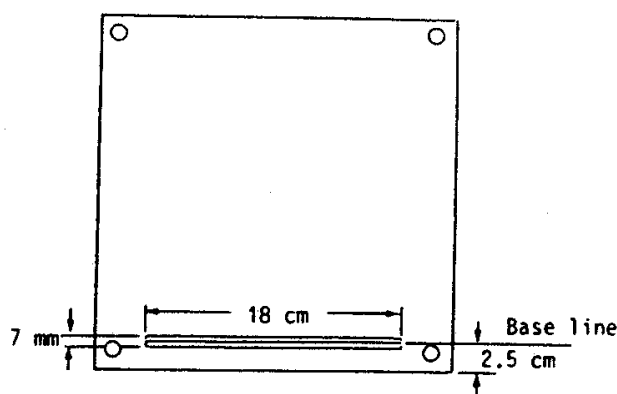


Figure 5. Method of Marking Chromatography Paper

Pour sufficient chromatography solvent into the solvent tray to bring the surface of the solvent about 1 cm below the base line of the chromatography sheets. The volume necessary will depend on the dimensions of the apparatus and should be predetermined. Put the supporting frame into position and replace the cover. Allow the solvent front to ascend the distance above the base line noted in the specification monograph, then remove the supporting frame and transfer it to a drying cabinet at 50-60° for 10-15 min. Remove the sheets from the frame.

Cut each subsidiary band from each chromatogram sheet as a strip, and cut an equivalent strip from the corresponding position of the plain sheet. Place each strip, subdivided into a suitable number of approximately equal portions, in a separate test tube. Add 5.0 ml of water:acetone (1:1 by vol) to each test tube, swirl for 2 - 3 min, add 15.0 ml of 0.05 N sodium hydrogen carbonate solution, and shake the tube to ensure mixing. Filter the coloured extracts and blanks through 9-cm coarse porosity filter papers into clean test tubes and determine the absorbances of the coloured extracts at their wavelengths of maximum absorbance, using 40-mm closed cells, against a filtered mixture of 5.0 ml of water:acetone (1:1 by vol) and 15.0 ml of the 0.05 N sodium hydrogen carbonate solution. Measure the absorbances of the extracts of the blank strips at the wavelengths at which those of the corresponding coloured extracts were measured and correct the absorbances of the coloured extracts with the blank values.

Prepare a standard solution from the 1.0% sample solution, corresponding to  $L/100\%$  where  $L$  is the subsidiary colouring matters limit given in the specification monograph. Apply 0.10 ml of this solution to a sheet of chromatography paper by the technique outlined above, run a chromatogram and a blank, and dry at 50-60° for 10-15 min. Cut the band from the sheet as a

strip and cut an equivalent strip from the blank sheet. Proceed as detailed previously and determine the total absorbance ( $A_s$ ) of the standard corrected for the blank.

### Calculation

Calculate the percent subsidiary colouring matters in the sample using the following equation:

$$\% \text{ Subsidiary Colouring matters} = 100 \times L \times D \times (A_a + A_b + A_c \dots A_n) / A_s$$

where

L is the limit for subsidiary colouring matters given in the specification monograph;

D is the total colouring matters content of the sample;

$A_a + A_b + A_c \dots A_n$  is the sum of the absorbances of the subsidiary colouring matters corrected for the blank values; and

$A_s$  is the absorbance of the standard solution;

## **Ether-extractable Matter**

### Method I

#### *Apparatus*

- Upward displacement type liquid/liquid extractor with sintered glass distributor, 500 ml working capacity with a piece of bright copper wire suspended through the condenser
- Distillation flasks: 250 and 500 ml
- Small coils of copper wire (0.5 g) for placing in distillation flasks
- Oven, 0 to 200° range
- Desiccator

#### *Reagents*

- Aluminium oxide, powdered, chromatography grade
- Ferrous sulfate, reagent grade
- Ammonium thiocyanate, reagent grade
- Titanium trichloride, 0.1 N, standard solution
- Sodium hydroxide, 2 N and 0.1 N, reagent grade
- Hydrochloric acid, 3 N and 0.1 N, reagent grade
- Ethyl ether or isopropyl ether, freshly distilled or stabilized

#### *Ether purification*

Immediately before use, freshly distilled ether should be passed through a 30 cm column of aluminium oxide in order to remove peroxides and inhibitors. Test to ensure the absence of peroxides, as follows:

Prepare a colourless solution of ferrous thiocyanate by mixing equal volumes of 0.1 N solutions of ferrous sulfate and ammonium thiocyanate. Carefully discharge any red colouration, due to ferric ions, with titanium trichloride. To 50 ml of this solution add 10 ml of ether and shake the mixture vigorously for 2-3 min. No red colour should develop.

#### *Procedure*

Alkaline ether extract. Weigh accurately about 5.0 g of the colouring matter sample ( $W_s$ ). (For colouring matters with solubilities of less than 5 g/150 ml, use the lower weights

prescribed in the specification monograph under TESTS). Dissolve the sample in 150 ml of water, add 2.5 ml of 2 N sodium hydroxide and transfer the solution to a 500-ml distillation flask; dilute with water to approximately 200 ml. Add 200 ml of ether to the distillation flask and extract for 2 h with a reflux rate of about 15 ml/min. Reserve the colour solution. Transfer the ether extract to a separatory funnel and wash the ether extract with two 25-ml portions of 0.1 N sodium hydroxide and then with water. Transfer to a tared 150-ml distillation flask ( $W_1$ ) containing a clean copper coil and distil off the ether in portions, reducing the volume to about 5 ml.

*Acid ether extract.* To the colour solution reserved above, add 5 ml of 3 N hydrochloric acid, mix and extract with a further quantity of the ether as above. Wash the ether extract with two 25-ml portions of 0.1 N hydrochloric acid and then with water. Transfer in portions to the flask containing the evaporated alkaline extract and carefully evaporate all the ether. Complete the drying in an oven at 85° for 20 min, then allow the flask to cool in a desiccator for 30 min and weigh. Repeat the drying and cooling until constant weight is obtained ( $W_2$ ).

#### Calculation

The percent ether-extractable matter is  $100 \times (W_2 - W_1) / W_S$ .

#### Method II

##### Apparatus

- Soxhlet extractor - Suspend a piece of bright copper wire through the condenser. Place a small coil of copper wire (0.5 g) in the distillation flask.

##### Reagent

- Ethyl ether or isopropyl ether, freshly distilled or stabilized

##### Ether purification

Purify the ether and test to ensure the absence of peroxides as directed in Method I.

##### Procedure

Weigh accurately about 2 g of the colouring matter sample ( $W_S$ ). Transfer to the Soxhlet thimble and extract with 150 ml ether for 5 h. Concentrate the ether extract on a steam bath to about 5 ml. Dry the residue in a tared evaporating dish ( $W_1$ ) on a water bath and then dry at 105° until a constant weight is obtained ( $W_2$ ).

#### Calculation

The percent ether-extractable matter is  $100 \times (W_2 - W_1) / W_S$ .

### Hydrochloric acid-insoluble Matter in Lakes

#### Apparatus

- Oven, 0 - 200° range
- Sintered glass crucible, No. 4
- Desiccator

#### Reagents

- Hydrochloric acid, concentrated
- Hydrochloric acid, 0.5% v/v

Procedure

Accurately weigh approximately 5 g of the lake ( $W_S$ ) into a 500 ml beaker. Add 250 ml water and 60 ml concentrated hydrochloric acid. Boil until all the colour and alumina have dissolved. Filter through a tared sintered glass crucible ( $W_1$ ). Wash the crucible with hot 0.5% hydrochloric acid until the washings are colourless. Dry the crucible at 135° to constant weight ( $W_2$ ). Cool in a desiccator before weighing.

Calculation

The percent hydrochloric acid-insoluble matter is  $100 \times (W_2 - W_1) / W_S$ .

**Leuco Base in Sulfonated Triarylmethane Colours**Principle

Air is blown through an aqueous solution containing the colouring matter, copper(II) chloride, and dimethylformamide and the solution is analyzed spectrophotometrically. Under these conditions the leuco base is oxidized to the corresponding colouring matters and the increase in absorbance is equivalent to the amount of leuco base originally present.

Apparatus

- Visible range spectrophotometer
- Spectrophotometer cells, 1 cm path lengths (flow-through cells optional)

Reagents

- Copper (II) chloride [ $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ ], reagent grade
- Dimethylformamide (DMF), reagent grade

Procedure

**Note:** *The entire procedure should be completed as quickly as possible.*

*Solution A:* Weigh 10.0 g of copper (II) chloride and dissolve in 200 ml of DMF. Transfer to a 1-liter volumetric flask and make up to the mark with DMF.

*Solution B:* Accurately weigh the quantity of sample indicated in the specification monograph ( $W$ , in mg). Dissolve in approximately 100 ml water, transfer quantitatively to a 1-liter volumetric flask and make up to the mark with water.

*Solution a:* Pipet 50 ml DMF into a 250-ml volumetric flask. Cover with Parafilm™ (or equivalent covering) and place in the dark.

*Solution b:* Accurately pipet 10 ml of Solution B into a 250-ml volumetric flask. Add 50 ml DMF. Cover with Parafilm™ (or equivalent covering) and place in the dark.

*Solution c:* Pipet 50 ml of Solution A into a 250-ml volumetric flask. Bubble air through the solution for 30 min in the following manner: Insert a 5-ml pipette into flexible tubing attached to a bench air flow source. Slowly, turn on the air, insert the pipette into the solution in the flask and adjust the air flow to a rapid, but controlled, rate. After 30 min remove the pipette from the solution and rinse the sides of the pipette into the flask with water from a wash bottle. Then, turn off the air flow.

*Solutions  $d_1$  and  $d_2$  (duplicates):* Accurately pipet 10 ml of Solution B into each of two 250-ml volumetric flasks. Add 50 ml of Solution A to each flask. Bubble air through the solutions for 30 min, using the method given for preparation of Solution c. After stopping the air flow, dilute solutions a-  $d_2$  in the five flasks nearly to volume with water and place the flasks in a

water bath until they have cooled to room temperature, as heat is evolved when DMF and water are mixed. Do not leave them for longer than necessary; 5-10 min is normally enough. Dilute to volume with water. Immediately measure the absorbances of the solutions by spectrophotometry.

#### *Spectrophotometric Determination*

According to the table below, generate the absorbance curves for solutions a, b, c, d<sub>1</sub>, and d<sub>2</sub> between 700 and 500 nm, using solutions a and c as blanks. Rinse cells thoroughly with each sample solution between measurements. When using the flow-through cells, use 3 separate rinses of at least 40 ml of each sample solution to be measured.

Curve	Blank	Solution	Comments
1	a	a	Set zero at 700 nm, run curve; record absorbance at wavelength of maximum absorption for colouring matter standard
2	a	b	Run curve without readjusting zero setting; record absorbance at wavelength of maximum absorption
3	c	c	Set zero at 700 nm; record absorbance at wavelength of maximum absorption for colouring matter standard
4a	c	d <sub>1</sub>	Run curve without readjusting zero setting; record absorbance at wavelength of maximum absorption
4b	c	d <sub>2</sub>	Run curve without readjusting zero setting; record absorbance at wavelength of maximum absorption

#### Calculations

Calculate the percent leuco base in the sample using the following equation:

$$\% \text{ leuco base} = 100 \times [(A_4 - A_3) - (A_2 - A_1)] \times 1 \text{ liter} \times F / (a \times b \times W \times R)$$

where

A<sub>1</sub>...A<sub>4</sub> are the recorded absorbances of curves 1, 2, 3, and 4, respectively;

F is 250 ml/10 ml (dilution factor);

a is the absorptivity of 100% colouring matters in liter/ (mg·cm);

b is the cell path length (1 cm); and

R is the formula weight of colouring matter/formula weight of leuco base (given in the specification monograph).

### **Organic Compounds other than Colouring Matters**

#### General Note

For the separation and determination of organic compounds other than colouring matters (i.e., uncoloured impurities, such as uncombined intermediate starting materials), high-performance liquid chromatography (HPLC) has several advantages over other chromatographic techniques, viz. improved separations, speed (it can be automated) and accuracy. When determining named organic compounds, standards of each compound likely to be encountered are needed before any particular colour can be analyzed.

HPLC methods are generally outlined rather than described in detail. Column packing materials, capillary columns, and type and sensitivity of detectors should be chosen for optimum separation and quantitation of impurities currently listed in food colour specifications, as well as other impurities.

The alternative (traditional) method to HPLC is column chromatography (described further below), which involves collecting the eluate in fractions, using ultraviolet spectrophotometry to identify the compounds in each fraction, and calculating their concentrations.

### Determination by High Performance Liquid Chromatography

#### *Principle*

The organic compounds other than colouring matters are separated by HPLC using gradient elution and are quantitatively determined by comparison of their peak areas against those obtained from standards. The conditions prescribed must be treated as guidelines and minor modifications might be needed to achieve the separations. Deviations from the prescribed conditions, such as a different column length, other types of column packing and solvent system, and the use of paired ion procedures, can result in elution characteristics different from those for the conditions given here, such as order of elution and resolution.

#### *Apparatus*

- High-performance liquid chromatograph capable of gradient elution with
  - controller/integrator
  - pump(s), flow rate 1 ml/min
  - auto-sampler with a 20  $\mu$ l injector
  - detector, UV-visible absorption
  - printer/plotter
- Chromatography column, C-18 on silica gel, 5  $\mu$ m particle size, 250  $\times$  4.6 mm.
- Guard column, C-18 on silica gel, 5  $\mu$ m particle size, 15  $\times$  4.6 mm

#### *Reagents*

- Methanol, HPLC grade
- Ammonium acetate, HPLC grade
- Reference standards as required

#### *Instrument Parameters*

- Injection volume: 20  $\mu$ l.
- Eluents:
  - A: 0.2 N ammonium acetate;
  - B: methanol
- Gradient:
  - 0.0 (sample injection)
  - 0 to 35 min – 0 to 40% B (analysis)
  - 35 to 41 min – 100% B (wash)
  - 41.1 to 55 min – 100 to 0% B (return to initial gradient composition and equilibrate column)
- Flow rate: 1.0 ml per min
- Temperature: Ambient
- Pump pressure: minimum 300 psi, maximum 4000 psi
- Detector wavelengths: as required
- Integration: peak area

#### *Procedure*

Prepare 0.5% (w/w) colouring matter sample solutions in 0.02 M ammonium acetate. Prepare calibration solutions from standards of impurities named in the specification monograph.



Analyze, following the instructions given for the HPLC chromatograph and detector.

#### Determination by Column Chromatography

##### *Apparatus*

- Chromatography column (see Figure 6)
- UV range spectrophotometer
- Spectrophotometer cells, 1 cm path length
- Reference standards, as required

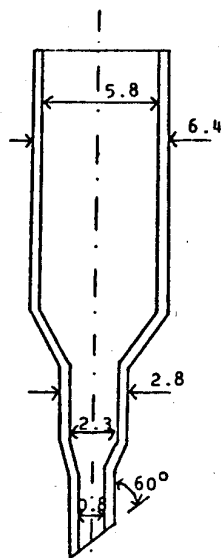


Figure 6. Chromatography Column (dimensions in cm)

##### *Reagents*

- Powdered cellulose, Whatman, or equivalent low iron cellulose
- Ammonium sulfate, reagent grade, very low in iron

##### *Column Preparation*

Prepare a 25% ammonium sulfate solution for use as the eluent. Prepare a slurry of powdered cellulose in the 25% ammonium sulfate solution, using about 75 g of cellulose to 500 ml of liquid. Place a small disk of stainless steel gauze in the constriction above the tip of the chromatography column. Pour a sufficient volume of the slurry into the column so that the height of the packing is about 5 cm from the top of the column. Tap the column occasionally to ensure efficient packing. Wash the column with 200 ml of the eluent.

Test the column by passing 200 ml of 25% ammonium sulfate solution through it and measuring the UV absorption of the solution by spectrophotometry. The absorption must be sufficiently low to avoid interference with the intended analysis.

##### *Procedure*

Weigh 0.200 g of the colouring matter sample (W) into a 150-ml beaker and dissolve in 20 ml of water. Add approximately 5 g of powdered cellulose. Add 50 g of ammonium sulfate to salt out the colour. Transfer the mixture to the chromatography column, rinse the beaker with

the 25% ammonium sulfate solution, and add the washings to the column. Allow the column to drain until flow ceases, or nearly so.

Add 25% ammonium sulfate solution to the column at a rate equal to the flow rate through the column. Collect the effluent in 100-ml fractions. Continue until twelve fractions have been collected. Reserve the column and contents until the last fraction has been examined by spectrophotometry.

Mix each fraction well, and obtain the UV absorption spectrum of each solution from 220 to 400 nm, using the eluent as the blank. If the UV spectrum of the twelfth fraction shows the presence of any compound, continue collecting fractions until the compound is eluted.

Absorptivities of the organic compounds, such as intermediate starting materials, collected in the separate fractions and expected to be present in the colouring matters are used to calculate the percent organic compounds other than colouring matters in the sample and can be found in the specification monograph of the food colour.

#### *Calculation*

Calculate the percent organic compounds other than colouring matters in the sample using the following equation:

$$\% \text{ organic compounds} = 100 \times (A \times 0.100 \text{ liter}) / (a \times 1 \text{ cm} \times W)$$

where

A is the total absorbance of eluted fractions corrected for absorbance of blank;

0.100 liter is the volume of one fraction; and

a is the absorptivity in liter/(g·cm).

**Note:** *Usually only one compound is encountered in each eluted fraction. When more than one compound is present in significant quantities in any fraction, the spectrophotometric data will so indicate. In such cases, the amounts of the various compounds must be determined by the procedure customarily used for the spectrophotometric analysis of mixtures of absorbing materials.*

*Some samples contain small amounts of various materials, particularly inorganic salts, which contribute "background absorption". Correction for this is made as follows: Determine the amount of background absorption of the fraction collected from the column immediately before and of the fraction immediately following those fractions in which the organic compounds are encountered. Subtract one-half of the sum of these two absorbances from the observed absorbance of the fractions containing the organic compounds. The remainder is taken as the absorbance due to inorganic salts.*

### **Sulfate as Sodium Sulfate**

**Note:** *This determination is done in connection with Water Content (Loss on Drying) for food colours and the result is included in that calculation.*

#### Reagents

- Sodium chloride, reagent grade, sulfate-free
- Hydrochloric acid, reagent grade
- Barium chloride, reagent grade

### Procedure

Weigh 5.0 g of the colouring matter sample, transfer it to a 250-ml conical flask and dissolve in about 100 ml of water by heating on a water bath. Add 35 g of sulfate-free sodium chloride, stopper the flask, and swirl at frequent intervals for 1 h. Cool the flask, transfer the contents with saturated sodium chloride solution to a 250-ml volumetric flask, allow the solution to cool further to 20°, and dilute to volume. Shake the flask, and filter the solution through a dry filter paper. Pipet 100 ml of the filtrate into a 500-ml beaker, dilute to 300 ml with water and acidify with hydrochloric acid, adding 1 ml in excess. Heat the solution to boiling, and add an excess of 0.25 N barium chloride solution, drop by drop, with stirring. Allow the mixture to stand on a hot plate for 4 h, or leave it overnight at room temperature. Heat the mixture to about 80° and allow the precipitate to settle. Filter off the precipitated barium sulfate, wash with hot water, and ignite at a dull red heat in a tared crucible until a constant weight is obtained. Carry out a blank determination using the above procedure and correct the weight of barium sulfate found.

### Calculation

Calculate the sulfate content of the sample as percent sodium sulfate:

$$\% \text{ sodium sulfate} = 100 \times (2.5 \times \text{corrected weight of barium sulfate found} \times 0.6086) / \text{Weight of sample.}$$

## **Un sulfonated Primary Aromatic Amines**

### Principle

Un sulfonated primary aromatic amines are extracted into toluene from an alkaline solution of the sample, re-extracted into acid, and then determined spectrophotometrically after diazotization and coupling. They are expressed as aniline unless they are known to be some other amine.

**Note:** *This method is not sufficiently sensitive for determining aniline at low mg/kg levels or below.*

### Apparatus

- Visible range spectrophotometer
- Spectrophotometer cells, 40 mm path length

### Reagents

- Toluene, reagent grade
- Hydrochloric acid, 1 N, reagent grade
- Hydrochloric acid, 3 N, reagent grade
- Potassium bromide, 50% solution, reagent grade
- Sodium carbonate solution, 2 N, reagent grade
- Sodium hydroxide, 1 N, reagent grade
- Sodium hydroxide, 0.1 N, reagent grade
- R salt (2-naphthol-3,6-disulfonic acid, disodium salt) solution, 0.05 N, reagent grade
- Sodium nitrite solution, 0.5 N, reagent grade
- Aniline, reagent grade

### Procedure

*Preparation of a Standard Aniline Solution*

Weigh 0.100 g of redistilled aniline into a small beaker and transfer to a 100-ml volumetric flask, rinsing the beaker several times with water. Add 30 ml of 3 N hydrochloric acid and dilute to the mark with water at room temperature. Dilute 10.0 ml of this solution to 100 ml with water and mix well; 1 ml of this solution is equivalent to 0.0001 g of aniline. Prepare the standard aniline solution freshly when required.

#### *Preparation of a Calibration Graph*

Measure the following volumes of the standard aniline solution into a series of 100-ml volumetric flasks: 5 ml, 10 ml, 15 ml, 20 ml, and 25 ml.

Dilute to 100 ml with 1 N hydrochloric acid and mix well. Pipet 10 ml of each solution into clean, dry test tubes; cool them for 10 min by immersion in a beaker of ice water. To each tube add 1 ml of the potassium bromide solution and 0.05 ml of the sodium nitrite solution. Mix and allow the tubes to stand for 10 min in the ice water bath while the aniline is diazotized. Into each of five 25-ml volumetric flasks, measure 1 ml of the R salt solution and 10 ml of the sodium carbonate solution. Pour each diazotized aniline solution into a separate flask containing R salt solution and sodium carbonate solution; rinse each test tube with a few drops of water. Dilute to the mark with water, stopper the flasks, mix the contents well and allow them to stand for 15 min in the dark.

Measure the absorbance of each coupled solution at 510 nm using 40 mm cells. As a reference solution, use a mixture of 10.0 ml of N hydrochloric acid, 10.0 ml of the sodium carbonate solution, and 2.0 ml of the R salt solution, diluted to 25.0 ml with water. Plot a graph relating absorbance to weight of aniline in each 100 ml of aniline solution.

#### *Preparation and Evaluation of a Test Solution*

Weigh, to the nearest 0.01 g, about 2.0 g of the colouring matter sample (W) into a separatory funnel containing 100 ml of water, rinse down the sides of the funnel with a further 50 ml of water, swirling to dissolve the sample, and add 5 ml of 1 N sodium hydroxide. Extract with two 50-ml portions of toluene and wash the combined toluene extracts with 10-ml portions of 0.1 N sodium hydroxide to remove traces of colour. Extract the washed toluene with three 10-ml portions of 3 N hydrochloric acid and dilute the combined extract to 100 ml with water. Mix well. Call this Solution T.

Pipet 10.0 ml of Solution T into a clean, dry test tube, cool for 10 min by immersion in a beaker of ice/water, add 1 ml of the potassium bromide solution and proceed as described above for the preparation of the calibration graph, starting with the addition of 0.05 ml of the sodium nitrite solution.

Measure the absorbance of the coupled test solution at 510 nm using a 40 mm cell. Use a reference solution prepared from 10.0 ml of Solution T, 10 ml of the sodium carbonate solution, and 2.0 ml of the R salt solution diluted to 25.0 ml with water.

From the calibration graph, read the weight of aniline ( $W_A$ ) corresponding to the observed absorbance of the test solution.

#### Calculation

$$\% \text{ unsulfonated primary aromatic amine (as aniline)} = 100 \times W_A / W$$

### **Water Content (Loss on Drying)**

**Note:** See the methods to determine Chloride as Sodium Chloride and Sulfate as Sodium Sulfate. Specifications for food colours include the results of those tests as part of the calculation of Loss on Drying.

Colouring materials containing  $-\text{SO}_3\text{Na}$  or  $-\text{COONa}$  groups are usually hygroscopic and any water retained from their manufacture (or subsequently absorbed from the atmosphere) is generally present in the form of a hydrate. When such colouring matters are dried at  $135^\circ$  the loss in weight can generally be equated to the total water content, but this is not always the case. For example, Erythrosine and Ponceau 4R each retain one molecule of water of crystallization at  $135^\circ$  and it is normal practice to take this into account when totalling the amounts of main components present in a sample.

#### Apparatus

- Oven,  $0 - 200^\circ$  range
- Weighing bottle, 50 mm in diameter and 30 mm high, with ground glass stopper

#### Procedure

Weigh 2.0 - 3.0 g of the sample ( $W_1$ ) in a tared weighing bottle plus stopper. Heat the unstoppered bottle in the oven at the temperature prescribed in the specification monograph ( $\pm 5^\circ$ ), until a constant weight is obtained. Cool the crucible and residue in a desiccator before each weighing.

#### Calculation

$$\text{Loss on drying of the sample (\%)} = 100 \times (1 - W_2/W_1)$$

where

$W_2$  is the weight of the dried sample. (See **Note** above)

### **Water-insoluble Matter**

#### Apparatus

- Oven,  $0 - 200^\circ$  range
- Porcelain filtering crucible
- Glass microfiber filter disc, Whatman type GF/C, compliant with BS 1752
- Desiccator

#### Procedure

Weigh 4.5 - 5.5 g of the sample ( $W_S$ ) into a 250 ml beaker. Add about 200 ml of hot water ( $80-90^\circ$ ), stir to dissolve, and allow the solution to cool to room temperature. Filter the solution through a tared porcelain crucible and filter disc and wash with cold water until the washings are colourless. Dry the crucible and residue at  $135^\circ$  until a constant weight is obtained. Cool the crucible and residue in a desiccator before weighing.

#### Calculation

$$\text{Water-insoluble matter in the sample (\%)} = 100 \times W_R/W_S,$$

where

$W_R$  is the weight of the residue.

### **Water-soluble Chlorides and Sulfates in Aluminium Lakes**

#### Reagents

- Nitric acid, 1.5 N, reagent grade
- Hydrochloric acid, reagent grade

Procedure

Accurately weigh 10 g of the sample into a 400 ml beaker. Add 250 ml of water. Stir to wet the sample and then stir occasionally during a period of 30 min. Filter.

Measure 50 ml of the filtrate, add 50 ml water and acidify with 5 ml of 1.5 N nitric acid. Determine the chloride content by the potentiometric method used for soluble colours (see Chloride as Sodium Chloride determination).

Measure 50 ml of the filtrate, dilute to 300 ml with water and acidify with hydrochloric acid, adding 1 ml in excess. Heat the solution to boiling and add an excess of 0.25 N barium chloride, drop by drop, with stirring. Complete the analysis by digesting, filtering, and igniting the precipitate as described in the method used for the determination of sulfate in soluble colours (see Sulfate as Sodium Sulfate determination).

## ASSAY METHODS

### Alginates Assay

(Carbon Dioxide Determination by Decarboxylation)

#### Apparatus

The apparatus required is shown in Figure 1 below. It consists essentially of a soda lime column, A, a mercury valve, B, connected through a side arm, C, to a reaction flask, D, by means of a rubber connection. Flask D is a 100-ml round-bottom, long-neck boiling flask, resting in a suitable heating mantle, E. The reaction flask is provided with a reflux condenser, F, to which is fitted a delivery tube, G, of 40-ml capacity, having a stopcock, H. On the reflux condenser is mounted a trap, I, containing 25 g of 20-mesh zinc or tin. The trap I should be connected with an absorption tower, J. The absorption tower consists of a 45-cm tube fitted with a medium-porosity fritted glass disk sealed to the inner part above the side arm and having a delivery tube sealed to it extending down to the end of the tube. A trap, consisting of a bulb of approximately 100-ml capacity, is blown above the fritted disk and the outer portion of a ground spherical joint is sealed on above the bulb. A 250-ml conical flask K, is connected to the bottom of the absorption tower. The top of the tower is connected to a soda lime tower, L, which is connected to a suitable pump to provide vacuum and air supply, the choice of which is made by a 3-way stopcock, M. The volume of air or vacuum is controlled by a capillary-tube regulator or needle valve, N. All joints are size 35/25, ground spherical type.

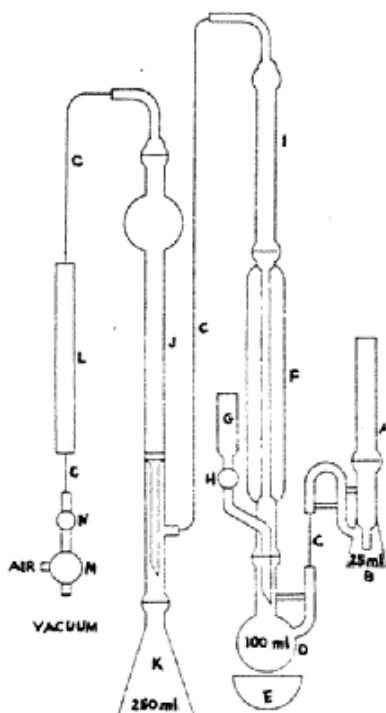


Figure 1. Apparatus for Carbon Dioxide determination by Decarboxylation

Procedure

Weigh to the nearest 0.1 mg, 250 mg of the sample, previously dried in vacuum for 4 h at 60°. Transfer into the reaction flask, D, add 25 ml of 0.1 N hydrochloric acid, insert several boiling chips, and connect the flask to the reflux condenser, F, using syrupy phosphoric acid as a lubricant.

**Note:** *Stopcock grease may be used for the other connections.*

Check the system for air leaks by forcing mercury up into the inner tube of the mercury valve, B, to a height of about 5 cm. Turn off the pressure using the stopcock, M. If the mercury level does not fall appreciably after 1 to 2 min, the apparatus may be considered to be free from leaks. Draw carbon dioxide-free air through the apparatus at a rate of 3,000 to 6,000 ml per h. Raise the heating mantle, E, to the flask, heat the sample to boiling, and boil gently for 2 min. Turn off and lower the mantle, and allow the sample to cool for 15 min. Charge the delivery tube, G, with 23 ml of concentrated hydrochloric acid. Disconnect the absorption tower, L, rapidly transfer 25.0 ml of 0.25 N sodium hydroxide into the tower, add 5 drops of n-butanol, and again connect the absorption tower. Draw carbon dioxide-free air through the apparatus at the rate of about 2,000 ml per h, add the hydrochloric acid to the reaction flask through the delivery tube, raise the heating mantle, and heat the reaction mixture to boiling.

After 2 h, discontinue the current of air and heating. Force the sodium hydroxide solution down into the flask, K, using gentle air pressure, and then rinse down the absorption tower with three 15-ml portions of water, forcing each washing into the flask with air pressure. Remove the flask, and add to it 10 ml of a 10% solution of barium chloride ( $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ ). Stopper the flask, shake gently for about 2 min, add phenolphthalein TS, and titrate with 0.1 N hydrochloric acid. Perform a blank determination.

Each ml of 0.25 N sodium hydroxide consumed is equivalent to 5.5 mg of carbon dioxide ( $\text{CO}_2$ ).

**Cellulose Derivatives Assay**

(Ethoxyl and Methoxyl Group Determination )

Apparatus

The apparatus used for the ethoxyl and methoxyl determination is shown in Figure 2. The boiling flask A, is fitted with a capillary side-arm, B, for the introduction of carbon dioxide and is connected to a column, C, which serves to separate aqueous hydriodic acid from the more volatile ethyl or methyl iodide. The volatile iodide passes through an aqueous red phosphorus suspension in a scrubber trap, D, and is finally absorbed in the bromine acetic acid solution in an absorption tube, F. The carbon dioxide is introduced from a device arranged to minimize pressure fluctuations and connected to the apparatus by a small capillary containing a small cotton plug.

Procedure

Prepare the apparatus by placing in trap D, through the funnel K or tube F and the connecting side-arm, a volume sufficient to make trap D half-full of a suspension of about 60 mg of red phosphorus in 100 ml of water. Rinse the tube F and the side-arm with water into trap D. Dry carefully the absorption tube F and pour down the funnel K 7 ml of bromine acetic acid TS. Weigh 0.05 g of the sample, to the nearest 0.1 mg, in a tared gelatin capsule, and place it in the boiling flask along with a few glass beads or pieces of porous plate. Add 6 ml of



hydriodic acid TS and attach the flask to the condenser, using a few drops of the acid to seal the junction. Bubble carbon dioxide through the apparatus at the rate of about 2 bubbles per sec. Place the boiling flask in an oil bath heated to  $150^{\circ}$ , and continue the reaction for 40 min. Drain the contents of the absorption tube F into a 500 ml conical flask containing 10 ml of a 1 in 4 solution of sodium acetate. Rinse tube F with water, adding the rinsings to the flask, and finally dilute with water to about 125 ml. Add formic acid, dropwise, with swirling, until the reddish-brown colour of the bromine is discharged, then add 3 additional drops. A total of 12 to 15 drops are usually required. Let stand for 3 min, and add 15 ml of dilute sulfuric acid TS and 3 g of potassium iodide, and titrate immediately with 0.1 N sodium thiosulfate, using starch TS as indicator near the endpoint. Perform a blank determination, including also a gelatin capsule and make any necessary correction.

Each ml of 0.1 N sodium thiosulfate is equivalent to 0.517 mg of  $(-\text{OCH}_3)$  or 0.751 mg of  $(-\text{OC}_2\text{H}_5)$ .

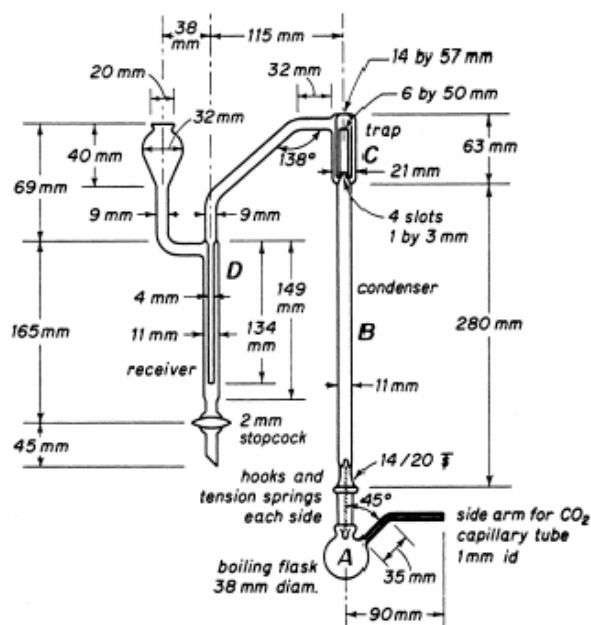


Figure 2. Apparatus for determination of ethoxyl and methoxyl groups

### Polysorbates Assay

(Oxyethylene Group Determination)

**Caution:** Use a safety shield and conduct the distillation in a hood.

#### Principle

The oxyethylene groups are converted to ethylene and ethyl iodide which can be determined by titration. By utilizing a conversion factor determined on a reference sample, it is possible to compute the polyoxyethylene ester content.

#### Apparatus

An arrangement of apparatus for the analysis is shown in Figure 3. It consists in part of the reaction flasks (A), condenser, trap (B), and first absorption tube (C) of a Clark alkoxyl apparatus. These are followed by an absorption tube (D) made from a section of a spiral from a Widmer distillation column and a standard-taper (24/40) gas inlet adapter. Dimensions of

the apparatus not readily determined from the diagram are as follows: carbon dioxide inlet, capillary, 1-mm inside diameter; flask A, 28-mm diameter, 12/18 standard-taper joint; condenser, 9-mm inside diameter; inlet to trap B, 2-mm inside diameter tube; inlet to trap C, 7/15 standard-taper joint, 2-mm inside diameter tube; trap C, 14-mm inside diameter; trap D, inner tube, 8-mm outside diameter, 2-mm opening at bottom of spiral; spiral, 1.75-mm rod, 23 turns, 8.5 rise per turn; trap D, outer tube, approximately 12.5-mm inside diameter, with side-arm 7 cm from top of spiral; side-arm, 3.5-mm inside diameter, 2 mm opening at bottom. The stopcock is lubricated with silicone grease. The absorption tubes may be conveniently suspended by a series of properly spaced sheet-metal clips attached to a stick clamped at an angle of about 60°.

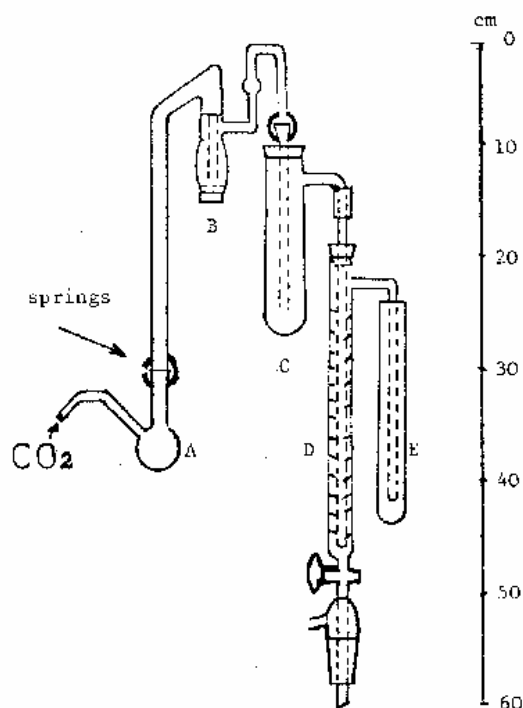


Figure 3. Apparatus for determination of oxyethylene groups

#### Procedure

Fill trap B with a suspension of a small amount of red phosphorus in enough water to cover the inlet tube. Pipet 10 ml of acid silver nitrate TS into tube C, pipet 15 ml of bromine-bromide TS into tube D, and place 10 ml of a 10% potassium iodide solution in trap E. Place about 0.05 g of the sample, accurately weighed, in the reaction flask A, together with a Hengar boiling granule and 10 ml of hydriodic acid TS. Connect the flask to the apparatus, pass a slow stream of carbon dioxide through (about 1 bubble per sec), and heat the flask slowly in an oil bath to 140-145°.

Maintain the flask at this temperature for at least 40 min, until there is no longer any cloudy reflux in the condenser above the reaction flask, and until the supernatant liquid in the silver nitrate trap C has clarified almost completely. Five min before the completion of the reaction, heat the silver nitrate trap C to 50-60° in a hot water bath to drive out any dissolved olefin.

On completion of the decomposition, disconnect tubes D and C cautiously in that order. Then disconnect the carbon dioxide source and remove the oil bath from flask A. Connect the spiral absorption tube, D, by its lower adapter to a 500-ml iodine-titration flask containing 10 ml of 10% potassium iodide solution and 150 ml of water. Remove the potassium iodide tube, E, and rinse the side-arm into it. Allow the bromine solution to run into the titration flask through the stopcock and rinse the tube and spiral with a few ml of water. Add the contents of the potassium iodide tube to the titration flask, stopper and allow to stand 5 min. Add 5 ml of dilute sulfuric acid TS and titrate at once with 0.05 N sodium thiosulfate, using 2 ml of starch TS as indicator.

Rinse the contents of the silver nitrate trap C into a flask, dilute to 150 ml with water, heat to boiling, cool to room temperature, and titrate with 0.05 N ammonium thiocyanate, using 3 ml of ferric ammonium sulfate TS as indicator.

Perform a blank determination omitting the sample.

#### Calculation

The volumes of sodium thiosulfate solution (S ml) of normality N and ammonium thiocyanate solution (S' ml) of normality N' used to titrate the contents of the bromine and silver nitrate traps are subtracted from the corresponding blank titrations (B and B' ml, respectively) and the following calculations made:

$$\% \text{C}_2\text{H}_4\text{O} = [(B - S) \times N \times 2.2] / \text{wt. of sample in g}$$

$$\% \text{C}_2\text{H}_4\text{O} = [(B' - S') \times N' \times 4.4] / \text{wt. of sample in g}$$

The sum of the values obtained from these calculations represents the total oxyethylene content of the sample. The % of polyoxyethylene ester can be estimated from the ratio of the % of oxyethylene in the unknown sample to that in a reference sample of known purity.

## MEDIA, REAGENTS AND SOLUTIONS

### MEDIA

The following media are used in the Microbiological Analyses Section of this volume and are referenced in that Section. The listing is alphabetical.

#### **Baird-Parker Medium**

Tryptone: 10 g  
Beef extract: 5 g  
Yeast extract: 1 g  
Sodium pyruvate: 10 g  
Glycine: 12 g  
Lithium chloride 6H<sub>2</sub>O: 5 g  
Agar: 20 g  
Distilled water: 1 litre

Autoclave 15 min at 121°. Final pH is 7.0 ± 0.2. If desired for immediate use, maintain melted medium at 48-50° before adding enrichment. Otherwise, store solidified medium at 4 ± 1° up to one month. Melt medium before use.

#### **Bismuth Sulfite Agar (Wilson and Blair)**

Polypeptone (or peptone): 10 g  
Beef extract: 5 g  
Dextrose: 5 g  
Na<sub>2</sub>HPO<sub>4</sub> (anhydrous): 4 g  
FeSO<sub>4</sub> (anhydrous): 0.3 g  
Bismuth sulfite (indicator): 8 g  
Brilliant green: 0.025 g  
Agar: 20 g  
Distilled water: 1 litre

Mix thoroughly and heat with agitation. Boil about 1 min to obtain uniform suspension. (Precipitate will not dissolve.) Cool to 45-50°. Suspend precipitate by gentle agitation, and pour 20 ml portions into sterile 15 x 100 mm petri dishes. Let plates dry about 2 h with lids partially removed; then close plates. Final pH, 7.7 ± 0.2. Do not autoclave. Prepare plates on day before streaking and store in dark. Selectivity decreases in 48 h. Store plates in dark.

#### **Brain Heart Infusion (BHI) Broth and Agar**

##### *Medium 1*

Calf brain infusion: 200 g  
Beef heart infusion: 250 g  
Proteose peptone or gelysate: 10 g  
NaCl: 5 g  
Na<sub>2</sub>HPO<sub>4</sub>·12 H<sub>2</sub>O: 2.5 g  
Dextrose: 2 g  
Distilled water: 1 litre

Dissolve ingredients in distilled water with gentle heat. Dispense broth into bottles or tubes for storage. Autoclave 15 min at 121°. Final pH,  $7.4 \pm 0.2$ .

*Medium 2*

Brain heart infusion : 6 g  
 Peptic digest of animal tissue: 6 g  
 NaCl: 5 g  
 Dextrose: 3 g  
 Pancreatic digest of gelatine: 14.5 g  
 Na<sub>2</sub>HPO<sub>4</sub>: 2.5 g  
 Distilled water: 1 litre

Suspend ingredients of *Medium 2* in distilled water and boil for 1 min to completely dissolve.

For both *Medium 1* and *Medium 2*, dispense broth into bottles or tubes for storage. Autoclave 15 min at 121°. Final pH,  $7.4 \pm 0.2$ . Commercially available BHI is acceptable.

To prepare brain heart infusion agar, add 15 g agar to 1 litre BHI broth. Heat to dissolve agar before dispensing into bottles or flasks. Autoclave 15 min at 121°.

**Brilliant Green Lactose Bile Broth**

Peptone: 10 g  
 Lactose: 10 g  
 Oxgall: 20 g  
 Brilliant green: 0.0133 g  
 Distilled water: 1 litre

Dissolve peptone and lactose in 500 ml distilled water. Add 20 g dehydrated oxgall dissolved in 200 ml distilled water. The pH of this solution should be 7.0-7.5. Mix and add water to make 975 ml. Adjust pH to 7.4. Add 13.3 ml 0.1% aqueous brilliant green in distilled water. Add distilled water to make 1 litre. Dispense into fermentation tubes, making certain that fluid level covers inverted vials. Autoclave 15 min at 121°. Final pH,  $7.2 \pm 0.1$ .

**Buffered Peptone Water (BPW)**

Peptone: 10g  
 NaCl: 5 g  
 Na<sub>2</sub>HPO<sub>4</sub>: 3.5 g  
 KH<sub>2</sub>PO<sub>4</sub>: 1.5 g  
 Distilled water: 1 litre

Autoclave at 121° for 15 min. Final pH  $7.2 \pm 0.2$ .

**Dichloran 18% Glycerol (DG18) Agar**

Glucose: 10 g  
 Bacteriological peptone: 5 g  
 KH<sub>2</sub>PO<sub>4</sub>: 1 g  
 MgSO<sub>4</sub>·7H<sub>2</sub>O: 0.5 g  
 Dichloran (0.2% in ethanol, w/v): 1 ml  
 Chloramphenicol: 0.1 g  
 Agar: 15 g  
 Distilled water: 1 litre

Mix above items and steam to dissolve agar, then bring volume to 1000 ml with distilled water. Add 220 g glycerol and sterilize by autoclaving at 121° for 15 min. The final pH should be 5.6 and the final  $a_w$ , 0.955.

This medium is used as a general purpose mould enumeration medium and is preferred when the  $a_w$  of the analyzed food is 0.95 or lower. The low water activity of this medium reduces interference by bacteria and fast-growing fungi. When both yeasts and moulds must be enumerated, DRBC agar should be used (see next media).

#### **Dichloran Rose Bengal Chloramphenicol (DRBC) Agar**

Glucose: 10 g  
 Bacteriological peptone: 5 g  
 KH<sub>2</sub>PO<sub>4</sub>: 1 g  
 MgSO<sub>4</sub>·7H<sub>2</sub>O: 0.5 g  
 Rose bengal (5% soln., w/v): 0.5 ml  
 Dichloran (2,6-dichloro-4-nitroaniline) solution (0.2%(w/v) in ethanol): 1 ml  
 Chloramphenicol: 0.1 g  
 Agar: 15 g  
 Distilled water: 1 litre

Final pH should be 5.6. Mix ingredients, heat to dissolve agar and sterilize by autoclaving at 121° for 15 min. Temper to 45 ± 1° in a water bath and pour plates.

#### **EC Broth**

Trypticase or tryptose: 20 g  
 Bile salts No. 3: 1.5 g  
 Lactose: 5 g  
 K<sub>2</sub>HPO<sub>4</sub>: 4 g  
 KH<sub>2</sub>PO<sub>4</sub>: 1.5 g  
 NaCl: 5 g  
 Distilled water: 1 litre

Distribute 8 ml portions to 16 x 150 mm test tubes containing inverted 10 x 75 mm fermentation tubes. Autoclave 15 min at 121°. Final pH 6.9 ± 0.2.

#### **Hektoen Enteric (HE) Agar**

Peptone: 12 g  
 Sodium thiosulfate: 5 g  
 Yeast extract: 3 g  
 Ferric ammonium citrate: 1.5 g  
 Bile salts: 9 g  
 Bromthymol blue: 0.064 g  
 Lactose: 12 g  
 Acid fuchsin: 0.1 g  
 Sucrose: 12 g  
 Agar: 13.5 g  
 Salicin: 2 g  
 Distilled water: 1 litre  
 NaCl: 5 g

Heat to boiling with frequent agitation to dissolve. Boil no longer than 1 min. Do not overheat. Cool in water bath. Pour 20 ml portions into sterile 15 x 100 mm petri dishes. Let dry 2 h with lids partially removed. Final pH,  $7.6 \pm 0.2$ . Do not store more than 1 day.

### **Koser's Citrate Broth**

NaNH<sub>4</sub>HPO<sub>4</sub>·4H<sub>2</sub>O: 1.5 g  
 K<sub>2</sub>HPO<sub>4</sub>: 1 g  
 MgSO<sub>4</sub>·7H<sub>2</sub>O: 0.2 g  
 Sodium citrate·2H<sub>2</sub>O: 3 g  
 Distilled water: 1 litre

Dispense into screw-cap tubes as desired. Autoclave 15 min at 121°. Final pH,  $6.2 \pm 0.2$ . This formulation is listed in *Official Methods of Analysis* of the AOAC and *Standard Methods for the Examination of Water and Wastewater* of the APHA. It differs from the composition of commercially available dehydrated media. The latter have been found to be satisfactory.

### **Lactose Broth**

Beef extract: 3 g  
 Peptone: 5 g  
 Lactose: 5 g  
 Distilled water: 1 litre

- For *E. coli*: Dissolve ingredients and dispense 10 ml portions into 20 x 150 mm tubes containing inverted 10 x 75 mm fermentation vials. Autoclave 15 min at 121°. Final pH  $6.9 \pm 0.2$ .
- For *Salmonella*: Dispense 225 ml portions into 500 ml Erlenmeyer flasks. After autoclaving 15 min at 121° and just before use, aseptically adjust volume to 225 ml. Final pH,  $6.9 \pm 0.2$ .

### **Lauryl Tryptose (LST) Broth**

Tryptose or trypticase: 20 g  
 Lactose: 5 g  
 K<sub>2</sub>HPO<sub>4</sub>: 2.75 g  
 KH<sub>2</sub>PO<sub>4</sub>: 2.75 g  
 NaCl: 5 g  
 Sodium lauryl sulfate: 0.1 g  
 Distilled water: 1 litre

Dispense 10 ml portions to 20 x 150 mm tubes containing inverted 10 x 75 mm fermentation tubes. Autoclave 15 min at 121°. Final pH,  $6.8 \pm 0.2$ .

### **Levine's Eosin-Methylene Blue (L-EMB) Agar**

Peptone: 10 g  
 Lactose: 10 g  
 K<sub>2</sub>HPO<sub>4</sub>: 2 g  
 Agar: 15 g  
 Eosin Y: 0.4 g  
 Methylene blue: 0.065 g  
 Distilled water: 1 litre

Boil to dissolve peptone, phosphate, and agar in 1 liter of water. Add water to make original volume. Dispense in 100 or 200 ml portions and autoclave 15 min at not over 121°. Final pH,  $7.1 \pm 0.2$ .

Before use, melt, and to each 100 ml portion add:

- a. 5 ml sterile 20% lactose solution;
- b. 2 ml aqueous 2% eosin Y solution; and
- c. 4.3 ml 0.15% aqueous methylene blue solution.

When using complete dehydrated product, boil to dissolve all ingredients in 1 liter water. Dispense in 100 or 200 ml portions and autoclave 15 min at 121°. Final pH,  $7.1 \pm 0.2$ .

#### **Lysine Decarboxylase Broth (Falkow) (for Salmonella)**

Gelysate or peptone: 5 g

Yeast extract: 3 g

Glucose: 1 g

L-Lysine: 5 g

Bromcresol purple: 0.02 g

Distilled water: 1 litre

Heat until dissolved. Dispense 5 ml portions into 16 x 125 mm screw-cap tubes. Autoclave loosely capped tubes 15 min at 121°. Screw the caps on tightly for storage and after inoculation. Final pH,  $6.8 \pm 0.2$ .

#### **Lysine Iron Agar (Edwards and Fife)**

Gelysate or peptone: 5 g

Yeast extract: 3 g

Dextrose: 1 g

L-Lysine hydrochloride: 10 g

Ferric ammonium citrate: 0.5 g

Sodium thiosulfate (anhydrous): 0.04 g

Bromcresol purple: 0.02 g

Agar: 15 g

Distilled water: 1 litre

Heat to dissolve ingredients. Dispense 4 ml portions into 13 x 100 mm screw-cap tubes. Autoclave 12 min at 121°. Let solidify in slanted position to form 4 cm butts and 2.5 cm slants. Final pH,  $6.7 \pm 0.2$ .

#### **MacConkey Agar**

Proteose peptone or polypeptone: 3 g

Peptone or gelysate: 17 g

Lactose: 10 g

Bile salts No. 3 (or bile salts mixture): 1.5 g

NaCl: 5 g

Neutral red: 0.03 g

Crystal violet: 0.001 g

Agar: 13.5 g

Distilled water: 1 litre



Suspend ingredients and heat with agitation to dissolve. Boil 1-2 min. Autoclave 15 min at 121°, cool to 45-50°, and pour 20 ml portions into sterile 15 x 100 mm petri dishes. Dry at room temperature with lids closed. Do not use wet plates. Final pH,  $7.1 \pm 0.2$ .

### **Malonate Broth**

Yeast extract: 1 g  
(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>: 2 g  
K<sub>2</sub>HPO<sub>4</sub>: 0.6 g  
KH<sub>2</sub>PO<sub>4</sub>: 0.4 g  
NaCl: 2 g  
Sodium malonate: 3 g  
Dextrose: 0.25 g  
Bromthymol blue: 0.025 g  
Distilled water: 1 litre

Dissolve by heating, if necessary. Dispense 3 ml portions into 13 x 100 mm test tubes. Autoclave 15 min at 121°. Final pH,  $6.7 \pm 0.2$ .

### **Malt Agar**

Malt extract, powdered: 20 g  
Agar: 20 g  
Distilled water: 1 litre

Mix ingredients, steam to dissolve agar and sterilize for 15 min at 121°. Temper medium to 45° and pour plates under aseptic conditions.

### **Malt Extract Agar**

Malt extract: 30 g  
Agar: 20 g  
Distilled water: 1 litre

Boil to dissolve ingredients. Autoclave 15 min at 121°. Dispense 20-25 ml into sterile 15 x 100 mm petri dishes. Final pH,  $5.5 \pm 0.2$ .

### **MR-VP Broth**

#### *Medium 1*

Buffered peptone-water powder: 7 g  
Glucose: 5 g  
K<sub>2</sub>HPO<sub>4</sub>: 5 g  
Distilled water: 1 litre

Dissolve ingredients in 800 ml water with gentle heat. Filter, cool to 20°, and dilute to 1 litre. Autoclave 12-15 min at 121°. Final pH,  $6.9 \pm 0.2$ .

#### *Medium 2*

Pancreatic digest of casein: 3.5 g  
Peptic digest of animal tissue: 3.5 g  
Dextrose: 5 g  
Potassium phosphate: 5 g  
Distilled water: 1 litre

Dissolve ingredients in water with gentle heat if necessary. Dispense 10 ml into 16 x 150 mm test tubes and autoclave 15 min at 118-121°. Final pH,  $6.9 \pm 0.2$ .

*Medium 3*

Peptone: 5 g  
 Glucose: 5 g  
 Phosphate buffer: 5 g  
 Distilled water: 1 litre

Dissolve ingredients in water. Dispense 10 ml into 16 x 150 mm test tubes and autoclave 15 min at 121°. Final pH,  $7.5 \pm 0.2$ .

*For Salmonella*: Dispense 10 ml into 16 x 150 mm test tubes, and autoclave 12-15 min at 121°.

**Motility Test Medium (semisolid)**

Beef extract: 3 g  
 Peptone or gelysate: 10 g  
 NaCl: 5 g  
 Agar: 4 g  
 Distilled water: 1 litre

Heat with agitation and boil 1-2 min to dissolve agar. Dispense 20 ml portions into 20 x 150 mm screw-cap tubes, replacing caps loosely. Autoclave 15 min at 121°. Cool to 45° after autoclaving. Tighten caps, and refrigerate at 5-8°. To use, re-melt in boiling water or flowing steam, and cool to 45°. Aseptically dispense 20 ml portions into sterile 15 x 100 mm petri plates. Cover plates and let solidify. Use same day as prepared. Final pH,  $7.4 \pm 0.2$ .

**Nutrient Broth**

Beef extract: 3 g  
 Peptone: 5 g  
 Distilled water: 1 litre

Heat to dissolve. Dispense 10 ml portions into tubes or 225 ml portions into 500 ml Erlenmeyer flasks. Autoclave 15 min at 121°. Final pH,  $6.8 \pm 0.2$ .

**Phenol Red Carbohydrate Broth**

Trypticase or proteose peptone No. 3: 10 g  
 NaCl: 5 g  
 Beef extract (optional): 1 g  
 Phenol red (7.2 ml of 0.25% phenol red solution): 0.018 g  
 Distilled water: 1 litre  
 Carbohydrate\*

\*Dissolve either 5 g dulcitol, 10 g lactose, or 10 g sucrose (as specified in the *Salmonella* test) in this basal broth. Dispense 2.5 ml portions into 13 x 100 mm test tubes containing inverted 6 x 50 mm fermentation tubes. Autoclave 10 min at 118°. Final pH,  $7.4 \pm 0.2$ . Alternatively, dissolve ingredients, omitting carbohydrate, in 800 ml distilled water with heat and occasional agitation. Dispense 2.0 ml portions into 13 x 100 mm test tubes containing inverted fermentation tubes. Autoclave 15 min at 118° and let cool. Dissolve carbohydrate in 200 ml distilled water and sterilize by passing solution through bacteria-retaining filter. Aseptically add 0.5 ml sterile filtrate to each

tube of sterilized broth after cooling to less than 45°. Shake gently to mix. Final pH, 7.4 ± 0.2.

*For viable yeasts and moulds:* Dispense 20-25 ml portions into sterile 15 x 100 mm petri dishes.

#### **Plate Count Agar (Standard Methods)**

Tryptone: 5 g  
Yeast extract: 2.5 g  
Dextrose: 1 g  
Agar: 15 g  
Distilled water: 1 litre

Heat to dissolve ingredients. Dispense into suitable tubes or flasks. Autoclave 15 min at 121°. Final pH 7.0 ± 0.2.

#### **Potassium Cyanide (KCN) Broth**

Potassium cyanide: 0.075 g  
Proteose peptone No. 3 or polypeptone: 3 g  
NaCl: 5 g  
KH<sub>2</sub>PO<sub>4</sub>: 0.225 g  
Na<sub>2</sub>HPO<sub>4</sub>: 5.64 g  
Distilled water: 1 litre

Dissolve above ingredients **except potassium cyanide** and autoclave 15 min at 121°. Cool and refrigerate at 5-8°. Final pH, 7.6 ± 0.2. Prepare KCN stock solution by dissolving 0.5 g KCN in 100 ml sterile distilled water cooled to 5-8°C. **Using bulb pipetter**, add 15 ml cold KCN stock solution to 1 litre cold, sterile base. **DO NOT PIPET BY MOUTH. Handle with gloves.**

Mix and aseptically dispense 1.0-1.5 ml portions to 13 x 100 mm sterile tubes. Using aseptic technique, stopper tubes with No. 2 corks impregnated with paraffin. Prepare corks by boiling in paraffin about 5 min. Place corks in tubes so that paraffin does not flow into broth but forms a seal between rim of tubes and cork. Store tubes at 5-8° **no longer than 2 weeks before use.**

#### **Potato Dextrose Agar**

Potato infusion: 200 ml  
Dextrose: 20 g  
Agar: 20 g  
Distilled water: 1 litre

To prepare potato infusion, boil 200 g sliced, unpeeled potatoes in 1 litre distilled water for 30 min. Filter through cheesecloth, saving effluent, which is potato infusion. Mix in other ingredients and boil to dissolve. Autoclave 15 min at 121°. Dispense 20-25 ml portions into sterile 15 x 100 mm petri dishes. Final pH, 5.6 ± 0.2. Medium should not be re-melted more than once.

*For potato dextrose salt agar,* prepare potato dextrose agar, as above, and add 75 g NaCl per litre.

**Purple Carbohydrate Broth**

Proteose peptone No. 3 : 10 g  
 Beef extract (optional): 1 g  
 NaCl: 5 g  
 Bromcresol purple: 0.02 g  
 Distilled water: 1 litre

Prepare as for phenol red carbohydrate broth (M109). Final pH,  $6.8 \pm 0.2$ .

**Rappaport-Vassiliadis Medium**

Tryptone: 5 g  
 NaCl: 8 g  
 KH<sub>2</sub>PO<sub>4</sub>: 1.6 g  
 Distilled water: 1 litre

*Magnesium chloride solution*

MgCl<sub>2</sub>·6H<sub>2</sub>O: 400 g  
 Distilled water: 1 litre

*Malachite green oxalate solution*

Malachite green oxalate: 0.4 g  
 Distilled water: 100 ml

To prepare the complete medium, combine 1000 ml broth base, 100 ml *magnesium chloride solution*, and 10 ml *malachite green oxalate solution* (total volume of complete medium is 1110 ml). Broth base must be prepared on same day that components are combined to make complete medium. Magnesium chloride solution may be stored in dark bottle at room temperature up to 1 year. To prepare solution, dissolve entire contents of MgCl<sub>2</sub>·6H<sub>2</sub>O from newly opened container according to formula, because this salt is very hygroscopic. Malachite green oxalate solution may be stored in dark bottle at room temperature up to 6 months. Merck analytically pure malachite green oxalate is recommended because other brands may not be equally effective. Dispense 10 ml volumes of complete medium into 16 x 150 mm test tubes. Autoclave 15 min at 115°. Final pH,  $5.5 \pm 0.2$ . Store in refrigerator and **use within 1 month**.

This medium must be made from its individual ingredients. Use of commercially available dehydrated media is not recommended. Users of this medium should be aware that there are formulations and incubation temperatures for this medium other than those recommended in this volume.

**Selenite Cystine Broth***Medium 1*

Tryptone or polypeptone: 5 g  
 Lactose: 4 g  
 Sodium selenite (NaHSeO<sub>3</sub>): 4 g  
 Na<sub>2</sub>HPO<sub>4</sub>: 10 g  
 L-Cystine: 0.01 g  
 Distilled water: 1 litre

Heat to boiling to dissolve. Dispense 10 ml portions into sterile 16 x 150 mm test tubes. Heat 10 min in flowing steam. Do not autoclave. Final pH,  $7.0 \pm 0.2$ . The medium is not sterile. Use same day as prepared.

*Medium 2 (North-Bartram modification)*

Polypeptone: 5 g  
Lactose: 4 g  
Sodium selenite ( $\text{NaHSeO}_3$ ): 4 g  
 $\text{Na}_2\text{HPO}_4$ : 5.5 g  
 $\text{KH}_2\text{PO}_4$ : 4.5 g  
L-Cystine: 0.01 g  
Distilled water : 1 litre

Heat with agitation to dissolve. Dispense 10 ml portions to sterile 16 x 150 mm test tubes. Heat 10 min in flowing steam. Do not autoclave. Use same day as prepared.

**Simmons Citrate Agar**

Sodium citrate· $2\text{H}_2\text{O}$ : 2 g  
NaCl: 5 g  
 $\text{K}_2\text{HPO}_4$ : 1 g  
 $\text{NH}_4\text{H}_2\text{PO}_4$ : 1 g  
 $\text{MgSO}_4$ : 0.2 g  
Bromthymol blue: 0.08 g  
Agar: 15 g  
Distilled water: 1 litre

Heat gently with occasional agitation. Boil 1-2 min until agar dissolves. Fill 13 x 100 or 16 x 150 mm screw-cap tubes 1/3 full. Autoclave 15 min at  $121^\circ$ . Before medium solidifies, incline tubes to obtain 4-5 cm slants and 2-3 cm butts. Final pH,  $6.8 \pm 0.2$ .

**Tetrathionate Broth**

Polypeptone: 5 g  
Bile salts: 1 g  
Calcium carbonate: 10 g  
Sodium thiosulfate· $5\text{H}_2\text{O}$ : 30 g  
Distilled water: 1 litre

Suspend ingredients in 1 litre distilled water, mix, and heat to boiling. (Precipitate will not dissolve completely.) Do not autoclave. Cool to less than  $45^\circ$ . Store at  $5-8^\circ$ . Final pH,  $8.4 \pm 0.2$ .

**Toluidine Blue – DNA Agar**

Deoxyribonucleic acid (DNA): 0.3 g  
Agar: 10 g  
Calcium chloride (anhydrous): 1.1 mg  
NaCl: 10 g  
Toluidine blue O: 83 mg  
Tris(hydroxymethyl)aminomethane: 6.1 g  
Distilled water: 1 litre

Dissolve the Tris(hydroxymethyl)aminomethane in 1 litre distilled water. Adjust the pH to 9.0. Add the remaining ingredients except the toluidine blue O and heat to boiling to dissolve.

Dissolve toluidine blue O in the medium. Dispense to rubber-stopped flasks. Sterilization is not necessary if used immediately. The sterile medium is stable at room temperature for 4 months and is satisfactory after several melting cycles.

### **Triple Sugar Iron (TSI) Agar**

Medium 1		Medium 2	
Polypeptone	20 g	Beef extract	3 g
NaCl	5 g	Yeast extract	3 g
Lactose	10 g	Peptone	15 g
Sucrose	10 g	Proteose peptone	5 g
Glucose	1 g	Glucose	1 g
Fe(NH <sub>4</sub> ) <sub>2</sub> (SO <sub>4</sub> )·6H <sub>2</sub> O	0.2 g	Lactose	10 g
Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub>	0.2 g	Sucrose	10 g
Phenol red	0.025 g	FeSO <sub>4</sub>	0.2 g
Agar	13 g	NaCl	5 g
Distilled water	1 litre	Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub>	0.3 g
		Phenol red	0.024 g
		Agar	12 g
		Distilled water	1 litre

These two media are interchangeable for general use. For use with *V. parahaemolyticus*, add 25 g NaCl per litre to either formula.

Suspend ingredients of Medium 1 in distilled water, mix thoroughly, and heat with occasional agitation. Boil about 1 min to dissolve ingredients. Fill 16 x 150 mm tubes 1/3 full and cap or plug to maintain aerobic conditions. Autoclave Medium 1 for 15 min at 118°. Prepare Medium 2 in the same manner as Medium 1, except autoclave 15 min at 121°. Before the media solidify, incline tubes to obtain 4-5 cm slant and 2-3 cm butt. Final pH, 7.3 ± 0.2 for Medium 1 and 7.4 ± 0.2 for Medium 2.

### **Trypticase (Tryptic) Soy Agar**

Trypticase peptone: 15 g  
 Phytone peptone: 5 g  
 NaCl: 5 g  
 Agar: 15 g  
 Distilled water: 1 litre

Heat with agitation to dissolve agar. Boil 1 min. Dispense into suitable tubes or flasks. Autoclave 15 min at 121°. Final pH, 7.3 ± 0.2.

### **Trypticase (Tryptic) Soy Broth**

Trypticase peptone: 17 g  
 Phytone peptone: 3 g  
 NaCl: 5 g

K<sub>2</sub>HPO<sub>4</sub>: 2.5 g  
 Glucose: 2.5 g  
 Distilled water: 1 litre

Heat with gentle agitation to dissolve. Dispense 225 ml into 500 ml Erlenmeyer flasks. Autoclave 15 min at 121°C. Final pH, 7.3 ± 0.2. For trypticase soy broth without glucose, prepare as above, but omit 2.5 g glucose.

#### **Trypticase (Tryptic) Soy Broth containing 10% NaCl and 1% Na pyruvate**

Trypticase or tryptose (pancreatic digest of casein): 17 g  
 Phytone peptone (pancreatic or enzymatic digest of casein): 3 g  
 NaCl: 100 g  
 K<sub>2</sub>HPO<sub>4</sub>: 2.5 g  
 Dextrose: 2.5 g  
 Na pyruvate: 10 g  
 Distilled water: 1 litre

Adjust to pH7.3. Heat gently if necessary. Dispense 10 ml into 16 x 150 mm tubes. Autoclave 15 min at 121°. Final pH, 7.3 ± 0.2. Store up to one month at 4 ± 1°.

#### **Trypticase (Tryptic) Soy Broth with ferrous sulphate**

Trypticase peptone: 17 g  
 Phytone peptone: 3 g  
 NaCl: 5 g  
 K<sub>2</sub>HPO<sub>4</sub>: 2.5 g  
 Glucose: 2.5 g  
 Ferrous sulphate: 35 mg  
 Distilled water: 1 litre

Heat with gentle agitation to dissolve. Dispense 225 ml into 500 ml Erlenmeyer flasks. Autoclave 15 min at 121°. Final pH, 7.3 ± 0.2.

#### **Trypticase Soy-Tryptose Broth**

Trypticase soy broth (commercial, dehydrated): 15 g  
 Tryptose broth (commercial, dehydrated): 13.5 g  
 Yeast extract: 3 g  
 Distilled water: 1 litre

Dissolve ingredients in 1 litre water. Heat gently to dissolve. Dispense 5 ml portions into 16 x 150 mm test tubes. Autoclave 15 min at 121°. Final pH, 7.2 ± 0.2.

#### **Tryptone Yeast Extract Agar**

Tryptone: 10 g  
 Yeast extract: 1 g  
 Carbohydrate: 10 g (glucose and mannitol are the carbohydrates used for identification of *Staphylococcus aureus*)  
 Bromcresol purple: 0.04 g  
 Agar: 2 g  
 Distilled water: 1 litre

Dissolve agar with heat and gentle agitation. Adjust pH to  $7.0 \pm 0.2$ . Fill 16 x 125 mm tubes 2/3 full. Autoclave 20 min at  $115^\circ$ . Before use, steam medium 10-15 min. Solidify by placing tubes in ice water.

### **Tryptone (Tryptophane) Broth, 1%**

Tryptone or trypticase: 10 g  
Distilled water: 1 litre

Dissolve and dispense 5 ml portions into 16 x 125 or 16 x 150 mm test tubes. Autoclave 15 min at  $121^\circ$ . Final pH,  $6.9 \pm 0.2$ . For use with *V. parahaemolyticus*, add 30 g NaCl.

### **Tryptose Blood Agar Base**

Tryptose: 10 g  
Beef extract: 3 g  
NaCl: 5 g  
Agar: 15 g  
Distilled water: 1 litre

Suspend ingredients in distilled water, mix thoroughly, and heat with occasional agitation. Boil about 1 min. Fill 16 x 150 mm tubes 1/3 full and cap or plug to maintain aerobic conditions. Autoclave 15 min at  $121^\circ$ . Before media solidify, incline tubes to obtain 4-5 cm slant and 2-3 cm butt.

### **Universal Preenrichment Broth**

Tryptone: 5 g  
Protease peptone: 5 g  
 $\text{KH}_2\text{PO}_4$ : 15 g  
 $\text{Na}_2\text{HPO}_4$ : 7 g  
NaCl: 5 g  
Dextrose: 0.5 g  
 $\text{MgSO}_4$ : 0.25 g  
Ferric ammonium sulphate: 0.1 g  
Sodium pyruvate: 0.2 g  
Distilled water: 1 litre

Heat with gentle agitation to dissolve. Autoclave 15 min at  $121^\circ$ . Final pH,  $6.3 \pm 0.2$ .

### **Urea Broth**

Urea: 20 g  
Yeast extract: 0.1 g  
 $\text{K}_2\text{HPO}_4$ : 9.1 g  
 $\text{Na}_2\text{HPO}_4$ : 9.5 g  
Phenol red: 0.01 g  
Distilled water: 1 litre

Dissolve ingredients in distilled water. Do not heat. Sterilize by filtration through 0.45  $\mu\text{m}$  membrane. Aseptically dispense 1.5-3.0 ml portions to 13 x 100 mm sterile test tubes. Final pH,  $6.8 \pm 0.2$ .

### **Urea Broth (Rapid)**

Urea: 20 g



Yeast extract: 0.1 g  
KH<sub>2</sub>PO<sub>4</sub>: 0.091 g  
Na<sub>2</sub>HPO<sub>4</sub>: 0.095 g  
Phenol red: 0.01 g  
Distilled water: 1 litre

Prepare as for urea broth, above.

#### **Violet Red Bile Agar (VRBA)**

Yeast extract: 3 g  
Peptone or gelysate: 7 g  
NaCl: 5 g  
Bile salts or bile salts No. 3: 1.5 g  
Lactose: 10 g  
Neutral red: 30 mg  
Crystal violet: 2 mg  
Agar: 15 g  
Distilled water: 1 litre

Suspend ingredients in distilled water and let stand for a few min. Mix thoroughly and adjust to pH  $7.4 \pm 0.2$ . Heat with agitation and boil for 2 min. Do not sterilize. Before use, cool to 45° and use as a plating medium. After solidification, add a cover layer above the agar of approximately 3.0 to 4.0 ml to prevent surface growth and spreading of colonies.

#### **Xylose Lysine Desoxycholate (XLD) Agar**

Yeast extract: 3 g  
Ferric ammonium citrate: 0.8 g  
L-lysine: 5 g  
Sodium thiosulfate: 6.8 g  
Xylose: 3.75 g  
NaCl: 5 g  
Lactose: 7.5 g  
Agar: 15 g  
Sucrose: 7.5 g  
Phenol red : 0.08 g  
Sodium desoxycholate: 2.5 g  
Distilled water: 1 litre

Heat with agitation just until medium boils. Do not overheat. Pour into plates when medium has cooled to 50°. Let dry about 2 h with covers partially removed. Then close plates. Final pH,  $7.4 \pm 0.2$ . Do not store more than 1 day.

## REAGENTS

The following reagents are used in the Microbiological Analyses Section of this Volume and are referenced in that Section. The listing is alphabetical.

### **Brilliant green solution**

Brilliant green dye, sterile: 0.1 g  
Distilled water, sterile: 100 ml

On day of use, add 20 ml I<sub>2</sub>-KI solution and 10 ml brilliant green solution to 1 litre base. Re-suspend precipitate by gentle agitation and aseptically dispense 10 ml portions into 20 x 150 or 16 x 150 mm sterile test tubes. Do not heat medium after addition of I<sub>2</sub>-KI and dye solutions.

### **Bromcresol Purple Dye Solution (0.2%)**

Bromcresol purple dye: 0.2 g  
Sterile distilled water: 100 ml

### **Butterfield's Phosphate-Buffered Dilution Water**

KH<sub>2</sub>PO<sub>4</sub>: 34 g  
Distilled water: 500 ml

Adjust pH to 7.2 with 1 N NaOH. Bring volume to 1 liter with distilled water. Sterilize 15 min at 121°. Store in refrigerator.

#### *Dilution blanks*

Take 1.25 ml of above stock solution and bring volume to 1 litre with distilled water. Dispense into bottles to 90 or 99 ± 1 ml. Sterilize 15 min at 121°.

### **Cellulase Solution**

Dissolve 1 g cellulase in 99 ml sterile distilled water. Filter sterilize through a 0.45 µm filter. Cellulase solution may be stored at 2-5° for 2 weeks.

### **Chlorine Solution (200 ppm)**

Commercial bleach (5.25% sodium hypochlorite): 8 ml  
Distilled water containing 1 g sodium dodecyl sulphate: 992 ml

Dissolve 1 g sodium dodecyl sulfate in 992 ml distilled water. Add 8 ml commercial bleach and mix well. Make immediately before use.

### **Ethanol Solution (70%)**

Ethanol (95%): 700 ml  
Distilled water: add to final volume of 950 ml

### **Formalinized Physiological Saline Solution**

Formaldehyde solution (36-38%): 6 ml  
NaCl: 8.5 g  
Distilled water: 1 litre

Dissolve 8.5 g NaCl in 1 liter distilled water. Autoclave 15 min at 121°. Cool to room temperature. Add 6 ml formaldehyde solution. Do not autoclave after addition of formaldehyde.

### **Hydrochloride Solution (1 N)**

HCl (concentrated): 89 ml  
Distilled water to make 1 litre

### **Kovac's Reagent**

p-Dimethylaminobenzaldehyde: 5 g  
Amyl alcohol (normal only): 75 ml  
HCl (concentrated): 25 ml

Dissolve p-dimethylaminobenzaldehyde in normal amyl alcohol. Slowly add HCl. Store at 4°. To test for indole, add 0.2-0.3 ml reagent to 5 ml of 24 h bacteria culture in tryptone broth. Dark red colour in surface layer is positive test for indole. For enteropathogenic *E. coli*, also test at 72 h if negative at 24 h.

### **Lysostaphin Solution**

Dissolve 2.5 mg of lysostaphin in 0.02M phosphate-saline buffer containing 1 % NaCl.

### **Methyl Red Indicator**

Methyl red: 0.1 g  
Ethanol (95%): 300 ml  
Distilled water to make 500 ml

### **Nonfat dry milk**

Nonfat dry milk: 100g  
Distilled water: 1 litre

For *Salmonella*:

Suspend 100 g dehydrated nonfat dry milk in 1 liter distilled water. Swirl until dissolved. Autoclave 15 min at 121°.

### **Papain**

Papain: 5 g  
Distilled water: 1 litre

Add papain to sterile, distilled water and swirl to dissolve completely. Dispense 100 ml portion into bottles.

### **Physiological Saline Solution Sterile (0.85%)**

NaCl: 8.5 g  
Distilled water: 1 litre

Dissolve 8.5 g NaCl in water. Autoclave 15 min at 121°. Cool to room temperature.

### **Potassium Hydroxide Solution (40%)**

KOH: 40 g

Distilled water to make 100 ml

**Sodium Hydroxide Solution (1 N)**

NaOH: 40 g

Distilled water to make 1 litre

**Voges-Proskauer (VP) Test Reagents**

*Solution 1*

alpha-Naphthol: 5 g

Alcohol (absolute): 100 ml

*Solution 2*

Potassium hydroxide: 40 g

Distilled water to make 100 ml

Voges-Proskauer (VP) test. At room temperature, transfer 1 ml of 48 h culture to test tube and add 0.6 ml solution 1 and 0.2 ml solution 2. Shake after adding each solution. To intensify and speed reaction, add a few creatine crystals to mixture. Read results 4 h after adding reagents. Development of eosin pink colour is a positive.



## BUFFER SOLUTIONS

### Buffer Test Solutions

#### Buffer TS (pH 2)

Combine 11.90 ml of 0.2 M hydrochloric acid and 88.10 ml of 0.2 M potassium chloride, and dilute to 200 ml with water.

#### Buffer TS (pH 5)

Add 51.5 ml of 0.2 M disodium hydrogen phosphate to 48.5 ml of 0.1 M citric acid.

#### Buffer TS (pH 5.45)

Dissolve 1.8360 g of citric acid and 3.198 g of disodium hydrogen phosphate in carbon dioxide-free water to make 200 ml.

#### Buffer TS (pH 6.5)

Combine 50 ml of 0.2 M potassium dihydrogen phosphate and 15.2 ml of 0.2 M sodium hydroxide, and dilute to 200 ml with water.

#### Buffer acetate TS (pH 5.0)

Add 4.6 g of anhydrous sodium acetate to 11.6 ml of 2 M acetic acid and dilute to 200 ml with water. Adjust the pH to  $5.0 \pm 0.1$  with glacial acetic acid or 10% sodium hydroxide solution.

#### Barbital buffer solution (pH 7.6)

Dissolve 4.3 g of barbital sodium in 200 ml of water, adjust the pH to 7.6 with dilute hydrochloric acid, and filter.

#### Citric acid buffer solution

Dissolve 21 g of citric acid in water to make 1,000 ml (Solution A). Dissolve 28.4 g of disodium hydrogen phosphate in water to make 1,000 ml (Solution B). Combine 11 volumes of Solution A and 389 volumes of Solution B.

#### Formic acid buffer solution (pH 2.5)

Add 18 ml of water to 0.8 ml of formic acid, adjust the pH to 2.5 with strong ammonia TS, and dilute to 200 ml with water.

#### Phosphate buffer solution (pH 7.0)

Combine 50 ml of 0.2 M potassium dihydrogen phosphate and 29.54 ml of 0.2 M sodium hydroxide, and dilute to 200 ml with water.

**Phosphate buffer solution (pH 7.3-7.4) (0.02M)****Stock solution 1:**

Sodium phosphate dibasic anhydrous: 28.4 g

NaCl: 85 g

Distilled water: 1 litre

**Stock solution 2:**

Sodium phosphate monobasic monohydrate: 27.6 g

NaCl: 85 g

Distilled water: 1 litre

Make 1:10 dilutions of each stock solution. For example:

Stock solution 1	50 ml	Stock solution 2	10 ml
Distilled water	450 ml	Distilled water	90 ml
Approximate pH	8.2	Approximate pH	5.6

Using a pH meter, titer diluted solution 1 to pH 7.3-7.4 by adding about 65 ml of solution 2. Use the resulting 0.02 M phosphate saline buffer solution in the lysostaphin susceptibility test on *S. aureus*.

**Note:** Do not titer 0.2 M phosphate buffer to pH 7.3-7.4 and then dilute to 0.02 M strength. This results in a drop in pH of approximately 0.25. Addition of 0.85% salt after pH adjustment also results in a drop of approximately 0.2.

**Phosphate buffer solution (pH 7.5)**

Dissolve 53.7 g of disodium hydrogen phosphate in water to make 1,000 ml (Solution A).

Dissolve 20.4 g of potassium dihydrogen phosphate in water to make 1,000 ml (Solution B).

Combine 21 volumes of Solution A and 4 volumes of Solution B, and adjust the pH to 7.5 with either Solution A or Solution B.

**Standard Buffer Solutions****Reagent Solutions**

Previously dry the crystalline reagents (except for boric acid), at 110° to 120°, and use water that has been previously boiled and cooled to prepare the solutions. Store the prepared reagent solutions in chemically resistant glass or polyethylene bottles, and use within 3 months. Discard if moulding is evident.

- *Boric acid/potassium chloride, 0.2 M*

Dissolve 12.366 g of boric acid (H<sub>3</sub>BO<sub>3</sub>) and 14.911 g of potassium chloride (KCl) in water to make 1,000 ml.

- *Hydrochloric acid, 0.2 M*

Dilute 19 ml of hydrochloric acid with water to make 1,000 ml and standardize the solution as follows: dissolve about 0.3 g, accurately weighed, of primary standard anhydrous sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>), previously dried at about 270° for 1 h in 100 ml of water. Titrate with the hydrochloric acid using 2 drops of methyl red TS. When

the solution becomes faintly pink, boil to expel carbon dioxide, cool, and continue the titration until the faint pink colour is no longer affected by continued boiling. Each 10.60 mg of  $\text{Na}_2\text{CO}_3$  is equivalent to 1 ml of 0.2 M hydrochloric acid.

- Potassium chloride, 0.2 M

Dissolve 14.911 g of potassium chloride (KCl) in water to make 1,000 ml.

- Potassium hydrogen phthalate, 0.2 M

Dissolve 40.844 g of potassium hydrogen phthalate [ $\text{KHC}_6\text{H}_4(\text{COO})_2$ ] in water to make 1,000 ml.

- Potassium dihydrogen phosphate, 0.2 M

Dissolve 27.218 g of potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) in water to make 1,000 ml.

- Sodium hydroxide, 0.2 M

Dissolve about 9 g of sodium hydroxide (NaOH) in about 950 ml of water, and add a freshly prepared saturated solution of barium hydroxide until no more precipitate forms. Shake the mixture thoroughly, and allow it to stand overnight in a stoppered bottle. Decant or filter the solution, and standardize the clear liquid as follows: Dissolve about 1 g, accurately weighed, of primary standard potassium hydrogen phthalate [ $\text{KHC}_6\text{H}_4(\text{COO})_2$ ], previously dried at  $105^\circ$  for 3 h in 75 ml of carbon dioxide-free water, and titrate with the sodium hydroxide solution to a permanent pink colour using 2 drops of phenolphthalein TS, as indicator. Each 40.84 mg of  $\text{KHC}_6\text{H}_4(\text{COO})_2$  is equivalent to 1 ml of 0.2 M sodium hydroxide.

#### Composition of Standard Buffer Solutions

To prepare a standard buffer solution having a pH within the range 1.2 to 10.0, combine the appropriate solutions, prepared above, as shown in the following table, and dilute with water to make 200 ml. The standard pH values given in this table are considered to be reproducible to within  $\pm 0.02$  of the pH unit specified at  $25^\circ$ .



Hydrochloric Acid Buffer		Acid Phthalate Buffer		Neutralized Phthalate Buffer		Phosphate Buffer		Alkaline Borate Buffer	
To 50.0 ml of 0.2 M KCl add the specified ml of 0.2 M HCl		To 50.0 ml of 0.2 M KHC <sub>6</sub> H <sub>4</sub> -(COO) <sub>2</sub> add the specified ml of 0.2 M HCl		To 50.0 ml of 0.2 M KHC <sub>6</sub> H <sub>4</sub> -(COO) <sub>2</sub> add the specified ml of 0.2 M NaOH		To 50.0 ml of 0.2 M KH <sub>2</sub> PO <sub>4</sub> add the specified ml of 0.2 M NaOH		To 50.0 ml of 0.2 M H <sub>3</sub> BO <sub>3</sub> KCl add the specified ml of 0.2 M NaOH	
pH	ml	pH	ml	pH	ml	pH	ml	pH	ml
1.2	85.0	2.2	49.5	4.2	3.0	5.8	3.6	8.0	3.9
1.3	67.2	2.4	42.2	4.4	6.6	6.0	5.6	8.2	6.0
1.4	53.2	2.6	35.4	4.6	11.1	6.2	8.1	8.4	8.6
1.5	41.4	2.8	28.9	4.8	16.5	6.4	11.6	8.6	11.8
1.6	32.4	3.0	22.3	5.0	22.6	6.6	16.4	8.8	15.8
1.7	26.0	3.2	15.7	5.2	28.8	6.8	22.4	9.0	20.8
1.8	20.4	3.4	10.4	5.4	34.1	7.0	29.1	9.2	26.4
1.9	16.2	3.6	6.3	5.6	38.8	7.2	34.7	9.4	32.1
2.0	13.0	3.8	2.9	5.8	42.3	7.4	39.1	9.6	36.9
2.1	10.2	4.0	0.1			7.6	42.4	9.8	40.6
2.2	7.8					7.8	44.5	10.0	43.7
						8.0	46.1		

## STANDARD SOLUTIONS

### Ammonium Standard Solution

Dissolve 296.0 mg of ammonium chloride,  $\text{NH}_4\text{Cl}$ , in sufficient water to make 100 ml. Transfer 10.0 ml of this solution into a 1,000-ml volumetric flask, dilute to volume with water. Each ml of this solution contains 0.01 mg of  $\text{NH}_4^+$ .

### Barium Standard Solution

Dissolve 177.9 mg of barium chloride,  $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ , in water in a 1,000-ml volumetric flask, dilute to volume with water, and mix. Each ml of this solution contains 0.1 mg of Ba.

### Barium Chloride Standard Solution

Dissolve 4.3 g of barium chloride in sufficient water to make 1,000 ml. Perform gravimetric analysis on the solution, and calculate the quantity of sodium sulfate ( $\text{Na}_2\text{SO}_4$ ) corresponding to 1 ml of the solution. Each ml of this solution corresponds to about 2.5 mg of  $\text{Na}_2\text{SO}_4$ .

### Chromium Standard Solution

To 0.934 g of potassium chromate, add 1 drop of 10% sodium hydroxide solution and water to 1,000 ml. To a 1.0 ml portion of the solution, add 1 drop of 10% sodium hydroxide solution and water to 1,000 ml. Each ml of this solution contains 0.25  $\mu\text{g}$  of Cr.

### Condensed Formaldehyde Standard Solution

Dilute 8.1 g of formalin (containing 37% of HCHO) with water to 1,000 ml. To a 10.0 ml portion of the solution, add water to 1,000 ml. Each ml of this solution contains 0.03 mg of HCHO. Prepare freshly before use.

### Dithizone Standard Solution

Dissolve 10 mg of dithizone in 1,000 ml of chloroform. Store in a stoppered bottle lead free and in a cold place.

### Formaldehyde Standard Solution

Dilute 2.7 g of formalin (containing 37% of HCHO) with water to 1,000 ml. To a 10 ml portion of the solution, add water to 1,000 ml. Each ml of this solution contains 0.01 mg of HCHO. Prepare the solution fresh.

### Iron Standard Solution

Dissolve 8.63 g of ferric ammonium sulfate in 20 ml of dilute nitric acid, and add water to 1,000 ml. To 10 ml of the solution add 20 ml of dilute nitric acid and water to 1,000 ml. Each ml of this solution contains 0.01 mg of Fe. Store in a dark bottle.

**Lead Standard Solution**

Dissolve 159.8 mg of lead nitrate in 10 ml of dilute nitric acid, and add water to 1,000 ml. Prepare and store this solution in lead-free glassware. Dilute 10 ml of the solution with water to 100 ml. Each ml of this solution contains 0.01 mg of Pb. Prepare the solution fresh.

**Lead Standard Solution for Dithizone test**

To 10 ml of lead standard solution, add 1% nitric acid to 100 ml. Each ml of this solution contains 1 µg of Pb. Prepare the solution fresh.

**Magnesium Standard Solution**

Dissolve 50.0 mg magnesium metal, Mg, in 1 ml of hydrochloric acid in a 1,000-ml volumetric flask, dilute to volume with water, and mix. Each ml of this solution contains 0.05 mg Mg.

**Mercury Standard Solution**

Dissolve 0.135 g of mercuric chloride in 10 ml of dilute nitric acid and sufficient water to make 1,000 ml. Dilute 10 ml of the solution with 10 ml of dilute nitric acid and water to make 1,000 ml. Dilute the second solution in same manner. Each ml of this final solution contains 0.1 µg of Hg in 1 ml. Prepare the solution fresh.

**Methanol Standard Solution**

To 5 ml of 0.1% methanol, add 2,5 ml of ethyl alcohol not containing methanol, and add water to 50 ml. Each ml of this solution contains 0.1 mg of CH<sub>3</sub>OH.

**Nitrate Standard Solution**

Dissolve 1.63 g of potassium nitrate in water to make 1,000 ml. To a 10 ml portion of the solution, add water to 100 ml. Each ml of this solution contains 0.1 mg of NO<sub>3</sub>.

**Phosphate Standard Solution**

Dissolve 143.3 mg of monobasic potassium phosphate, KH<sub>2</sub>PO<sub>4</sub>, in water in a 100 ml volumetric flask, dilute to volume with water, and mix. Transfer 10.0 ml of this solution into a 1,000-ml volumetric flask, dilute to volume with water, and mix. Each ml of this solution contains 10 µg phosphate.

**Potassium Phosphate, Monobasic, Standard Solution**

Dissolve 4.394 g of potassium phosphate monobasic in sufficient water to make 1,000 ml. Each ml of this solution contains 1 mg of phosphate.

**Selenium Standard Solution**

Add 10 ml of dilute sulfuric acid (1 in 2) to 1 g of selenium. Heat to dissolve, and evaporate to dryness on a water bath. Dissolve the residue in sufficient water to make 1,000 ml. To a 10

ml portion of the solution, add water to 1,000 ml. Each ml of this solution contains 0.01 mg of Se.

**Thiamine Hydrochloride Standard Solution**

Dissolve 0.1 g of vitamin B<sub>1</sub> hydrochloride reference standard previously dried at 105° for 2 h, in water to make 1,000 ml. To a 10 ml portion of the solution, add water to 1,000 ml. Each ml of this solution contains 1 µg of vitamin B<sub>1</sub> hydrochloride reference standard.

**Zinc Standard Solution**

Dissolve 4.4 g of zinc sulfate in water to make 1,000 ml. To a 10 ml portion of the solution, add water to 1,000 ml. Each ml of this solution contains 0.01 mg of Zn.



## TEST SOLUTIONS

For the preparation of Test Solutions (TS), analytical grade reagents are to be used.

Certain of the following Test Solutions are intended for use as acid-base indicators in volumetric analyses. Such solutions should be adjusted so that when 0.15 ml of indicator solution is added to 25 ml of carbon dioxide-free water, 0.25 ml of 0.02 N acid or alkali, respectively, will produce the characteristic colour change.

The notation "PbT" indicates a lead-free solution.

In general, the directive to use a freshly prepared solution indicates that the solution is of limited stability and must be prepared on the day of use.

### **Acetic Acid TS**

A solution containing approximately 30% w/v of  $\text{CH}_3\text{COOH}$  in water (approximately 5N).

### **Acetic Acid TS, Dilute**

A solution containing approximately 6% w/v of  $\text{CH}_3\text{COOH}$  (approximately N).

### **Acetic Acid TS, Strong**

See acetic acid TS.

### **Acetic Anhydride/Benzol TS**

To 10 ml of acetic anhydride add sufficient benzol to make 100 ml.

### **Acetic Anhydride/Pyridine TS**

To 25 g of acetic anhydride add sufficient dehydrated pyridine to make 100 ml. Prepare freshly before use.

### **Acetic Periodic Acid TS**

Dissolve 5.4 g of periodic acid in 100 ml of distilled water and then add 1900 ml of glacial acetic acid and mix thoroughly. Store the solution in a dark glass-stoppered bottle or store in the dark in a clear glass-stoppered bottle.

### **Alcoholic Potassium Hydroxide TS**

See potassium hydroxide TS, ethanolic.

### **Alizarin Yellow GG TS**

Dissolve 0.1 g of alizarin yellow GG in 100 ml ethanol. Filter if necessary.

**Alizarin Yellow GG/Thymolphthalein TS**

Prepare by mixing 10 ml of alizarin yellow GG TS with 20 ml of thymolphthalein TS.

**Alkaline Cupric Tartrate TS**

(Fehling's TS). See cupric tartrate TS, alkaline.

**Alkaline Mercuric-Potassium Iodide TS**

(Nessler's TS). See mercuric-potassium iodide TS, alkaline.

**Alkaline Tartrate Solution TS**

See tartrate solution TS, alkaline.

**1-Amino-2-Naphthol-4-Sulfonic Acid TS**

Dissolve 0.2 g of 1-amino-2-naphthol-4-sulfonic acid in 195 ml of sodium bisulfite solution (3 in 20) and 5 ml of anhydrous sodium sulfite solution (1 in 5), and filter if necessary. Stopper tightly, and store in a dark, cold place. Use within 10 days of preparation.

**Ammonia TS**

A solution containing between 9.5% and 10.5% of  $\text{NH}_3$  (approximately 6 N). Prepare by diluting 400 ml of ammonium hydroxide (28%) with sufficient water to make 1,000 ml.

**Ammonia TS, Dilute**

See ammonia TS.

**Ammonia TS, Strong**

A solution containing approximately 25% w/v of  $\text{NH}_3$  in water (approximately 15 N).

**Ammonia TS, Dilute (PbT)**

Dilute ammonia TS, which complies with the following test: to 20 ml of ammonia TS add 1 ml of potassium cyanide TS (PbT), dilute to 50 ml with water and add 2 drops of sodium sulfide TS (PbT); no darkening should be produced.

**Ammonia TS, Ethanolic**

A 9 to 11% w/v solution of  $\text{NH}_3$  in ethanol. A transparent, colourless liquid having a strong odour of ammonia. Specific gravity is about 0.80. Store in a rubber-stoppered container and in a cold place.

**Ammonia/Ammonium Chloride Buffer TS**

(Approx. pH 10). Dissolve 67.5 g of ammonium chloride ( $\text{NH}_4\text{Cl}$ ) in water, add 570 ml of ammonium hydroxide (28%) and dilute with water to 1,000 ml.

**Ammoniacal Silver Nitrate TS**

Add ammonia TS, dropwise, to a 1 in 20 solution of silver nitrate until the precipitate that first forms is almost, but not entirely, dissolved. Filter the solution, and store in a dark bottle.

*(Note: Ammoniacal silver nitrate TS forms explosive compounds on standing. Do not store this solution, but prepare a fresh quantity for each series of determination. Neutralize the excess reagent and rinse all glassware with hydrochloric acid immediately after completing a test.)*

**Ammonium Acetate TS**

A 10% w/v solution of ammonium acetate ( $\text{CH}_3\text{COONH}_4$ ) in water.

**Ammonium Acetate Citrate TS (PbT)**

Dissolve 12.5 g of ammonium acetate ( $\text{CH}_3\text{COONH}_4$ ) and 12.5 g of ammonium citrate [ $\text{C}_3\text{H}_4\text{OH}(\text{COOH})(\text{COONH}_4)_2$ ] in water, add strong ammonia TS until the solution is alkaline to thymol blue paper and add water to 100 ml. Purify with a 0.002% w/v solution of dithizone in chloroform, and finally shake the solution with chloroform to remove excess of dithizone.

**Ammonium Carbonate TS**

Dissolve 20 g of ammonium carbonate and 20 ml of ammonia TS in sufficient water to make 100 ml.

**Ammonium Chloride TS**

10.5% w/v of ammonium chloride in water (approximately 2 N).

**Ammonium Chloride/Ammonium Hydroxide TS**

Mix equal volumes of water and strong ammonia TS, and saturate with ammonium chloride.

**Ammonium Citrate TS (PbT)**

Dissolve 40 g of citric acid in 90 ml of water. Add 2 or 3 drops of phenol red TS, then cautiously add strong ammonia TS until the solution acquires a reddish colour. Remove any lead that may be present by extracting the solution with 20-ml portions of dithizone extraction TS until the dithizone solution retains its orange-green colour.

**Ammonium Molybdate TS**

Dissolve 6.5 g of finely powdered molybdic acid (85%) in a mixture of 14 ml of water and 14.5 ml of strong ammonia TS. Cool the solution, and add it slowly, with stirring, to a well-



cooled mixture of 32 ml of nitric acid and 40 ml of water. Allow to stand for 48 h, and filter through glass wool. This solution deteriorates upon standing and is unsuitable for use if, upon the addition of 2 ml of sodium phosphate TS to 5 ml of the solution, an abundant yellow precipitate does not form at once or after slight warming. Store it in the dark. If a precipitate forms during storage use only the clear, supernatant solution.

#### **Ammonium Molybdate/Sulfuric Acid TS**

Dissolve 18.8 g of ammonium molybdate in 300 ml of water, and add 150 ml of sulfuric acid and sufficient water to make 500 ml.

#### **Ammonium Oxalate TS**

A 3.0% w/v solution of ammonium oxalate  $[(\text{COONH}_4)_2]$  in water (approximately 0.5 N).

#### **Ammonium Sulfanilate TS**

To 2.5 g of sulfanilic acid add 15 ml of water and 3 ml of ammonia TS and mix. If necessary, add with stirring, more ammonia TS, until the acid dissolves. Adjust the pH of the solution to about 4.5 with dilute hydrochloric acid TS, using bromocresol green TS as an outside indicator, and dilute to 25 ml.

#### **Ammonium Sulfide TS**

Saturate ammonia TS with hydrogen sulfide ( $\text{H}_2\text{S}$ ), and add two-thirds of its volume of ammonia TS. Residue on ignition: not more than 0.05%. The solution is not rendered turbid either by magnesium sulfate TS or by calcium chloride TS (carbonate). This solution is unsuitable for use if an abundant precipitate of sulfur is present. Store it in a small, well-filled, dark amber-coloured bottle, in a cold, dark place.

#### **Ammonium Thiocyanate TS**

A 7.6% w/v solution of ammonium thiocyanate ( $\text{NH}_4\text{SCN}$ ) in water (approximately N).

#### **Ammonium Thiocyanate/Cobalt Nitrate TS**

Dissolve 17.4 g of ammonium thiocyanate and 2.8 g of cobalt nitrate in sufficient water to make 100 ml.

#### **Amylase TS**

To 0.2 g of amylase (crystal), add 100 ml of water, shake well and filter. Prepare freshly before use.

#### **Anthrone TS**

Dissolve about 0.1 g of anthrone in 100 g of sulfuric acid. Prepare freshly before use.

**Antimony TS, Standard**

Dissolve 2.742 g of antimony potassium tartrate in water, and dilute to 100 ml; dilute 5 ml of this solution to 500 ml with water. Each ml of the solution contains 0.001 mg of Sb.

**Antimony Trichloride TS**

Wash the surface of antimony trichloride with anhydrous chloroform until the washings become transparent. Add anhydrous chloroform to antimony trichloride to make a saturated solution. Store in a tight container shaded from light and in a cold place. Prepare freshly before use.

**Arsenic TS, Dilute**

Mix 1 ml of strong arsenic TS with sufficient water to produce 100 ml. The dilute solution of arsenic must be freshly prepared. 1 ml contains 0.01 mg of arsenic.

**Arsenic TS, Strong**

Dissolve 0.132 g of arsenic trioxide in 50 ml of hydrochloric acid (a 25% w/v solution of HCl in water) and add sufficient water to 100 ml.

**Arsenous Acid TS**

Dissolve 1 g of arsenous acid in 30 ml of sodium hydroxide solution (1 in 40), and heat. Cool, and slowly add sufficient glacial acetic acid to 100 ml.

**Barium Chloride TS**

A 12% w/v solution of barium chloride ( $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ ) in water (approximately N).

**Barium Diphenylamine Sulfonate TS**

A 0.3% w/v solution of p-diphenylamine sulfonic acid barium salt in water.

**Benedict's Qualitative Reagent**

See cupric citrate TS, alkaline.

**Benzidine TS**

Dissolve 50 mg of benzidine in 10 ml of glacial acetic acid, dilute to 100 ml with water and mix. (**Caution:** *benzidine is toxic.*)

**Bertrand's TS, A**

Dissolve 40 g of fine cupric sulfate in sufficient water to make 1,000 ml. Fill a glass-stoppered container almost to the top, and store.

**Bertrand's TS, B**

Dissolve 200 g of potassium sodium tartrate and 150 g of sodium hydroxide in sufficient water to make 1,000 ml. Store in a rubber-stoppered container.

**Bertrand's TS, C**

Dissolve 50 g of ferric sulfate (shall not reduce potassium permanganate solution) in sufficient water. Add 200 ml of sulfuric acid, and add sufficient water to make 1,000 ml.

**Bertrand's TS, D**

Dissolve 5 g of potassium permanganate in sufficient water to make 1,000 ml.

*(Standardization: Dissolve 0.25 g of ammonium oxalate in 100 ml of water, and add 2 ml of sulfuric acid. Heat this solution to the temperature of 60° to 70°, and titrate with Bertrand's TS, D. If the volume of Bertrand's TS, D consumed is designated as a ml, each 1 ml of Bertrand's TS, D is equivalent to (0.2238/a)g of Cu).*

**2,2'-Bipyridine TS**

Dissolve 0.100 g of 2,2'-bipyridine in 50 ml of purified absolute ethanol TS.

**Bismuth Nitrate TS (I)**

Reflux 5 g of bismuth nitrate ( $\text{Bi}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$ ), in 7.5 ml of nitric acid and 10 ml of water until dissolved, cool, filter and dilute the solution to 250 ml.

**Bismuth Nitrate TS (II)**

Dissolve 5 g of bismuth nitrate ( $\text{Bi}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$ ) in 25 ml of water and 25 ml of glacial acetic acid and dilute to 250 ml.

**Borax Buffer (PbT)**

Dissolve 3.0 g of borax in 90 ml of water, and extract with successive portions, each of 5 ml of 1 volume of diphenylthiocarbazone solution PbT and 4 volumes of chloroform, with vigorous shaking, until the extract is blue or purple in colour; continue the extraction with successive portions, each of 10 ml of chloroform, until the extract is colourless; reject the extracts, and dilute the solution to 100 ml with water.

**Boric Acid TS**

Dissolve 5 g boric acid in 500 ml distilled water in a 1,000-ml measuring flask. Add 25 ml alcoholic indicator solution (67 mg methyl red and 33 mg bromocresol green in 100 ml 96% ethanol) and 200 ml ethanol. Make up to volume with distilled water. The boric acid indicator solution is red. 5 ml must turn green with not more than 3 drops of 0.01 N NaOH.

**Bromide/Bromate TS**

(About 0.1 N bromine) (7.991 g Br per litre). Dissolve 3 g of potassium bromate ( $\text{KBrO}_3$ ) and 15 g of potassium bromide ( $\text{KBr}$ ) in sufficient water to make 1,000 ml and standardize the solution as follows: transfer about 25 ml of the solution, accurately measured, into a 500-ml iodine flask and dilute with 120 ml of water. Add 5 ml of hydrochloric acid, stopper the flask and shake it gently. Then add 5 ml of potassium iodide TS, re-stopper, shake the mixture, allow it to stand for 5 min and titrate the liberated iodine with 0.1 N sodium thiosulfate, adding starch TS near the end of the titration. Calculate the normality. Store this solution in a dark amber coloured, glass-stoppered bottle.

**Bromine TS**

(Bromine water). A saturated solution of bromine, prepared by agitating 2 to 3 ml of bromine ( $\text{Br}_2$ ) with 100 ml of cold water in a glass-stoppered bottle, the stopper of which should be lubricated with petrolatum. Store it in a cold place, protected from light.

**Bromine/Acetic Acid TS**

Dissolve 5 ml of bromine in 145 ml of potassium acetate in acetic acid TS. Prepare this solution fresh daily.

**Bromine/Bromide TS**

Add 1 ml of bromine to 300 ml of glacial acetic acid saturated with dry potassium bromide (5 g). 15 ml of this solution require about 50 ml of 0.05 N sodium thiosulfate. This solution is stored in a dark bottle and kept in the dark. It is standardized at least once a day during use.

**Bromine/Glacial Acetic Acid TS**

Dissolve about 1.5 g of bromine in sufficient glacial acetic acid to make about 100 ml. Each 1 ml of this solution is equivalent to about 2 ml of 0.1 N sodium thiosulfate.

**Bromine/Hydrochloric Acid TS**

Mix 1 ml of bromine/potassium bromide TS with 100 ml of hydrochloric acid, arsenic-free.

**Bromine/Potassium Bromide TS**

Dissolve 30 g of bromine and 30 g of potassium bromide in sufficient water to make 100 ml.

**Bromocresol Blue TS**

Use bromocresol green TS.

**Bromocresol Green TS**

Dissolve 0.05 g of bromocresol green in 100 ml of ethanol, and filter if necessary. For pH determinations, dissolve 0.05 g in 1.4 ml of 0.05 N sodium hydroxide, and dilute with carbon dioxide-free water to 100 ml.

**Bromocresol Green/Methyl Red TS**

Mix equal volumes of bromocresol green TS and methyl red TS.

**Bromocresol Purple TS**

Dissolve 0.25 g of bromocresol purple in 20 ml of 0.05 N sodium hydroxide, and dilute with water to 250 ml.

**Bromophenol Blue TS**

Dissolve 0.1 g of bromophenol blue in 100 ml of dilute ethanol (1 in 2), and filter if necessary. For pH determinations, dissolve 0.1 g in 3.0 ml of 0.05 N sodium hydroxide, and dilute with carbon dioxide-free water to 200 ml.

**Bromophenol Blue TS**

(For citric acid). Mix bromophenol blue TS with equal volume of ethanol, adjust pH to 7.0 by adding 0.01 N sodium hydroxide solution.

**Bromophenol Blue/Sodium Hydroxide TS**

Dissolve 0.1 g of bromophenol blue in 3 ml of 0.05 N sodium hydroxide by mixing well, and add sufficient water to 25 ml.

**Bromothymol Blue TS**

Dissolve 0.1 g of bromothymol blue in 100 ml of dilute ethanol (1 in 2), and filter if necessary. For pH determinations, dissolve 0.1 g in 3.2 ml of 0.05 N sodium hydroxide, and dilute with carbon dioxide-free water to 200 ml.

**Buffer TS**

See Buffer Test Solutions.

**Calcium Chloride TS**

A 7.5% w/v solution of calcium chloride ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ) in water (approximately N).

**Calcium Hydroxide TS**

A solution containing approximately 0.14 g of  $\text{Ca}(\text{OH})_2$  in each 100 ml. To prepare, add 3 g of calcium hydroxide [ $\text{Ca}(\text{OH})_2$ ] to 1,000 ml of water, and agitate the mixture vigorously and repeatedly during 1 h. Allow the excess calcium hydroxide to settle, and decant or draw off the clear supernatant liquid.

**Carr-Price TS**

Weigh an unopened (100 g) bottle of antimony trichloride. Open the bottle and empty the contents into a wide-mouthed, glass-stoppered amber bottle containing approximately 100 ml

of chloroform. By difference, obtain the weight of antimony trichloride and then add sufficient chloroform to supply 100 ml for each 25 g. Dissolve by warming or shaking for several hours and filter through sodium sulfate into a clean, dry, amber bottle with ground glass stopper. This solution may be stored at room temperature but should be kept in the dark when not in use. The reagent is apparently stable for long periods of time, but it is convenient to make up sufficient amounts to last for one month. Rinse all glassware coming in contact with this reagent with chloroform, a mixture of ethanol and ether or dilute or concentrated hydrochloric acid before washing, since the antimony oxychloride which forms is insoluble in water.

#### **Ceric Ammonium Nitrate TS**

Dissolve 6.25 g of ceric ammonium nitrate  $[(\text{NH}_4)_2\text{Ce}(\text{NO}_3)_6]$  in 100 ml of 0.25 N nitric acid. Prepare the solution fresh every third day.

#### **Chloral Hydrate TS**

Dissolve 50 g of chloral hydrate in a mixture of 15 ml of water and 10 ml of glycerol.

#### **Chlorine TS**

(Chlorine water). A saturated solution of chlorine in water. Place the solution in a small, completely filled, light-resistant container. Chlorine TS, even when kept from light and air, is apt to deteriorate. Store it in a cold, dark place. For full strength, prepare this solution fresh.

#### **Chromate TS, Standard**

Dissolve 0.0566 g potassium dichromate ( $\text{K}_2\text{Cr}_2\text{O}_7$ ) in 1,000 ml of water. Each ml contains 0.02 mg of Cr.

#### **Chromic Acid TS**

See Chromium trioxide TS.

#### **Chromium Trioxide TS**

A 3% w/v solution of chromium trioxide in water.

#### **Chromotropic Acid TS**

Dissolve 2.0 g of chromotropic acid (4,5-dihydroxy-2,7-naphthalene-disulfonic acid, disodium salt) in 40 ml of water in a 1-litre volumetric flask. Dilute to volume with 15 M sulfuric acid.

#### **Citric Acid Buffer Solution**

See standard buffer solutions.

**Cobaltous Chloride TS**

Dissolve 2 g of cobaltous chloride ( $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ) in 1 ml of hydrochloric acid and sufficient water to make 100 ml.

**Cobaltous Chloride TSC**

Dissolve about 65 g cobaltous chloride ( $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ) in enough of a mixture of 25 ml of hydrochloric acid and 975 ml of water to make 1,000 ml. Place exactly 5 ml of this solution in a 250-ml iodine flask, add 5 ml of hydrogen peroxide TS and 15 ml of 20% sodium hydroxide solution. Boil for 10 min, cool, and add 2 g of potassium iodide and 20 ml of 25% sulfuric acid. When the precipitate has dissolved, titrate the liberated iodine with 0.1 N sodium thiosulfate, using starch TS as indicator. Each ml of 0.1 N sodium thiosulfate is equivalent to 23.8 mg of  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ . Adjust the final volume of the solution by adding enough of the hydrochloric acid and water mixture so that each ml contain 59.5 mg of  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ .

**Cobalt-Uranyl Acetate TS**

Dissolve, with warming, 40 g of uranyl acetate [ $\text{UO}_2(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 2\text{H}_2\text{O}$ ] in a mixture of 30 g glacial acetic acid and sufficient water to make 500 ml. Similarly, prepare a solution containing 200 g of cobaltous acetate [ $\text{Co}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 4\text{H}_2\text{O}$ ] in a mixture of 30 g of glacial acetic acid and sufficient water to make 500 ml. Mix the two solutions while still warm, and cool to 20°. Maintain the temperature at 20° for about 2 h to separate the excess salts from solution, and then filter through a dry filter.

**Congo Red TS**

Dissolve 0.10 g of congo red (sodium diphenyl-diazo-bis-alpha-naphthylaminesulfonate) ( $\text{C}_{32}\text{H}_{22}\text{N}_6\text{O}_6\text{S}_2\text{Na}_2$ ) in 20 ml of 90% ethanol and add sufficient water to make 100 ml.

**Copper Sulfate Solution TS**

Dissolve 34.639 g of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  in water, dilute to 500 ml, and filter through glass wool or paper. Determine the Cu content of the solution (preferably by electrolysis), and adjust the content to 440.9 mg Cu/25 ml.

**Cresol Red TS**

Triturate 0.10 g of cresol red in a mortar with 26.2 ml of 0.01 N sodium hydroxide until solution is complete, then dilute the solution with water to 250 ml.

**Cresol Red/Thymol Blue TS**

Add 15 ml of thymol blue TS to 5 ml of cresol red TS, and mix.

**Crystal Violet TS**

A 1% solution of methyl violet (methyl-rosaniline chloride; crystal violet) in glacial acetic acid.

**Cupric Acetate TS, Strong**

Dissolve 13.3 g of cupric acetate in 5 ml of acetic acid and 195 ml of water.

**Cupric Citrate TS, Alkaline**

(Benedict's qualitative reagent). With the aid of heat, dissolve 173 g of sodium citrate ( $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7 \cdot 2\text{H}_2\text{O}$ ) and 117 g of sodium carbonate ( $\text{Na}_2\text{CO}_3 \cdot \text{H}_2\text{O}$ ) in about 700 ml of water, and filter through paper, if necessary. In a separate container dissolve 17.3 g of cupric sulfate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ) in about 100 ml of water, and slowly add this solution, with constant stirring, to the first solution. Cool the mixture, dilute to 100 ml, and mix.

**Cupric Nitrate TS**

A 2.4% w/v solution of cupric nitrate [ $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$ ] in water.

**Cupric Sulfate TS**

A 12.5% w/v solution of cupric sulfate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ) in water.

**Cupric Sulfate TSC**

Dissolve about 65 g of cupric sulfate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ) in enough of a mixture of 25 ml of hydrochloric acid and 975 ml of water to make 1,000 ml. Pipet 10.0 ml of this solution in a 250-ml iodine flask, add 40 ml of water, 4 ml of acetic acid, and 3 g of potassium iodide. Titrate the liberated iodine with 0.1 N sodium thiosulfate, using starch TS as indicator. Each ml of 0.1 N sodium thiosulfate is equivalent to 24.97 mg of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ . Adjust the final volume of the solution by adding enough of the mixture of hydrochloric acid and water to make each ml contain 62.4 mg of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ .

**Cupric Sulfate/Ammonia TS**

Dissolve 0.4 g of cupric sulfate in 50 ml mixture of ammonia TS and solution of citric acid (1 in 5) in the ratio of 2:3.

**Cupric Tartrate TS, Alkaline**

(Fehling's TS). [The Copper Solution (A)]. Dissolve 34.66 g of carefully selected, small crystals of cupric sulfate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ) showing no trace of efflorescence or of adhering moisture, in sufficient water to make 500 ml. Store this solution in a small, tight container. [The Alkaline Tartrate Solution (B)]. Dissolve 173 g of crystallized potassium sodium tartrate ( $\text{KNaC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$ ) and 50 g of sodium hydroxide (NaOH) in sufficient water to make 500 ml. Store this solution in a small, alkali-resistant container. For use, mix exactly equal volumes of Solutions A and B at the time required.

**Cyanogen Bromide TS**

Dissolve 5 g of cyanogen bromide in water to make 50 ml. (**Caution:** Prepare this solution under a hood, as cyanogen bromide volatilizes at room temperature and the vapour is highly irritating and poisonous).



**Denigès' Reagent**

See mercuric sulfate TS.

**4,4'-Diaminodiphenylamine TS**

Triturate 4,4'-diaminodiphenylamine sulfate with a small amount of ethanol, and add ethanol again. Transfer this solution to a flask connected to a reflux condenser, heat on a water bath, and prepare a saturated solution.

**Di-β-Naphthylthiocarbazono/Chloroform TS**

Add 0.1 g of di-β-naphthylthiocarbazono to 100 ml of carbon tetra-chloride. Dilute this solution 1:40 with chloroform.

**2,6-Dichlorophenol-Indophenol TS**

Warm 0.1 g of 2,6-dichlorophenol-indophenol sodium ( $C_{12}H_6Cl_2NNaO_2$ ) with 100 ml of water and filter. The solution must be used within 3 days of preparation.

**2,7-Dihydroxynaphthalene TS**

Dissolve 0.1 g of 2,7-dihydroxynaphthalene in 1,000 ml of sulfuric acid and allow the solution to stand until the initial yellow colour disappears. If the solution is very dark, discard it and prepare a new solution from a different supply of sulfuric acid. This solution is stable for approximately one month if stored in a dark bottle.

**p-Dimethylaminobenzaldehyde TS**

Dissolve 0.125 g of p-dimethylaminobenzaldehyde in a cooled mixture of 65 ml of sulfuric acid and 35 ml of water, and add 0.05 ml of ferric chloride TS. Use within 7 days after preparation.

**Dimethylglyoxime TS**

A 1% w/v solution of dimethylglyoxime in ethanol.

**2,4-Dinitrophenylhydrazine TS**

Dissolve 0.2 g of 2,4-dinitrophenylhydrazine in 100 ml of 85% sulfuric acid. Filter through a glass-filter (G3) if necessary. Store in a light-shaded bottle and in a dark, cold place. Use within 2 weeks of preparation.

**Diphenylamine TS**

A 1% w/v solution of diphenylamine  $[(C_6H_5)_2NH]$  in sulfuric acid. The solution should be colourless.

**Diphenylcarbazide TS**

Dissolve 0.125 g of diphenylcarbazide  $[(C_6H_5 \cdot NH \cdot NH)_2CO]$  in a mixture of 25 ml acetone and 25 ml water. To be prepared immediately before use.

**Diphenylcarbazone TS**

An approximately 1% w/v solution of diphenylcarbazone ( $C_{13}H_{12}N_4O$ ) in ethanol. Store this solution in a brown bottle.

**Diphenylthiocarbazone Solution (PbT)**

Extract 15 ml of a 0.1% w/v solution of diphenylthiocarbazone ( $C_6H_4 \cdot N : N \cdot CS \cdot NH \cdot NH \cdot C_6H_5$ ) in chloroform, with 2 successive portions, each of 50 ml of water containing 5 ml of dilute ammonia TS; acidify the combined extracts with dilute hydrochloric acid PbT, and extract with 100 ml of chloroform; wash the extract with 2 successive portions, each of 10 ml of water, and filter through a dry filter. Determine the approximate strength of this solution by the method for determination of zinc (see titanium dioxide monograph), using 5 ml of standard zinc sulfate TS diluted to 25 ml with water in place of the 25 ml of acid solution used in the determination; dilute with chloroform so that 3 ml is approximately equivalent to each ml of standard zinc sulfate TS. This solution must be freshly prepared.

 **$\alpha, \alpha$ -Dipyridyl TS**

A 0.2% w/v solution of alpha, alpha-dipyridyl ( $C_{10}H_8N_2$ ) in absolute ethanol.

**Dithizone TS**

Dissolve 25.6 mg of dithizone in 100 ml of ethanol.

**Dithizone TS, Extraction**

Dissolve 30 mg of dithizone in 1,000 ml of chloroform, and add 5 ml of ethanol. Store the solution in a refrigerator. Before use shake a suitable volume of the dithizone extraction solution with about half its volume of 1% nitric acid, discarding the nitric acid. Do not use more than 1 month old.

**Dithizone TS, Standard**

Dissolve 10 mg of dithizone in 1,000 ml of chloroform. Keep the solution in a glass-stoppered, lead-free bottle, suitably wrapped to protect it from light, and store in a refrigerator.

**Dragendorff TS**

Solution 1: Weigh 0.85 g of basic bismuth nitrate, and dissolve in 10 ml of acetic acid and 40 ml of water.

Solution 2: Weigh 8 g of potassium iodide, and dissolve in 20 ml of water. Mix 5 ml of Solution 1, 5 ml of Solution 2, 20 ml of acetic acid, and 100 ml of water before use.

**Eosin Y TS**

(Adsorption indicator) A 0.5% solution of eosin Y in water.

**Eriochrome Black TS**

Dissolve 0.2 g of eriochrome black T and 2 g of hydroxylamine hydrochloride ( $\text{NH}_2\text{OH}\cdot\text{HCl}$ ) in sufficient methanol to make 50 ml, and filter. Store the solution in a light-resistant container and use within 2 weeks.

**Ethanol, Aldehyde-free**

To 1,000 ml of ethanol, add 5 ml of sulfuric acid and 20 ml of water, and distil. Add 10 g of silver nitrate and 1 g of potassium hydroxide to a 1,000 ml portion of this distillate, boil for 3 h by connecting a reflux condenser, and recover the ethanol by distillation.

**Ethanol TS, Purified Absolute**

Add about 0.1% potassium permanganate and 0.1% potassium hydroxide to absolute ethanol and distil in an all-glass apparatus.

**Ethanol TS, 72%**

Mix 360 ml of purified absolute ethanol TS with 150 ml of water.

**Ethanol Potassium Hydroxide TS**

See potassium hydroxide TS, ethanolic.

**p-Ethoxychrysoidin TS**

Dissolve 50 mg of p-ethoxychrysoidin monohydrochloride in a mixture of 25 ml of water and 25 ml of ethanol, add 3 drops of hydrochloric acid, stir vigorously, and filter if necessary to obtain a clear solution.

**Fehling's TS**

See cupric tartrate TS, alkaline.

**Ferric Ammonium Sulfate TS**

An 8% w/v solution of ferric ammonium sulfate [ $\text{FeNH}_4(\text{SO}_4)_2\cdot 12\text{H}_2\text{O}$ ] in water.

**Ferric Ammonium Sulfate/Hydrochloric Acid TS**

A 0.1% w/v solution of ferric ammonium sulfate [ $\text{FeNH}_4(\text{SO}_4)_2\cdot 12\text{H}_2\text{O}$ ] in hydrochloric acid.

**Ferric Chloride TS**

A 9% w/v solution of ferric chloride ( $\text{FeCl}_3\cdot 6\text{H}_2\text{O}$ ) in water (approximately N).

**Ferric Chloride TSC**

Dissolve about 55 g of ferric chloride ( $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ) in sufficient of a mixture of 25 ml of hydrochloric acid and 975 ml of water to make 1,000 ml. Pipet 10 ml of this solution in a 250-ml iodine flask, add 15 ml of water and 3 g of potassium iodide, and allow the mixture to stand for 15 min. Dilute with 100 ml of water, and titrate the liberated iodine with 0.1 N sodium thiosulfate, adding starch TS. Each ml of 0.1 N sodium thiosulfate is equivalent to 27.03 mg of  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ . Adjust the final volume of the solution by the addition of enough of the mixture of hydrochloric acid and water, so that each ml contains 45.0 mg of  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ .

**Ferric Chloride TS, Ethanolic**

A 0.2% w/v solution of ferric chloride ( $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ) in absolute ethanol. Prepare this solution fresh.

**Ferric Chloride TS, Dilute**

To 2 ml of ferric chloride TS, add sufficient water to make 100 ml. Prepare freshly before use.

**Ferric Chloride/Hydrochloric Acid TS**

Dissolve 5 g of ferric chloride ( $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ) in 5 ml of hydrochloric acid and sufficient water to make 100 ml.

**Ferric Sulfate TS**

Add 500 ml of water to 50 g of ferric sulfate, and mix thoroughly. To this mixture, add 200 ml of sulfuric acid, dissolve by shaking well, and add sufficient water to make 1,000 ml.

**Ferric Sulfate TS, Acid**

Add 7.5 ml of sulfuric acid to 100 ml of water, and dissolve 80 g of ferrous sulfate in the mixture with the aid of heat. Mix 7.5 ml of nitric acid and 20 ml of water, warm, and add to this the ferrous sulfate solution. Concentrate the mixture until, with the sudden emission of a red coloured vapour, the black colour of the liquid changes to red. Test for the absence of ferrous iron, and, if necessary, add a few drops of nitric acid and boil again. When the solution is cold, add sufficient water to make 110 ml.

**Ferrous Sulfate TS**

Dissolve 8 g of clear crystals of ferrous sulfate ( $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ) in about 100 ml of recently boiled and thoroughly cooled water. Prepare this solution fresh.

**Ferrous Sulfate TS, Acid**

Dissolve 7 g of ferrous sulfate crystals in 90 ml of recently boiled and thoroughly cooled water, and add sufficient sulfuric acid to make 100 ml. Standardize frequently with 0.1 N potassium permanganate (approximately 0.25 N).

**Fluorescein TS**

A 0.1% w/v solution of sodium fluorescein in 50% ethanol.

**Folin-Ciocalteu TS**

Into a 150-ml flask introduce 10 g of sodium tungstate ( $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$ ), 2.5 g of sodium molybdate ( $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ ), 70 ml of water, 5 ml of phosphoric acid, and 10 ml of hydrochloric acid. Reflux the mixture gently for about 10 h, and add 15 g of lithium sulfate ( $\text{Li}_2\text{SO}_4 \cdot \text{H}_2\text{O}$ ), 50 ml of water, and few drops of bromine. Boil the mixture, without the condenser, for 15 min or until the excess bromine is expelled. Cool, dilute with water to 100 ml, and filter. The filtrate has no greenish tint. Before use, dilute 1 part of filtrate with 1 part of water.

**Formaldehyde TS**

A solution containing approximately 37.0% w/v of HCHO. It may contain methanol to prevent polymerization.

**Formalin/Sulfuric Acid TS**

Mix 0.2 ml of formaldehyde TS with 10 ml of sulfuric acid. Prepare freshly before use.

**Fuchsin/Sulfurous Acid TS**

Dissolve 0.5 g of basic fuchsin in 300 ml of hot water, and cool. Add a solution of 5 g anhydrous sodium sulfite dissolved in 50 ml of water while stirring, and add 5 ml of hydrochloric acid with shaking. Dilute with water to 500 ml, and allow to stand for 5 h. Store in a light-shaded bottle, and in a cold place.

**Hydriodic Acid TS**

Distil hydriodic acid over red phosphorus, passing carbon dioxide through the apparatus during the distillation. The constant-boiling mixture distilling over a  $126^\circ\text{-}127^\circ$ , which is colourless or nearly colourless, is used. Place the acid in a small, brown, glass-stoppered bottle previously swept out with carbon dioxide, seal with paraffin, and store in a cool, dark place.

**Hydrochloric Acid TS, Brominated**

Mix 1 ml of solution of bromine with 100 ml of hydrochloric acid.

**Hydrochloric Acid TS, Dilute**

A solution containing 10% w/v of HCl. Prepare by diluting 266 ml of hydrochloric acid (36%) with sufficient water to make 1,000 ml.

**Hydrochloric Acid, Dilute (PbT)**

A solution containing approximately 10% w/v of HCl; it complies with the following test: make 10 ml alkaline with ammonia dilute, PbT add 1 ml of potassium cyanide solution PbT, dilute to 50 ml with water, and add 2 drops of a 10% sodium sulfide solution in water. No darkening is produced.

**Hydrochloric Acid TS, Stannated**

Mix 1 ml of a solution of stannous chloride TS with 100 ml of hydrochloric acid (a 25% w/v solution of HCl in water).

**Hydrogen Peroxide TS**

A solution containing between 2.5 and 3.5 g of H<sub>2</sub>O<sub>2</sub> in each 100 ml. It may contain suitable preservatives, totalling not more than 0.05%.

**Hydrogen Sulfide TS**

A saturated solution of hydrogen sulfide made by passing H<sub>2</sub>S into cold water. Store it in a small, dark, amber-coloured bottle, filled nearly to the top. It is unsuitable unless it possesses a strong odour of H<sub>2</sub>S, and unless it produces at once a copious precipitate of sulfur when added to an equal volume of ferric chloride TS. Store in a cold dark place.

**Hydroxylamine Hydrochloride TS**

Dissolve 20 g of hydroxylamine hydrochloride (HONH<sub>2</sub>·HCl) in sufficient water to make approximately 65 ml. Transfer to a separatory funnel, add a few drops of thymol blue pH indicator, then add strong ammonia TS until the solution assumes a yellow colour. Add 10 ml of a 4% solution of sodium diethyldithiocarbamate, mix well, and allow to stand for 5 min. Extract this solution with successive 10 to 15-ml portions of chloroform until a 5-ml portion of the chloroform extract does not assume a yellow colour when shaken with a dilute cupric sulfate solution. Add diluted hydrochloric acid PbT until the solution is pink, and then dilute with sufficient water to make 100 ml.

**8-Hydroxyquinoline TS**

A 5% w/v solution of 8-hydroxyquinoline (oxine) in ethanol.

**Indigo Carmine TS**

Dissolve a quantity of indigo carmine (sodium indigotindisulfonate) equivalent to 0.18 g of C<sub>16</sub>H<sub>8</sub>O<sub>8</sub>N<sub>2</sub>S<sub>2</sub>Na<sub>2</sub>, in sufficient water to make 100 ml. This solution should be used within 60 days of preparation.

**Iodine TS**

Dissolve 14 g of iodine in a solution of 36 g of potassium iodide in 100 ml of water, add 3 drops of hydrochloric acid, and dilute with water to 1,000 ml.

**Iron Indicator TS**

Place 62.5 g of ferric ammonium sulfate in a one-litre bottle, dissolve in 500 ml of water, add 450 ml of concentrated nitric acid, and mix.

**Iron TS, Standard**

Dissolve 0.70 g of  $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$  in 50 ml of water and add 20 ml of dilute  $\text{H}_2\text{SO}_4(1:15)$ . Dilute to 1,000 ml with water and mix thoroughly. Dilute 10 ml of this solution to 100 ml with water. Each ml contains 0.01 mg of Fe.

**Lead TS, Standard**

Dissolve 0.1598 g of lead nitrate  $[\text{Pb}(\text{NO}_3)_2]$  in water to which has been added 1 ml of nitric acid and dilute to 1,000 ml. Then dilute 10 ml of this solution to 100 ml. Each ml contains 0.01 mg of lead. This solution must be freshly prepared.

**Lead Acetate TS**

Dissolve 9.5 g of clear, transparent crystals of lead acetate  $[\text{Pb}(\text{COOCH}_3)_2 \cdot 3\text{H}_2\text{O}]$  in sufficient recently boiled water to make 100 ml. Store in a well-stoppered bottle.

**Lead Acetate TS, Basic**

Mix 10 parts of finely pulverized lead oxide (PbO) with 30 parts of lead acetate  $[\text{Pb}(\text{COOCH}_3)_2 \cdot 3\text{H}_2\text{O}]$  and 5 parts of water and heat gently in a closed vessel shaking repeatedly, until the mixture is white. Add 95 parts of water, heat for 1 h, shaking repeatedly, allow to cool and filter. Add water if necessary to obtain a solution of specific gravity 1.225-1.230.

**Lead Subacetate TS**

Triturate 14 g of lead monoxide (PbO) to a smooth paste with 10 ml of water, and transfer the mixture to a bottle, using an additional 10 ml of water for rinsing. Dissolve 22 g of lead acetate  $[\text{Pb}(\text{COOCH}_3)_2 \cdot 3\text{H}_2\text{O}]$  in 70 ml of water, and add the solution to the lead oxide mixture. Shake it vigorously for 5 min, then set it aside, shaking it frequently over 7 days. Finally filter, and add enough recently boiled water through the filter to make 100 ml.

**Lead Subacetate TS, Dilute**

Dilute 3.25 ml of lead subacetate TS with sufficient water, recently boiled and cooled, to make 100 ml. Store in a small, well-filled, tight container.

**Litmus TS**

Boil 10 g of litmus of reagent purity with 40 ml of ethanol (90%) for 1 h and pour away the clear liquid, repeat this operation twice with 30 ml of ethanol (90%). Digest the washed litmus with 100 ml of boiling water for 1 h, cool and filter.

**Manganese Sulfate TS**

Dissolve 90 g of manganese sulfate in 200 ml of water, 175 ml of phosphoric acid and 350 ml of diluted sulfuric acid (1 in 2). Add sufficient water to 1,000 ml.

**Magnesia Mixture TS**

Dissolve 5.5 g of magnesium chloride ( $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ ) and 7 g of ammonium chloride ( $\text{NH}_4\text{Cl}$ ) in 65 ml of water, add 35 ml of ammonia TS, set the mixture aside for a few days in a well-stoppered bottle, and then filter. If the solution is not perfectly clear, filter again before use.

**Magnesium Sulfate TS**

Dissolve 12 g of crystals of magnesium sulfate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ), selected for freedom from efflorescence, in water to make 100 ml.

**Malachite Green TS**

A 1% w/v solution of malachite green oxalate in glacial acetic acid.

**Mayer's TS**

See mercuric-potassium iodide TS.

**Mercuric Acetate TS**

A 6% w/v solution of mercuric acetate [ $\text{Hg}(\text{COOCH}_3)_2$ ] in glacial acetic acid. Store in a tight container protected from direct sunlight.

**Mercuric Chloride TS**

A 6.5% w/v solution of mercuric chloride ( $\text{HgCl}_2$ ) in water (approximately 0.5 N).

**Mercuric-Potassium Iodide TS**

(Mayer's TS). Dissolve 1.358 g of mercuric chloride ( $\text{HgCl}_2$ ) in 60 ml of water. Dissolve 5 g of potassium iodide (KI) in 10 ml of water. Mix the two solutions, and add water to make 100 ml.

**Mercuric-Potassium Iodide TS, Alkaline**

(Nessler's TS). Dissolve 10 g of potassium iodide (KI) in 10 ml of water, and add slowly with stirring, a saturated solution of mercuric chloride until a slight red precipitate remains undissolved. To this mixture add an ice-cold solution of 30 g of potassium hydroxide (KOH) in 60 ml of water, then add 1 ml more of the saturated solution of mercuric chloride. Dilute with water to 200 ml. Allow the precipitate to settle, and draw off the clear liquid. A 2-ml portion of this reagent, when added to 100 ml of a 1 in 300,000 solution of ammonium chloride in ammonia-free water, produces at once a yellowish brown colour.



**Mercuric Nitrate TS**

Dissolve 40 g of yellow mercuric oxide (HgO) in a mixture of 32 ml of nitric acid and 15 ml of water. Store in a light-shaded, glass-stoppered bottle (approximately 4 N).

**Mercuric Sulfate TS**

(Denigès' TS). Mix 5 g of yellow mercuric oxide (HgO) with 40 ml of water, and while stirring slowly add 20 ml of sulfuric acid, then add another 40 ml of water, and stir until completely dissolved (approximately 0.5 N).

**Mercurous Nitrate TS**

Dissolve 200 g of mercury in nitric acid and add sufficient water to produce 1,000 ml. Mercurous nitrate TS should be kept in a bottle containing a little metallic mercury.

**p-Methylaminophenol Sulfate TS**

Dissolve 2 g of p-methylaminophenol sulfate  $[(HO\cdot C_6H_4\cdot NHCH_3)_2\cdot H_2SO_4]$  in 100 ml of water. To 10 ml of this solution add 90 ml of water and 20 g of sodium bisulfite. Confirm the suitability of this solution by the following test: add 1 ml of the solution to each of four tubes containing 25 ml of 0.5 N sulfuric acid and 1 ml of ammonium molybdate TS. Add 5  $\mu$ g of phosphate ( $PO_4$ ) to one tube, 10  $\mu$ g to a second, and 20  $\mu$ g to a third, using 0.5, 1.0, and 2.0 ml, respectively, of Phosphate Standard Solution, and allow to stand for 2 h. The solutions in the three tubes should show readily perceptible differences in blue colour corresponding to the relative amounts of phosphate added, and the one to which 5  $\mu$ g of phosphate was added should be perceptibly bluer than the blank.

**Methylene Blue TS**

Dissolve 0.125 g of methylene blue in 100 ml of ethanol, and dilute with ethanol to 250 ml.

**Methylene Blue TS, Diluted**

To 1 ml of methylene blue TS, add sufficient water to make 100 ml.

**Methyl Orange TS**

Dissolve 0.1 g of methyl orange in 100 ml of water and filter if necessary.

**Methyl Orange/Xylencyanol FF TS**

Dissolve 1 g of methyl orange and 1.4 g of xylencyanol FF in 500 ml of 50% v/v ethanol.

**Methyl Red TS**

Dissolve 0.1 g of methyl red in 100 ml of ethanol, and filter if necessary. For pH determinations, dissolve 0.1 g in 7.4 ml of 0.05 N sodium hydroxide, and dilute with carbon dioxide-free water to 200 ml.

**Methyl Red/Methylene Blue TS**

Add 10 ml of methyl red TS to 10 ml of methylene blue TS, and mix.

**Methylrosaniline Chloride TS**

See crystal violet TS.

**Methyl Violet TS**

See crystal violet TS.

**Millon's TS**

To 2 ml of mercury in an Erlenmeyer flask add 20 ml of nitric acid. Shake the flask under a hood to break up the mercury into small globules. After about 10 min add 35 ml of water and, if a precipitate or crystals appear, add sufficient dilute nitric acid (1 in 5, prepared from nitric acid free from the oxides which have been removed by blowing air through it until it is colourless) to dissolve the separated solid. Add sodium hydroxide solution (1 in 10), dropwise, with thorough mixing, until the curdy precipitate that forms after the addition of each drop no longer redissolves but is dispersed to form a suspension. Add 5 ml more of the dilute nitric acid, and mix well. Prepare this solution fresh.

**Murexide Indicator Preparation**

Add 0.4 g of murexide to 40 g of powdered potassium sulfate, and grind in a glass mortar to a homogeneous mixture. (Tablets containing 0.4 mg of murexide admixed with potassium sulfate or potassium chloride are available commercially.)

**Naphthalenediol TS**

Dissolve 0.1 g of 2,7-dihydroxynaphthalene in 1,000 ml of sulfuric acid and allow the solution to stand in the dark until the yellow colour has disappeared (at least 18 h).

**1-Naphthol TS**

Dissolve 1 g of 1-naphthol in 25 ml of methanol. Prepare this solution freshly.

**Naphthol Green TS**

A 0.05% w/v solution of naphthol green in water.

**alpha-Naphtholbenzein TS**

A 1% w/v solution of alpha-naphtholbenzein in benzol.

**Nessler's TS**

See mercuric-potassium iodide TS, alkaline.

**Neutral Red TS**

A 0.1% w/v solution of neutral red in 50% ethanol.

**Ninhydrin TS**

A 0.2% w/v solution of ninhydrin (triketohydrindene hydrate,  $C_9H_4O_3 \cdot H_2O$ ) in water. Prepare this solution fresh.

**Nitric Acid TS, Dilute**

A solution containing about 10% w/v of  $HNO_3$ . Prepared by diluting 105 ml of nitric acid (70%) with water to make 1,000 ml.

**Nitric Acid/Sulfuric Acid TS**

Prepare about 1,000 ml of nitric acid (32-35% w/v of  $HNO_3$ ) by diluting 420 ml of nitric acid (70%) with 580 ml of distilled water, and add 30 ml of sulfuric acid.

**Nitrite Standard TS**

Dissolve 1.5 g of sodium nitrite ( $NaNO_2$ ) in 1,000 ml of carbon dioxide- and ammonia-free water. Each contains 1 mg of  $NO_2$ .

**o-Nitrobenzaldehyde TS**

Saturate a 2 N sodium hydroxide solution with o-nitrobenzaldehyde ( $NO_2C_6H_4CHO$ ).

**Orthophenanthroline TS**

Dissolve 0.15 g of orthophenanthroline ( $C_{12}H_8N_2 \cdot H_2O$ ) in 10 ml of a solution of ferrous sulfate, prepared by dissolving 1.48 g of clear crystals of ferrous sulfate ( $FeSO_4 \cdot 7H_2O$ ) in 100 ml water. The ferrous sulfate solution must be prepared immediately before dissolving the orthophenanthroline. Store the solution in well-closed containers.

**Oxalic Acid TS**

A 6.3% w/v solution of oxalic acid ( $C_2H_2O_4 \cdot 2H_2O$ ) in water (approximately N).

**Oxalic Acid/Sulfuric Acid TS**

Add an equal volume of sulfuric acid to water, and cool. To a 500 ml portion of the solution, add 25 g of oxalic acid.

**8-Oxyquinoline TS**

Dissolve 2 g of 8-oxyquinoline in 6 ml of glacial acetic acid. Add sufficient water to 100 ml. Prepare freshly before use.

**Phenol Red TS**

(Phenolsulfonphthalein TS). Dissolve 0.1 g of phenolsulfonphthalein in 100 ml of ethanol, and filter if necessary. For pH determinations, dissolve 0.1 g in 5.7 ml of 0.05 N sodium hydroxide, and dilute with carbon dioxide-free water to 200 ml.

**Phenolphthalein TS**

Dissolve 0.2 g of phenolphthalein ( $C_{20}H_{14}O_4$ ) in 60 ml of 90% ethanol and add sufficient water to make 100 ml.

**Phenolphthalein/Thymol Blue TS**

Dissolve 2 g of phenolphthalein and 0.1 g of thymol blue in 100 ml of absolute ethanol, and filter if necessary. Prepare freshly before use.

**Phenolsulfonphthalein TS**

See phenol red TS.

**Phenylhydrazine Hydrochloride/Sodium Acetate TS**

Dissolve 0.5 g of phenylhydrazine hydrochloride in 10 ml of sodium acetate TS, and filter if necessary. Prepare freshly before use.

**p-Phenylphenol TS**

On the day of use, dissolve 0.75 g of p-phenylphenol in 50 ml of sodium hydroxide TS.

**Phloroglucin/Hydrochloric Acid TS**

Dissolve 0.1 g of phloroglucin in 1 ml of ethanol, add 9 ml of hydrochloric acid, and mix well. Store in a dark place.

**Phosphomolybdic Acid TS**

Dissolve 5 g of phosphomolybdic acid ( $20MoO_3 \cdot 2H_3PO_4 \cdot 48H_2O$ ) in water, filter and dilute to 100 ml with water.

**Phosphotungstic Acid TS**

A 1% w/v solution of phosphotungstic acid (approximately  $24WO_3 \cdot 2H_3PO_4 \cdot 48H_2O$ ) in water.

**Picric Acid TS**

See trinitrophenol TS.

**Platinic Chloride TS**

A 13% w/v solution of platinic chloride in water (approximately 0.5 N).

**Platinum/Cobalt TSC**

Transfer 1.246 g of potassium chloroplatinate ( $K_2PtCl_6$ ), and 1.00 g of crystallized cobaltous chloride, ( $CoCl_2 \cdot 6H_2O$ ), into a 1,000-ml volumetric flask, dissolve in about 200 ml of water and 100 ml of hydrochloric acid, dilute to volume with water, and mix. (Use this solution only when specified in an individual monograph.)

**Potassium Acetate TS**

A 10% w/v solution of potassium acetate ( $KCOOCH_3$ ) in water.

**Potassium Acetate in Acetic Acid TS**

Dissolve 10 g of potassium acetate in 100 ml of a solution consisting of 90 ml of glacial acetic acid and 10 ml of acetic anhydride.

**Potassium Bichromate TS**

See potassium dichromate TS.

**Potassium Bromate/Potassium Bromide TS**

Dissolve 1.4 g of potassium bromate and 8.1 g of potassium bromide in sufficient water to make 100 ml.

**Potassium Chloride/Hydrochloric Acid TS**

Dissolve 25 g of potassium chloride in 0.85 ml of hydrochloric acid and 75 ml of water.

**Potassium Chromate TS**

A 10% w/v solution of potassium chromate ( $K_2CrO_4$ ) in water.

**Potassium Cyanate TS**

Dissolve 1 g of potassium cyanate in 9 ml of water. Prepare freshly before use.

**Potassium Cyanide TS (PbT)**

Dissolve 50 g of potassium cyanide in sufficient purified water to make 100 ml. Remove the lead by shaking with portions of the dithizone extraction TS. Part of the dithizone remains in the aqueous phase but can be removed, if desired, by washing with chloroform. The strong potassium cyanide solution is then diluted to a concentration of 10 g per 100 ml.

**Potassium Dichromate TS**

A 7.5% w/v solution of potassium dichromate ( $K_2Cr_2O_7$ ) in water.

**Potassium Ferricyanide TS**

Dissolve 1 g of potassium ferricyanide [ $\text{K}_3\text{Fe}(\text{CN})_6$ ] in 10 ml of water. Prepare this solution fresh.

**Potassium Ferrocyanide TS**

Dissolve 1 g of potassium ferrocyanide [ $\text{K}_4\text{Fe}(\text{CN})_6 \cdot 3\text{H}_2\text{O}$ ] in 10 ml of water. Prepare this solution fresh.

**Potassium Hydroxide TS**

A 6.5% w/v solution of potassium hydroxide (KOH) in water (approximately N).

**Potassium Hydroxide TS, Ethanolic**

Place a few g (5 to 10) of potassium hydroxide in a 2-litre flask, add 1 to 1.5 L of 95% ethanol and boil on a water bath under reflux condenser from 30 to 60 min. Distil and collect the ethanol. Dissolve 40 g of potassium hydroxide, low in carbonate, in 1,000 ml of the distilled ethanol keeping the temperature below  $15.5^\circ$  while the alkali is being dissolved. This solution should remain clear.

**Potassium Iodate TS**

A 0.71% w/v solution of potassium iodate in water. Preserve in the dark.

**Potassium Iodide TS**

A 16.5% w/v solution of potassium iodide (KI) in water (approximately N). Store in a light-resistant container.

**Potassium Permanganate TS**

A 1.0% w/v solution of potassium permanganate ( $\text{KMnO}_4$ ) in water.

**Potassium Permanganate/Phosphoric Acid TS**

To 75 ml of phosphoric acid, add sufficient water to 500 ml, and dissolve 15 g of potassium permanganate in the solution.

**Potassium Pyroantimonate TS**

To 2 g of potassium pyroantimonate, add 100 ml of water. Boil the solution for about 5 min, cool quickly, and add 10 ml of 15% potassium hydroxide solution. Allow to stand for one day, and filter.

**Potassium Sodium Tartrate TS**

A 14.1% w/v solution of potassium sodium tartrate ( $\text{KNaC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$ ) in water.

**Potassium Sulfate TS**

A 1% w/v solution of potassium sulfate ( $K_2SO_4$ ) in water.

**Pyridine/Acetic Anhydride TS**

Mix 3 volumes of pyridine with 1 volume of acetic anhydride. Prepare freshly before use.

**Pyridinium Chloride/Chloroform TS**

Place 75 g of anhydrous pyridine ( $C_5H_5N$ ) and approximately 400 ml of chloroform in a 2-litre graduated cylinder. Weigh the cylinder and cool it in an ice water bath. Bubble dry hydrogen chloride slowly through the solution. At intervals of several min interrupt the flow of gas, remove, dry and weigh the cylinder and its contents to determine the rate of flow of the gas. When approximately 35 g have been added, stop the flow of gas, warm the mixture to room temperature and expel the vapours with a stream of dry air. Add 100 ml of anhydrous pyridine and dilute to 1,000 ml with chloroform. When most of the reagent has been used, discard the last 100 ml.

**Quimociac TS**

Dissolve 70 g of sodium molybdate ( $Na_2MoO_4 \cdot 2H_2O$ ) in 150 ml of water (Solution A). Dissolve 60 g of citric acid in a mixture of 85 ml of nitric acid and 150 ml of water, and cool (Solution B). Gradually add Solution A to Solution B, with stirring, to produce Solution C. Dissolve 5.0 ml of synthetic quinoline in a mixture of 35 ml of nitric acid and 100 ml of water (Solution D). Gradually add Solution D to Solution C, mix well, and allow to stand overnight. Filter the mixture, add 280 ml of acetone to the filtrate, dilute to 1,000 ml with water, and mix. Store in a polyethylene bottle. Caution. This reagent contains acetone. Do not use near an open flame. Operations involving heating or boiling should be conducted in a well-ventilated hood.

**Quinaldine Red TS**

A 0.1% w/v solution of quinaldine red in glacial acetic acid.

**Salicylaldehyde TS**

A 20% v/v solution of salicylaldehyde in ethanol.

**Schiff's TS**

Aqueous solution of 0.125 g of crystalline rosamine chlorohydrate in 1,000 ml and discoloured with sulfurous acid.

**Schiff's TS, Modified**

Dissolve 0.2 g of rosaniline hydrochloride ( $C_{20}H_{20}ClN_3$ ) in 120 ml of hot water. Cool, add 2 g of sodium bisulfite ( $NaHSO_3$ ) followed by 2 ml of hydrochloric acid, and dilute to 200 ml with water. Store in a brown bottle at  $15^\circ$  or lower.

**Silicotungstic Acid TS**

Dissolve 10 g of silicotungstic acid ( $\text{SiO}_2 \cdot 12\text{WO}_3 \cdot 26\text{H}_2\text{O}$ ) in water and neutralize with 10% sodium hydroxide solution to a methyl red endpoint. Dilute to approximately 100 ml.

**Silver Ammonionitrate TS**

See Silver ammonium nitrate TS.

**Silver Ammonium Nitrate TS**

Dissolve 1 g of silver nitrate in 20 ml of water. Add ammonia TS, dropwise, with constant stirring, until the precipitate is almost but not entirely dissolved. Filter, and store in a tight, light-resistant container.

**Silver Nitrate TS**

A 4.2% w/v solution of silver nitrate ( $\text{AgNO}_3$ ) in water (approximately 0.25 N).

**Silver Nitrate TS, Acid**

Dissolve 15 g of silver nitrate in 50 ml of water, add 400 ml of ethanol and several drops of concentrated nitric acid. This solution is standardized against 0.05 N ammonium thiocyanate by the Volhard method. The solution is very stable.

**Silver Nitrate Spray TS**

Prepare the following two solutions:

- (a) Dissolve 50 g of silver nitrate in 450 ml of distilled water. Store in an amber bottle.
- (b) Add 120 ml of concentrated ammonium hydroxide to 330 ml of distilled water.

When required combine equal volumes of solutions (a) and (b) for use as spray reagent.

**Sodium Acetate TS**

A 13.6% w/v solution of sodium acetate in water (approximately N).

**Sodium Azide TS**

A 5% w/v solution of sodium azide in water.

**Sodium Bisulfite TS**

Prepare a solution of sodium bisulfite in water (approximately 0.5 N). Check the pH and if necessary, adjust to the range 3.0 to 4.5 with dilute sulfuric acid or sodium hydroxide.



**Sodium Bitartrate TS**

A 1% w/v solution of sodium bitartrate ( $\text{NaHC}_4\text{H}_4\text{O}_6 \cdot \text{H}_2\text{O}$ ) in water (approximately N). Prepare this solution fresh.

**Sodium Borate TS**

A 2% w/v solution of sodium borate ( $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ ) in water.

**Sodium Carbonate TS**

A 10.6% w/v solution of anhydrous sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) in water.

**Sodium Chloride TS**

A 10% w/v solution of sodium chloride in water.

**Sodium Cobaltinitrite TS**

Dissolve 10 g of sodium cobaltinitrite [ $\text{Na}_3\text{Co}(\text{NO}_2)_6$ ] in water to make 50 ml and filter if necessary.

**Sodium Ethoxide TS**

Dissolve 10 g of sodium in 120 ml of absolute ethanol, using the following method: remove surplus oil from the sodium metal with filter paper, weigh in benzol and again dry on a filter paper. Cut the weighed metal into small pieces about the size of a pea and carefully add one or two pieces at a time to a 500-ml conical flask which is fitted with a water-cooled reflux condenser and contains the 120 ml of ethanol.

**Sodium Fluorescein TS**

A 0.1% w/v solution of sodium fluorescein in 50% ethanol.

**Sodium Fluoride TS**

Dry about 0.5 g of sodium fluoride ( $\text{NaF}$ ) at  $200^\circ$  for 4 h. Weigh accurately 0.222 g of the dried sodium fluoride, and dissolve it in sufficient water to make exactly 100 ml. Transfer 10.0 ml of this solution into a 1,000-ml volumetric flask, dilute to volume with water, and mix. Each ml of this final solution corresponds to 0.01 mg of fluorine (F).

**Sodium Hydrogen Sulfite TS**

A 33.3% w/v solution of sodium hydrogen sulfite in water. Prepare freshly before use.

**Sodium Hydroxide TS**

Dissolve 4.3 g of sodium hydroxide in water to make 100 ml (approximately N).

**Sodium Hydroxide TS (5%), Methanolic**

Dissolve 5 g of sodium hydroxide in 5 ml of water, then add sufficient methanol to make 100 ml. Use supernatant.

**Sodium Indigotindisulfonate TS**

See indigo carmine TS.

**Sodium Nitrite TS**

A 10% w/v solution of sodium nitrite in water. Prepare freshly before use.

**Sodium Nitroferricyanide TS**

A 5% w/v solution of sodium nitroferricyanide  $[\text{Na}_2\text{Fe}(\text{NO})(\text{CN})_5 \cdot 2\text{H}_2\text{O}]$  in water. Prepare this solution fresh.

**Sodium Nitroprusside TS**

See sodium nitroferricyanide TS.

**Sodium Phosphate TS**

See sodium phosphate TS, dibasic.

**Sodium Phosphate TS, Dibasic**

A 12% w/v solution of clear crystals of dibasic sodium phosphate ( $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ ) in water.

**Sodium Phosphate TS, Monobasic**

A 62.4% w/v solution of monobasic sodium phosphate ( $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ ) in water (approximately 4 M).

**Sodium Starch Glycolate TS (5%)**

Moisten 5 g of sodium starch glycolate with a few drops of ethanol, add 100 ml of water and boil for 2-3 min, and cool.

**Sodium Starch Glycolate TS (1%)**

Dilute 10 ml of sodium starch glycolate TS (5%) to 50 ml with distilled water. Prepare freshly before use.

**Sodium Sulfide TS**

A 10% w/v solution of sodium sulfide ( $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ ) in water. Prepare this solution fresh.

**Sodium Sulfide TS (PbT)**

Dissolve 10 g of sodium sulfide (PbT) in sufficient water to make 100 ml and filter.

**Sodium Thiosulfate TS**

Use 0.1 N sodium thiosulfate.

**Stannous Chloride TS**

Dissolve 3.2 g of stannous chloride ( $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ ) in 40 ml of 0.3 N hydrochloric acid. Transfer the solution to a 100-ml volumetric flask and dilute to the mark with 0.3 N hydrochloric acid. Prepare fresh daily. The stannous chloride solution should be titrated with sulfuric periodic acid TS before use and adjusted so that 10.0 ml of the stannous chloride reagent will titrate 10.2 ml of the periodic acid reagent. For the titration, 5 ml of concentrated hydrochloric acid are added to 10 ml of stannous chloride plus 1 ml of starch indicator (a blue colour indicates the endpoint).

**Starch TS**

Triturate 1 g of arrowroot starch with 10 ml of cold water, and pour slowly, with constant stirring, into 200 ml of boiling water. Boil the mixture until a thin, translucent fluid is obtained. (Longer boiling than necessary renders the solution less sensitive.) Allow to settle, and use only the clear, supernatant liquid. Prepare this solution fresh.

**Starch Iodide Paste TS**

Heat 100 ml of water in a 250-ml beaker to boiling, add a solution of 0.75 g of potassium iodide (KI) in 5 ml of water, then add 2 g of zinc chloride ( $\text{ZnCl}_2$ ) dissolved in 10 ml of water, and, while the solution is boiling, add with stirring a smooth suspension of 5 g of potato starch in 30 ml of cold water. Continue to boil for 2 min, then cool. Store in a well-closed container in a cool place. This mixture must show a definite blue streak when a glass rod dipped in a mixture of 1 ml of 0.1 M sodium nitrite, 500 ml of water, and 10 ml of hydrochloric acid, is streaked on a smear of the paste.

**Starch Mucilage TS**

See Starch TS.

**Sulfanilic Acid TS**

A 0.8% w/v solution of sulfanilic acid ( $\text{p-NH}_2 \cdot \text{C}_6\text{H}_4\text{SO}_3\text{H} \cdot \text{H}_2\text{O}$ ) in acetic acid. Store in a tight container.

**Sulfanilic Acid/ $\alpha$ -Naphthylamine TS**

Dissolve 0.5 g of sulfanilic acid in 150 ml of acetic acid. Dissolve 0.1 g of  $\alpha$ -naphthylamine in 0.26 g of hydrochloric acid and 150 ml of acetic acid, and mix. When a pink colour is produced upon standing, add zinc dust to decolourize.

**Sulfuric Acid TS**

Add a quantity of sulfuric acid of known concentration to sufficient water to adjust the final concentration to between 94.5 and 95.5% of H<sub>2</sub>SO<sub>4</sub>.

**Sulfuric Acid TS, Dilute**

A solution containing 10% w/v of H<sub>2</sub>SO<sub>4</sub>. Prepare by cautiously adding 57 ml of sulfuric acid (95-98%) or sulfuric acid TS to about 100 ml of water, then cool to room temperature, and dilute with water to 1,000 ml (approximately 2 N).

**Sulfuric Acid/Periodic Acid TS**

Dissolve 3.42 g of periodic acid (H<sub>5</sub>IO<sub>6</sub>) in 100 ml of 0.25 M sulfuric acid. Transfer the solution to a 500-ml volumetric flask and dilute to the mark with 0.25 M sulfuric acid (approximately 0.03 M sulfuric acid/periodic acid).

**Tannic Acid TS**

Dissolve 1 g of tannic acid (tannin) in 1 ml of ethanol, and add water to make 10 ml. Prepare this solution fresh.

**Tannic Acid/Glacial Acetic Acid TS**

Dissolve 10 mg of tannic acid in 80 ml of glacial acetic acid while shaking, and add 32 ml of phosphoric acid. Prepare freshly before use.

**Tartrate Solution TS, Alkaline**

Dissolve 34.6 g of potassium sodium tartrate (Rochelle salt) and 10 g of sodium hydroxide in water, dilute to 100 ml, let stand two days, and filter through glass wool.

**Thymol Blue TS**

Dissolve 0.1 g of thymol blue in 100 ml of ethanol, and filter if necessary. For pH determinations, dissolve 0.1 g in 4.3 ml of 0.05 N sodium hydroxide, and dilute with carbon dioxide-free water to 200 ml.

**Thiourea TS**

A 10% w/v solution of thiourea in water.

**Thymolphthalein TS**

Dissolve 0.1 g of thymolphthalein in 100 ml of ethanol, and filter if necessary.

**Tin (II) Sulfate TS**

Add 10 g of tin (II) sulfate to 100 ml of 1% sulfuric acid. Agitate continuously for several hours decanting the solution from the insoluble fraction at frequent intervals.

**Triketohydrindene Hydrate TS**

See Ninhydrin TS.

**Trinitrophenol TS**

(Picric acid TS). Dissolve the equivalent of 1 g of anhydrous trinitrophenol in 100 ml of hot water. Cool the solution, and filter if necessary.

**Uranyl Acetate TS**

Dissolve 1 g of uranyl acetate in 20 ml of water by shaking well and filter.

**Uranyl Zinc Acetate TS**

Dissolve 10 g of uranyl acetate  $[(\text{CH}_3\text{COO})_2\text{UO}_2 \cdot 2\text{H}_2\text{O}]$  by heating with 50 ml of water and 5 ml of acetic acid (a solution containing approximately 30% w/v of  $\text{CH}_3\text{COOH}$ , in water, approximately 5 N). Dissolve 3 g of zinc acetate  $[(\text{CH}_3\text{COO})_2\text{Zn}]$  in 30 ml of water and 3 ml of 30% w/v acetic acid (approximately 5 N). Mix the two solutions, allow to cool to room temperature, and remove by filtration any solid material which separates.

**Vanadic Acid/Molybdic Acid TS**

Dissolve 1.12 g of ammonium metavanadate in about 300 ml of warm water, add 250 ml of nitric acid. Combine the cooled solution with another solution of 27 g of ammonium molybdate in about 400 ml of warm water, then add sufficient amount of water to make 1,000 ml. Use after 3 to 4 days of preservation in a dark-coloured bottle.

**Xylenol Orange TS**

A 0.1% w/v of xylenol orange in ethanol.

**Zinc Amalgam TS**

Add about 10 g of granulated zinc to sufficient mercury, about 20 ml, to produce a liquid amalgam on cooling, and heat at  $150^\circ$ , with stirring, until the zinc is dissolved. Zinc amalgam may be used repeatedly until the content of zinc is reduced to 0.2% w/w, as determined by the following process. Fill a pycnometer with mercury at  $25^\circ \pm 1^\circ$ , and weigh. Repeat the operation, using the amalgam. Calculate the proportion of zinc from the formula:

$$\text{Percentage w/w of zinc} = (13.534 - A) / 0.000875$$

where  $A = (\text{wt of amalgam} - 13.534) / \text{wt of mercury}$

**Zinc Sulfate TS**

A 10% w/w solution of zinc sulfate ( $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ) in water.

**Zinc Sulfate TS, Standard**

Dissolve 0.440 g of zinc sulfate in sufficient water to produce 1,000 ml, and dilute 50 ml of the solution to 1,000 ml with water. Each ml of the solution contains 5 µg of zinc.

**Zirconium/Alizarin TS**

Dissolve 0.80 g of zirconium nitrate [Zr(NO<sub>3</sub>)<sub>2</sub>·5H<sub>2</sub>O] in water, add a few drops of 4 N nitric acid, and make up to 100 ml with water. Dissolve 0.10 g of alizarin sulfonate monohydrate in 20 ml of water, and make up to 100 ml with ethanol. Mix 1 ml of the first solution with 1 ml of the second solution and add 18 ml of water. This solution must be clear and the dilution should be freshly prepared.

**Zwikker's TS**

Mix 1 ml of pyridine with 4 ml of a 10% aqueous solution of copper sulfate and 5 ml of water.



## VOLUMETRIC SOLUTIONS

### Normal Solutions

A normal solution contains 1 g equivalent weight of the solute per litre of solution. The normalities of solutions used in volumetric determinations are designated as 1 N; 0.1 N; 0.05 N; etc.

### Molar Solutions

A molar solution contains 1 g molecular weight of the solute per litre of solution. The molarities of such solutions are designated as 1 M; 0.1 M; 0.05 M; etc.

### Preparation and Methods of Standardization

The details for the preparation and standardization of solutions used in several normalities are usually given only for those most frequently required. Solutions of other normalities are prepared and standardized in the same general manner as described. Solutions of lower normalities may be prepared accurately by making an exact dilution of a stronger solution, but solutions prepared in this way should be restandardized before use.

Dilute solutions that are not stable, such as 0.01 N potassium permanganate and sodium thiosulfate, are preferably prepared by diluting exactly the higher normality with thoroughly boiled and cooled water on the same day they are to be used.

All volumetric solutions should be prepared, standardized, and used at the standard temperature of 20°, if practicable. When a titration must be carried out at a markedly different temperature, the volumetric solution should be standardized at that same temperature, or a suitable temperature correction should be made. Since the strength of a standard solution may change upon standing, the normality or molarity factor should be redetermined frequently.

Although the directions provide only one method of standardization, other methods of equal or greater accuracy may be used. For substances available as certified primary standards, or of comparable quality, the final standard solution may be prepared by weighing accurately a suitable quantity of the substance and dissolving it to produce a specific volume solution of known concentration. Hydrochloric and sulfuric acids may be standardized against a certified primary standard.

In volumetric assays described, the number of mg of the test substance equivalent to 1 ml of the primary volumetric solution is given. In general, these equivalents may be derived by simple calculation.

#### **0.1 N Ammonia, (3.505 g of NH<sub>4</sub>OH per litre)**

Add sufficient water to about 35 ml of ammonia TS to 1,000 ml. Standardize the solution with 0.1 N hydrochloric acid, using bromophenol blue TS as the indicator.

#### **0.1 N Ammonium Thiocyanate, (7.612 g of NH<sub>4</sub>SCN per litre)**

Dissolve about 8 g of ammonium thiocyanate, NH<sub>4</sub>SCN, in 1,000 ml of water, and standardize by titrating the solution against 0.1 N silver nitrate as follows: transfer about 30



ml of 0.1 N silver nitrate, accurately measured, into a glass-stoppered flask. Dilute with 50 ml of water, then add 2 ml ferric ammonium sulfate TS and 2 ml of nitric acid, and titrate with the ammonium thiocyanate solution to the first appearance of a red-brown colour. Calculate the normality, and, if desired, adjust the solution to exactly 0.1 N. If desired, 0.1 N ammonium thiocyanate may be replaced by 0.1 N potassium thiocyanate where the former is directed in various tests and assays.

#### **0.01 M Barium Chloride**

Dissolve 2.44 g of barium chloride in sufficient water, freshly boiled and cooled, to make 1,000 ml.

#### **0.1 N Bromine, (7.990 g of Br per litre)**

Dissolve 3 g of potassium bromate,  $\text{KBrO}_3$ , and 15 g of potassium bromide,  $\text{KBr}$ , in sufficient water to make 1,000 ml, and standardize the solution as follows: transfer about 25 ml of the solution, accurately measured, into a 500-ml iodine flask, and dilute with 120 ml of water. Add 5 ml of hydrochloric acid, stopper the flask, and shake it gently. Then add 5 ml of potassium iodide TS, re-stopper, shake the mixture, allow it to stand for 5 min, and titrate the liberated iodine with 0.1 N sodium thiosulfate, adding starch TS near the end of the titration. Calculate the normality. Store this solution in dark, glass-stoppered bottles.

#### **0.1 N Ceric Sulfate, (33.22 g of $\text{Ce}(\text{SO}_4)_2$ per litre)**

Transfer 59 g of ceric ammonium nitrate,  $\text{Ce}(\text{NO}_3)_4 \cdot 2\text{NH}_4\text{NO}_3 \cdot 2\text{H}_2\text{O}$ , to a beaker, add 31 ml of sulfuric acid, mix, and cautiously add water, in 20 ml portions, until solution is complete. Cover the beaker, let stand overnight, filter through a sintered-glass crucible of fine porosity, add water to make 1,000 ml, and mix.

Standardize the solution as follows: weigh accurately 200 mg of primary standard arsenic trioxide,  $\text{As}_2\text{O}_3$ , previously dried at  $100^\circ$  for 1 h, and transfer to a 500-ml Erlenmeyer flask. Wash down the inner walls of the flask with 25 ml of sodium hydroxide solution (2 in 5), swirl to dissolve the sample, and when solution is complete add 100 ml of water, and mix. Add 10 ml of dilute sulfuric acid (1 in 3) and 2 drops each of orthophenanthroline TS and a solution of osmium tetroxide in 0.1 N sulfuric acid (1 in 400), and slowly titrate with the ceric sulfate solution until the pink colour is changed to a very pale blue. Calculate the normality. Each 4.964 mg of  $\text{As}_2\text{O}_3$  is equivalent to 1 ml of 0.1 N ceric sulfate.

#### **0.01 N Ceric Sulfate, for Tocopherol Assay (3.322 g of $\text{Ce}(\text{SO}_4)_2$ per litre)**

Dissolve 4.2 g of ceric sulfate,  $\text{Ce}(\text{SO}_4)_2 \cdot 4\text{H}_2\text{O}$ , or 5.5 g of the acid sulfate  $\text{Ce}(\text{HSO}_4)_4$ , in about 500 ml of water containing 28 ml of sulfuric acid, and dilute to 1,000 ml. Allow the solution to stand overnight, and filter.

Standardize this solution daily as follows: weigh accurately about 275 mg of hydroquinone,  $\text{C}_6\text{H}_6\text{O}_2$ , dissolve it in sufficient 0.5 N ethanolic sulfuric acid to make 500 ml, and mix. To 25 ml of this solution add 75 ml of 0.5 N sulfuric acid, 20 ml of water, and 2 drops of diphenylamine TS. Titrate with the ceric sulfate solution at a rate of about 25 drops per 10 sec until the red point is reached which persists for 10 sec. Perform a blank determination using 100 ml of 0.5 N ethanolic sulfuric acid, 20 ml of water, and 2 drops of diphenylamine TS, and make any necessary correction. Calculate the normality of the ceric sulfate solution by

the formula  $0.05W/55.057V$ , in which  $W$  is the weight, in mg, of the hydroquinone sample taken, and  $V$  is the volume, in ml, of the ceric sulfate solution consumed in the titration.

**0.05 M Disodium Ethylenediaminetetraacetate (EDTA), (16.811 g of  $C_{10}H_{14}O_8N_2Na_2$  per litre)**

Dissolve 18.7 g of disodium ethylenediaminetetraacetate in sufficient water, freshly boiled and cooled, to make 1,000 ml.

Standardize the solution as follows: weigh accurately about 0.2 g of chelometric standard calcium carbonate,  $CaCO_3$ , transfer to a 400-ml beaker, add 10 ml of water, and swirl to form a slurry. Cover the beaker with a watch glass, and introduce 2 ml of dilute hydrochloric acid TS from a pipet inserted between the lip of the beaker and the edge of the watch glass. Swirl the contents of the beaker to dissolve the calcium carbonate. Wash down the sides of the beaker, the outer surface of the pipet, and the watch glass, and dilute to about 100 ml with water. While stirring preferably with a magnetic stirrer, add about 30 ml of the disodium EDTA solution from a 50-ml buret, then add 15 ml of sodium hydroxide TS and 300 mg of hydroxynaphthol blue indicator, and continue the titration to a blue end-point. Calculate the molarity by the formula  $W/100.09V$ , in which  $W$  is the weight, in mg, of  $CaCO_3$  in the sample of calcium carbonate taken, and  $V$  is the volume, in ml, of disodium EDTA solution consumed. Each 5.004 mg of  $CaCO_3$  is equivalent to 1 ml 0.05 M disodium EDTA.

**0.1 N Ferrous Ammonium Sulfate, (28.405 g of  $FeSO_4(NH_4)_2SO_4$  per litre)**

Dissolve 40 g of ferrous ammonium sulfate hexahydrate in a 100 ml portion of a mixture of 100 ml of sulfuric acid and 100 ml of water previously cooled, add water to 1,000 ml, and standardize as follows:

Titrate 25 ml of this solution with 0.1 N ceric sulfate, using 2 drops of orthophenanthroline TS as the indicator, until a red colour of the solution changes to pale blue. Calculate the normality from the volume of 0.1 N ceric sulfate consumed.

**10 N Hydrochloric Acid**

Prepare and standardize, as directed under 1 N hydrochloric acid, using 950 ml of hydrochloric acid.

**6 N Hydrochloric Acid**

Prepare and standardize, as directed under 1 N hydrochloric acid, using 570 ml of hydrochloric acid.

**2 N Hydrochloric Acid**

Prepare and standardize, as directed under 1 N hydrochloric acid, using 190 ml of hydrochloric acid.

**1 N Hydrochloric Acid, (36.461 g of HCl per litre)**

Dilute 95 ml of hydrochloric acid with water to 1,000 ml. Standardize by one of the following methods:

Method I: Dissolve about 1.5 g sodium carbonate (standard reagent) previously dried at about 270° for 1 h and accurately weighed, in 100 ml of water, and titrate with hydrochloric acid, using 2 drops of bromophenol blue TS as the indicator. Near the endpoint, boil to expel carbon dioxide, cool and continue to titrate. Calculate the normality.

Method II: Add 130 ml of water and 5 drops of nitric acid to 20 ml of 1 N hydrochloric acid. While stirring constantly, add about 40 ml of silver nitrate solution (1 in 10) or even more if necessary, until the precipitation is completed. Boil the mixture gently for 5 min, allow to stand in the dark until the precipitate settles. Transfer the precipitate completely into a tared Gooch crucible, dry to constant weight at 110°, and wash with water, slightly acidified with nitric acid, until the washings give no reaction for silver. Dry to constant weight at about 110°. From the weight of silver chloride obtained, calculate the normality of hydrochloric acid.

### **0.5 N Hydrochloric Acid**

Using 47.5 ml of hydrochloric acid, prepare and standardize, as directed under 1 N hydrochloric acid.

### **0.1 N Hydrochloric Acid**

Prepare this solution by diluting 1 N hydrochloric acid with water to 10 volumes, or using 9.5 ml of hydrochloric acid, prepare as directed under 1 N hydrochloric acid. Standardize as directed under 1 N hydrochloric acid.

### **0.02 N Hydrochloric Acid**

Dilute 0.1 N hydrochloric acid with water to 5 volumes, and standardize as directed under 1 N hydrochloric acid.

### **0.01 N Hydrochloric Acid**

Dilute 0.1 N hydrochloric acid with water to 10 volumes, and standardize as directed under 1 N hydrochloric acid.

### **0.002 N Hydrochloric Acid**

Dilute 0.1 N hydrochloric acid with water to 50 volumes.

### **0.001 N Hydrochloric Acid**

Dilute 0.1 N hydrochloric acid with water to 100 volumes.

### **0.5 N Hydroxylamine Hydrochloride, (34.745 g of NH<sub>2</sub>OH·HCl per litre)**

Dissolve 35 g of hydroxylamine hydrochloride in 150 ml of water, and dilute to 1,000 ml with anhydrous methanol. To 500 ml of this solution add 15 ml of a 0.04% solution of bromophenol blue in ethanol, and titrate with 0.5 N triethanolamine until the solution appears greenish blue by transmitted light. Prepare this solution fresh before use.

**0.1 N Iodine, (12.690 g of iodine per litre)**

Dissolve 14 g of iodine in a solution of 36 g of potassium iodide dissolved in 100 ml of water. Add 3 drops of hydrochloric acid and water to 1,000 ml, and standardize as follows.

Weight accurately about 0.15 g of arsenic trioxide previously pulverized and dried to constant weight at 100°, and dissolve in 20 ml of 1 N sodium hydroxide by heating if necessary. Dilute with about 40 ml of water, add 2 drops of methyl orange TS, and add dilute hydrochloric acid until the yellow colour changes to pale pink. Add 2 g of sodium bicarbonate, dilute with 50 ml of water, and add 3 ml of starch TS. Titrate with 0.1 N iodine until a sustaining blue colour is produced. Store in a glass stoppered bottle and restandardize frequently. Calculate the normality. Each 4.946 mg of  $\text{As}_2\text{O}_3$  is equivalent to 1 ml of 0.1 N iodine.

**0.1 N Lithium Methoxide, (3.797 g of  $\text{CH}_3\text{OLi}$  per litre)**

Dissolve 600 mg of freshly cut lithium metal in a mixture of 150 ml of absolute methanol and 850 ml of benzene. Filter the resulting solution if it is cloudy, and standardize it as follows: dissolve about 80 mg of benzoic acid, accurately weighed, in 35 ml of dimethylformamide, add 5 drops of thymol blue TS, and titrate with the lithium methoxide solution to a dark blue endpoint. (Caution. Protect the solution from absorption of carbon dioxide and moisture by covering the titration vessel with aluminium foil while dissolving the benzoic acid sample and during the titration.) Each ml of 0.1 N lithium methoxide is equivalent to 12.21 mg of benzoic acid.

**0.1 N Magnesium Chloride, (4.761 g of  $\text{MgCl}_2$  per litre)**

Dissolve 10.5 g of magnesium chloride in freshly boiled and cooled water to make 1,000 ml.

**0.1 M Mercuric Nitrate, (32.46 g of  $\text{Hg}(\text{NO}_3)_2$  per litre)**

Dissolve about 35 g of mercuric nitrate,  $\text{Hg}(\text{NO}_3)_2 \cdot \text{H}_2\text{O}$ , in a mixture of 5 ml of nitric acid and 500 ml of water, and dilute with water to 1,000 ml. Standardize the solution as follows: transfer an accurately measured volume of about 20 ml of the solution into an Erlenmeyer flask, and add 2 ml of ferric ammonium sulfate TS. Cool to below 20°, and titrate with 0.1 N ammonium thiocyanate to the first appearance of a permanent brownish colour. Calculate the molarity.

**0.1 N Oxalic Acid, (4.502 g of  $\text{H}_2\text{C}_2\text{O}_4$  per litre)**

Dissolve 6.45 g of oxalic acid,  $\text{H}_2\text{C}_2\text{O}_4 \cdot 2\text{H}_2\text{O}$ , in sufficient water to make 1,000 ml. Standardize by titration against freshly standardized 0.1 N potassium permanganate as directed under Potassium Permanganate 0.1 N. Store this solution in glass-stoppered bottles, protected from light.

**0.1 N Perchloric Acid, (10.046 g of  $\text{HClO}_4$  per litre)**

Transfer about 8.5 ml of 70% perchloric acid into a 1,000-ml flask, add 950 ml of glacial acetic acid, and shake well. Add 15 ml of acetic anhydride gradually by dividing into 1 ml portions, and then dilute with acetic acid to 1,000 ml. Allow the solution to stand overnight.

Standardize as follows: add 50 ml of glacial acetic acid to 0.4 g of potassium biphthalate, previously dried at 120° for 1 h and accurately weighed, and heat to dissolve on a water bath. Titrate with 0.1 N perchloric acid to the end point, at which the colour changes from violet to blue, using 1 ml of 0.05% acetic anhydride solution of crystal violet as the indicator, and calculate the normality by the following formula:

Normality factor = (Weight of potassium biphthalate (g) x 1,000 x 10) / (The number of ml of 0.1 N perchloric acid consumed x 204.22)

**0.1 N Potassium Acid Phthalate, (20.42 of  $\text{KHC}_6\text{H}_4(\text{COO})_2$  per litre)**

Dissolve 20.42 g of primary standard potassium biphthalate,  $\text{KHC}_6\text{H}_4(\text{COO})_2$ , in glacial acetic acid in a 1,000-ml volumetric flask, warming on a steam bath if necessary to effect solution and protecting the solution from contamination by moisture. Cool to room temperature, dilute to volume with glacial acetic acid, and mix.

**0.1 N Potassium Bromate, (2.784 g of  $\text{KBrO}_3$  per litre)**

Dissolve 2.8 g of potassium bromate in sufficient water to make 1,000 ml.

Standardize as follows: transfer 40 ml of the solution into a glass-stoppered flask, and add 3 g of potassium iodide and 3 ml of hydrochloric acid. Stopper tightly, and allow to stand for 5 min in the dark. Titrate the free iodine with 0.1 N sodium thiosulfate, using starch TS as the indicator. Perform a blank test in the same manner as the sample.

**0.1 N Potassium Dichromate, (4.903 g of  $\text{K}_2\text{Cr}_2\text{O}_7$  per litre)**

Dissolve 4.904 g of potassium dichromate previously pulverized and dried to constant weight at 120°, in sufficient water to make 1,000 ml.

**0.5 N Potassium Hydroxide, Ethanolic**

Dissolve about 35 g of potassium hydroxide, KOH, in 20 ml of water, and sufficient aldehyde-free alcohol to make 1,000 ml. Allow the solution to stand in a tightly stoppered bottle for 24 h. Then quickly decant the clear supernatant liquid into a suitable, tight container, and standardize as follows: transfer quantitatively 25 ml of 0.5 N hydrochloric acid into a flask, dilute with 50 ml of water, add 2 drops of phenolphthalein TS, and titrate with the ethanolic potassium hydroxide solution until a permanent, pale pink colour is produced. Calculate the normality. Store this solution in a tightly stoppered bottle protected from light.

**1 N Potassium Hydroxide, (56.109 g of KOH per litre)**

Using about 70 g of potassium hydroxide, prepare and standardize as directed under 1 N sodium hydroxide. Each 204.2 mg of  $\text{KHC}_6\text{H}_4(\text{COO})_2$  is equivalent to 1 ml of 1 N potassium hydroxide.

**0.5 N Potassium Hydroxide**

Dilute 1 N potassium hydroxide with water freshly boiled and cooled to 5 volumes, or using about 35 g of potassium hydroxide, prepare as directed under 1 N potassium hydroxide.

**0.1 N Potassium Hydroxide**

Dilute 1 N potassium hydroxide with water, freshly boiled and cooled, to 10 volumes, or using about 7 g of potassium hydroxide, prepare as directed under 1 N potassium hydroxide. Standardize as directed under 1 N potassium hydroxide.

**0.05 M Potassium Iodate, (10.70 g of KIO<sub>3</sub> per litre)**

Dissolve 10.700 g of potassium iodate of primary standard quality, KIO<sub>3</sub>, previously dried at 110° to constant weight, in sufficient water to make 1,000 ml.

**0.1 N Potassium Permanganate, (3.161 g of KMnO<sub>4</sub> per litre)**

Dissolve about 3.3 g of potassium permanganate in 1,000 ml of water, and boil for about 15 min. Allow to stand in a tightly closed flask for at least 2 days, and filter through a fine porosity sintered glass crucible. Store in a glass-stoppered, light-resistant bottle, and restandardize before use.

Standardize as follows: dissolve 0.2 g of sodium oxalate previously dried at 110° to constant weight and accurately weighed, in about 250 ml of water. Add 7 ml of sulfuric acid, heat to about 70° and titrate with 0.1 N potassium permanganate while hot. Each 6.700 mg of Na<sub>2</sub>C<sub>2</sub>O<sub>4</sub> is equivalent to 1 ml of 0.1 N potassium permanganate.

**0.01 M Potassium Sulfate, (1.743 g of K<sub>2</sub>SO<sub>4</sub> per litre)**

Dissolve 1.743 g of potassium sulfate, previously dried at 110° for 4 h, in sufficient water, freshly boiled and cooled, to make 1,000 ml.

**0.1 N Silver Nitrate, (16.99 g of AgNO<sub>3</sub> per litre)**

Dissolve about 17.5 g of silver nitrate, AgNO<sub>3</sub>, in 1,000 ml of water, and standardize the solution as follows: dilute about 40 ml, accurately measured, of the silver nitrate solution with about 100 ml of water, heat the solution, and add slowly, with continuous stirring, dilute hydrochloric acid TS until precipitation of the silver is complete. Boil the mixture cautiously for about 5 min, then allow it to stand in the dark until the precipitate has settled and the supernatant liquid has become clear. Transfer the precipitate completely to a tared filtering crucible, and wash it with small portions of water slightly acidified with nitric acid. Dry the precipitate at 110° to constant weight. Each 14.332 mg of silver chloride obtained is equivalent to 1 ml of 0.1 N silver nitrate. Protect the silver chloride from light as much as possible during the determination.

**0.05 N Sodium Arsenite, (3.248 g of NaAsO<sub>2</sub> per litre)**

Transfer 2.4725 g of arsenic trioxide, which has been pulverized and dried at 100° to constant weight, to a 1,000-ml volumetric flask, dissolve it in 20 ml of 1 N sodium hydroxide, and add 1 N sulfuric acid or 1 N hydrochloric acid until the solution is neutral or only slightly acid to litmus. Add 15 g of sodium bicarbonate, dilute to volume with water, and mix.

**0.1 N Sodium Chloride, (5.844 g of NaCl per litre)**

Dissolve 5.845 g of sodium chloride, previously dried at 110° for 2 h, in sufficient water to make 1,000 ml.

**1 N Sodium Hydroxide, (39.997 g of NaOH per litre)**

Dissolve 45 g of sodium hydroxide in about 950 ml of water, and add a saturated barium hydroxide solution, freshly prepared, until not further precipitate is formed. Shake the mixture thoroughly, and allow to stand overnight in a stoppered bottle. Decant the supernatant liquid or filter the solution, and standardize by one of the following methods. Store in a well-fitted, rubber-stoppered bottle, or in a bottle with a soda-lime tube, and restandardize frequently.

Method I: Dilute 25 ml of 1 N hydrochloric acid or 1 N sulfuric acid with 50 ml of water, freshly boiled and cooled, and titrate with 1 N sodium hydroxide, using 2 drops of phenolphthalein TS as the indicator.

Method II: Dissolve about 5 g of potassium biphthalate previously powdered, dried at 100° for 3 h and weighed accurately, in 75 ml of water, freshly boiled and cooled, and titrate with 1 N sodium hydroxide solution, using drops of phenolphthalein Ts as the indicator. Each 204.2 mg of potassium biphthalate is equivalent to 1 ml of 1 N sodium hydroxide.

**0.5 N Sodium Hydroxide**

Using about 22 g of sodium hydroxide, prepare, standardize and store, as directed under 1 N sodium hydroxide. Restandardize frequently.

**0.2 N Sodium Hydroxide**

Dilute 1 N sodium hydroxide with water, freshly boiled and cooled, to 5 volumes, or use about 9 g of sodium hydroxide and prepare as directed under 1 N sodium hydroxide. Standardize and store, as directed under 1 N sodium hydroxide. Restandardize frequently.

**0.1 N Sodium Hydroxide**

Dilute 1 N sodium hydroxide with water, freshly boiled and cooled, to 10 volumes, or use about 4.5 g of sodium hydroxide and prepare as directed under 1 N sodium hydroxide. Standardize and store, as directed under 1 N sodium hydroxide. Restandardize frequently.

**0.05 N Sodium Hydroxide**

Dilute 1 N sodium hydroxide with water, freshly boiled and cooled, to 20 volumes. Standardize and store, as directed under 1 N sodium hydroxide. Restandardize frequently.

**0.02 N Sodium Hydroxide**

Dilute 0.1 N sodium hydroxide with water, freshly boiled and cooled, to 5 volumes. Standardize and store, as directed under 1 N sodium hydroxide. Restandardize frequently.

**0.01 N Sodium Hydroxide**

Dilute 0.1 N sodium hydroxide with water, freshly boiled and cooled, to 10 volumes. Standardize and store, as directed under 1 N sodium hydroxide. Restandardize frequently.

**0.1 N Sodium Methoxide, in Pyridine, (5.40 g of CH<sub>3</sub>ONa per litre)**

Weigh 14 g of freshly cut sodium metal, and cut into small cubes. Place about 0.5 ml of anhydrous methanol in a round-bottom 250-ml flask equipped with a ground-glass joint, add 1 cube of the sodium metal, and when the reaction subsides, add the remaining sodium metal to the flask. Connect a water-cooled condenser to the flask, and slowly add 100 ml of anhydrous methanol, in small portions, through the top of the condenser. Regulate the addition of the methanol so that the vapours are condensed and do not escape through the top of the condenser. After addition of the methanol is complete, connect a drying tube to the top of the condenser, and allow the solution to cool. Transfer 17.5 ml of this solution (approximately 6 N) into a 1,000-ml volumetric flask containing 70 ml of anhydrous methanol, and dilute to volume with freshly distilled pyridine. Store preferably in the reservoir of an automatic buret suitably protected from carbon dioxide and moisture. Standardize the solution as follows: weigh accurately about 400 mg of benzoic acid, transfer it into a 250 ml wide-mouth Erlenmeyer flask, and dissolve it in 50 ml of freshly distilled pyridine. Add a few drops of thymolphthalein TS, and titrate immediately with the sodium methoxide solution to a blue endpoint. During the titration, direct a gentle stream of nitrogen into the flask through a short piece of 6-mm glass tubing fastened near the tip of the buret. Perform a blank determination, correct for the volume of sodium methoxide solution consumed by the blank, and calculate the normality. Each 12.21 mg of benzoic acid is equivalent to 1 ml of 0.1 N sodium methoxide in pyridine.

**0.1 M Sodium Nitrite, (7.900 g of NaNO<sub>2</sub> per litre)**

Dissolve 7.5 g of sodium nitrite, NaNO<sub>2</sub>, in sufficient water to make 1,000 ml, and standardize the solution as follows: Weigh accurately about 500 mg of U.S.P. Sulfanilamide Reference Standard, previously dried at 105° for 3 h, and transfer to a beaker or a casserole. Add 50 ml of water and 5 ml of hydrochloric acid, and stir well until dissolved. Cool to 15°, and add about 25 g of crushed ice, then titrate slowly with the sodium nitrite solution, stirring vigorously until a blue colour is produced immediately when a glass rod dipped in the titrated solution is streaked on a smear of starch iodide paste TS. When the titration is complete, the endpoint should be reproducible after the mixture has been standing for 1 min. Calculate the molarity. Each 17.22 mg of sulfanilamide is equivalent to 1 ml of 0.1 M sodium nitrite.

**0.1 N Sodium Thiosulfate, (15.82 g of Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> per litre)**

Dissolve about 26 g of sodium thiosulfate, Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>·5H<sub>2</sub>O, and 200 mg of sodium carbonate, Na<sub>2</sub>CO<sub>3</sub>, in 1,000 ml of recently boiled and cooled water. Standardize the solution as follows: weigh accurately about 210 mg of primary standard potassium dichromate, previously pulverized and dried at 120° for 4 h, and dissolve in 100 ml of water in a 500 ml glass-stoppered flask. Swirl to dissolve the sample, remove the stopper and quickly add 3 g of potassium iodide, KI, and 5 ml of hydrochloric acid. Stopper the flask, swirl to mix, and let stand in the dark for 10 min. Rinse the stopper and inner walls of the flask with water, and titrate the liberated iodine with the sodium thiosulfate solution until the solution is only faint



yellow in colour. Add starch TS, and continue the titration to the discharge of the blue colour. Calculate the normality.

#### **0.01 N Sodium Thiosulfate**

Dilute 0.1 N sodium thiosulfate with water, freshly boiled and cooled, to 10 volumes. Standardize as directed under 0.1 N sodium thiosulfate before use.

#### **4 N Sulfuric Acid**

This solution contains 196.155 g of  $\text{H}_2\text{SO}_4$  per 1,000 ml.

Using 120 ml of sulfuric acid, prepare and standardize, as directed under 1 N sulfuric acid.

#### **1 N Sulfuric Acid, (49.039 g of $\text{H}_2\text{SO}_4$ per litre)**

While stirring, slowly add 30 ml of sulfuric acid to about 1,000 ml of water, allow to cool to  $20^\circ$ , and standardize with sodium carbonate (standard reagent) as directed under 1 N hydrochloric acid. Each 52.99 mg of  $\text{Na}_2\text{CO}_3$  is equivalent to 1 ml of 1 N sulfuric acid.

#### **0.5 N Sulfuric Acid**

Using 15 ml of sulfuric acid, prepare and standardize, as directed under 1 N sulfuric acid.

#### **0.2 N Sulfuric Acid**

Using 6 ml of sulfuric acid, prepare and standardize, as directed under 1 N sulfuric acid.

#### **0.1 N Sulfuric Acid**

Dilute 1 N sulfuric acid with water to 10 volumes, or using 3 ml of sulfuric acid, prepare as directed under 1 N sulfuric acid. Standardize as directed under 1 N sulfuric acid.

#### **0.02 N Sulfuric Acid**

Dilute 0.1 N sulfuric acid with water to 5 volumes, and standardize as directed under 1 N sulfuric acid.

#### **0.01 N Sulfuric Acid**

Dilute 0.1 N sulfuric acid with water to 10 volumes, and standardize as directed under 1 N sulfuric acid.

#### **0.5 N Sulfuric Acid, Ethanolic**

Add cautiously, with stirring, 13.9 ml of sulfuric acid to a sufficient quantity of absolute ethanol to make 1,000 ml. Alternatively, this solution may be prepared by diluting 100 ml of 5 N sulfuric acid with absolute ethanol to make 1,000 ml.

**0.1 M Thorium Nitrate, (48.01 g of Th(NO<sub>3</sub>)<sub>4</sub> per litre)**

Weigh accurately 55.21 g of thorium nitrate Th(NO<sub>3</sub>)<sub>4</sub>·4H<sub>2</sub>O, dissolve it in water, dilute to 1,000 ml, and mix. Standardize the solution as follows: transfer 50 ml into a 500-ml volumetric flask, dilute to volume with water, and mix. Transfer 50 ml of the diluted solution into a 400-ml beaker, add 150 ml of water and 5 ml of hydrochloric acid, and heat to boiling. While stirring, add 25 ml of a saturated solution of oxalic acid, then digest the mixture for 1 h just below the boiling point and allow to stand overnight. Decant through Whatman No. 42, or equivalent, filter paper, and transfer the precipitate to the filter using about 100 ml of a wash solution consisting of 70 ml of the saturated oxalic acid solution, 430 ml of water, and 5 ml of hydrochloric acid. Transfer the precipitate and filter paper to a tared tall-form porcelain crucible, dry, char the paper, and ignite at 950° for 1.5 h or to constant weight. Cool in a desiccator, weigh, and calculate the molarity of the solution by the formula  $200W/264.04$ , in which W is the weight, in g, of thorium oxide obtained.

**0.1 N Titanous Chloride, (15.426 g of TiCl<sub>3</sub> per litre)**

Mix 200 ml of 15% titanous chloride and 150 ml of hydrochloric acid, and dilute with freshly boiled and cooled water to 2,000 ml. Transfer into a light-shaded bottle equipped with a buret, replace the air in the bottle with hydrogen, and allow to stand for 2 days before use. Standardize as follows: place 3 g of ferrous ammonium sulfate in a wide-mouthed 500 ml flask, dissolve in 50 ml of freshly boiled and cooled water in an atmosphere of carbon dioxide and add 25 ml dilute sulfuric acid (27 in 100). Pass carbon dioxide through the solution, then quickly add 40 ml of 0.1 N potassium permanganate and add 0.1 N titanous chloride until the endpoint is nearly reached. Immediately add 5 g of ammonium thiocyanate, and titrate the solution with 0.1 N titanous chloride to the end point, when the colour of the solution disappears. Perform a blank test in the same manner as the sample.

Normality factor = Volume of 0.1 N potassium permanganate added (ml) / The number of ml of 0.1 N titanous chloride consumed

**0.5 N Triethanolamine, (74 g of N(CH<sub>2</sub>CH<sub>2</sub>OH)<sub>3</sub> per litre)**

Transfer 65 ml (74 g) of 98% triethanolamine into a 1,000 ml volumetric flask, dilute to volume with water, stopper the flask, and mix thoroughly.

**0.01 M Zinc Acetate, (1.835 g of Zn(CH<sub>3</sub>COO)<sub>2</sub> per litre)**

Dissolve 2 g of zinc acetate in sufficient water to make 1,000 ml. Standardize as follows: to 25 ml of 0.01 M zinc acetate, add 2 ml of ammonia/ammonium chloride buffer solution and sufficient water to about 100 ml. Titrate the solution with 0.01 M disodium ethylenediaminetetraacetate, using 3 drops of eriochrome black TS as the indicator.

**0.025 M Zinc Chloride, (3.407 g of ZnCl<sub>2</sub> per litre)**

Place about 1.6 g of zinc in a beaker, add 30 ml of dilute hydrochloric acid, cover with a watch glass, and allow to stand. Dissolve by heating gently on a water bath after the initial rapid release of hydrogen gas. Wash the watch glass and the inside wall of the beaker with water, evaporate to almost dryness on a water bath, cool, and add water to 1,000 ml. Standardize as directed for 0.05 M zinc sulfate. Calculate the molarity.

**0.05 M Zinc Sulfate, (8.072 g of ZnSO<sub>4</sub> per litre)**

Dissolve about 15 g of zinc sulfate, ZnSO<sub>4</sub>·7H<sub>2</sub>O in sufficient water to make 1,000 ml, and standardize the solution as follows: dilute about 35 ml 0.05 M zinc sulfate accurately measured, with 75 ml of water, add 5 ml of ammonia/ammonium chloride buffer TS and 0.1 ml of eriochrome black TS, and titrate with 0.05 M disodium ethylenediaminetetraacetate until the solution is deep blue in colour. Calculate the molarity.