Food and Agriculture Organization of the United Nations

84th JECFA - Chemical and Technical Assessment (CTA), 2017

YEAST EXTRACTS CONTAINING MANNOPROTEINS

Chemical and Technical Assessment (CTA)

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1. Summary

This Chemical and Technical Assessment (CTA) summarizes data and information on yeast mannoproteins submitted to the 84th meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) upon request by the 48th Codex Committee on Food Additives (FAO/WHO, 2016). At the present meeting, JECFA was asked to evaluate all data necessary for the assessment of safety, dietary intake and specifications related to the use of yeast mannoproteins as a stabilizer in wine.

Yeast extracts containing mannoproteins have not been previously evaluated by JECFA. Yeast mannoproteins have been approved for use in wine as a stabilizer in the following countries: Argentina, Australia, Canada, EU, New Zealand and the USA.

Argentina has approved the use of yeast mannoproteins as a food additive for wine stabilization (Resolución C 19/2006), 2006. It is also approved by the Organization Internationale de la Vigne et du Vin (OIV).

Australia and New Zealand have approved the use of yeast mannoproteins as a food additive for wine stabilization in Standards 1.3.1 – Food Additives (listed in Schedule 5 Technological functions which may be performed by food additives) and 4.5.1 – Wine Production Requirements (Australia only) of the Australia New Zealand Food Standards Code. It is approved at a maximum permitted level of 400 mg/kg is considered to be appropriate, which takes into account both added mannoproteins and naturally occurring mannoproteins in wine and aligns with the Agreement between Australia and the European Community on Trade in Wine, and Protocol (1994). It allows for the use of preparations of yeast cell wall, up to 40 g/hl (400 mg/l) for wines originating in Australia and separately for wines originating in the Community.

Health Canada on 6 January 2015 also published a Proposal to enable the use of a new food additive, yeast mannoproteins, to inhibit crystal formation in wine in the List of permitted food additives with other generally accepted food uses, which came into force on 15 June 2015 at a maximum level of use of 0.04% (400 parts per million).

In the USA, a Generally Recognized as Safe (GRAS) notice for baker's yeast mannoprotein (GRN 000284) was submitted to the US Food and Drug Administration (FDA) for review on March 6,

2009. In a response letter dated August 28, 2009, the US FDA had no further questions regarding the petitioner's determination that its baker's yeast mannoprotein was GRAS for use as a stabilizing agent in wines, at levels ranging from 50 – 400 mg/l, to prevent tartaric acid precipitation. The OIV International Oenological Codex (OIV Codex) includes a specification for yeast mannoproteins in OIV Resolution Oeno 26/2004. This specification indicates that yeast mannoproteins can be used for tartaric and/or protein stabilization of wine. The European Union Council Regulation (EC) No. 2165/2005, which amends Regulation (EC) No. 1493/1999 permits 'the addition of yeast mannoproteins to ensure the tartaric and protein stabilization of wines'.

This document discusses published information relevant to yeast extracts containing mannoproteins, the production methodologies, and the specifications. The name was changed from "yeast mannoproteins" to "yeast extracts containing mannoproteins" because the name "yeast mannoproteins" was not adequately descriptive. The products in commerce are extracts containing yeast components and mannoproteins, and not pure mannoproteins.

JECFA at its 84th meeting prepared new tentative specifications for yeast extracts containing mannoproteins.

2. Description

Yeast mannoproteins are extracted from purified yeast (*Saccharomyces cerevisiae*) cell walls by enzymatic treatment with β-glucosidase or by physicochemical extraction with thermal treatment. Yeast mannoprotein represents a large family of natural compounds in which polysaccharide chains are bound to proteins and peptides by covalent and non-covalent linkages (e.g. ionic interactions). The structure and molecular weight of mannoproteins vary depending on the degree and type of glycosylation. The polysaccharide chains consist almost exclusively of mannose units linked by α-links forming a long α-1→6 linked backbone containing short α-1→2 and α-1→3 linked side-chains. Several of the side chains may have phosphodiester linkages to other mannosyl residues. The molecular weight of yeast mannoprotein ranges from 20 kDa to more than 450 kDa (Food Standards Australia New Zealand 2008). Yeast mannoproteins are a white or beige, odourless powder (powder form), or yellow, translucid colloidal solution (liquid form). In liquid form, yeast mannoproteins precipitate when one volume of ethanol is added. Yeast mannoproteins decompose by excessive heating. Storage temperatures should be 4 - 20° for powder form and 4 – 12° for liquid form.

3. Manufacturing

Yeast mannoproteins are extracted from purified yeast (*S. cerevisiae*) cell walls, via enzymatic extraction using glucan 1,3-β-glucosidase (EC 3.2.1.58), which is an approved enzyme or by physico-chemical extraction. During enzyme hydrolyses the yeast cell wall which then allows the mannoproteins to be solubilized. Figure 1 depicts the scheme of this operation. The *S. cerevisiae* cell walls, all media and devices used in the yeast mannoprotein production process are of food grade. The *S. cerevisiae* hydrolysis conditions are monitored through the enzymatic process. Each batch of yeast extracts containing mannoproteins is sterilized by ultrafiltration or pasteurization and analysed to certify the levels of chemical and microbiological contaminants.

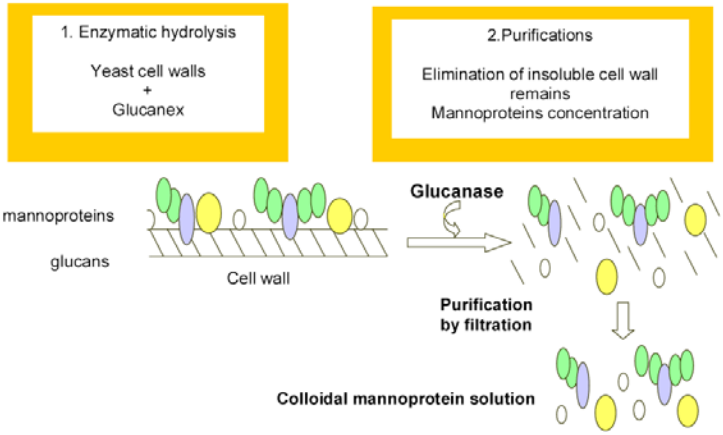

Figure 1: Depicting the enzymatic process that mimics the natural yeast lysis during fermentation and/or digestion.

Likewise, a thermal treatment applied on yeast hulls can also break the links with β-glucans to allow solubilisation of the mannoproteins, which are then separated from the insoluble cell wall material, concentrated and eventually micro- or ultra-filtered. Figure 2 depicts the scheme of this operation. The *S. cerevisiae* cell walls, all media and devices used in the yeast mannoprotein production process are of food grade. The *S. cerevisiae* hydrolysis conditions are monitored through the thermal process. Each batch of yeast extracts containing mannoproteins is sterilized by pasteurization and analysed to certify the levels of chemical and microbiological contaminants

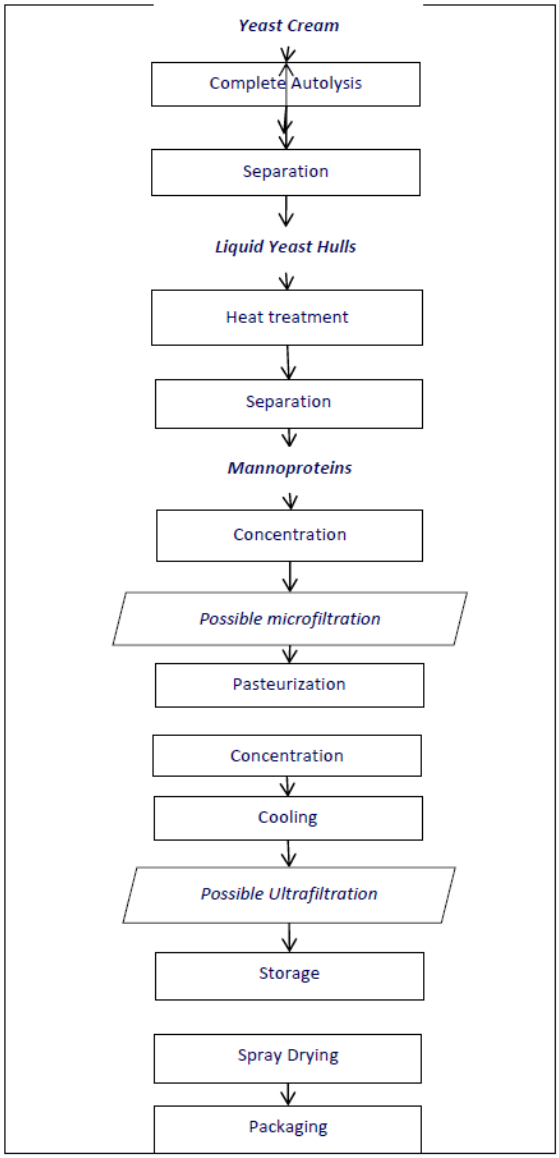

Figure 2: Thermal extraction process of mannoproteins from *S. cerevisae*

The mannoprotein products produced are commercialized in solid form or in solution. The dry product has a long lifespan, being stable for a minimum of two years if kept sealed in a dry location at 12°. The liquid product has a long lifespan, being stable for a minimum of two years if kept sealed in a cool location at below 12°.

4. Chemical characterization

4.1. Composition of the food additive

The external protein layer of the cell wall of *S. cerevisiae* may at any time consist of at least 20 different glycoproteins and the composition of this protein layer may vary depending on growth conditions (Klis et al., 2006).

Yeast mannoproteins have different structures depending on their molecular weights and the degree and type of glycosylation. Their molecular weight ranges from 20 kDa to more than 450 kDa. Many mannoproteins carry *N*-linked glycans with a core structure of Man10–14GlcNAc2-Asn. Outer chains present on many yeast *N*-glycans consist of 50 to 200 additional α-linked mannose units, with a long α-1,6-linked backbone decorated with short α-1,2- and α-1,3-linked side chains. There are often several large *N*-glycans per glycopeptide, so that *N*-linked sugar can add 50-100 kDa to the size of the mannoproteins. Phosphorylation of the mannosyl side chains gives yeast its anionic surface charge.
Commercial yeast extracts containing mannoproteins contain more than 60% polysaccharides on the dried basis, more than 70% mannose of the total polysaccharides, and 0.5-7.5%of total nitrogen on the dried basis.

4.2. Possible impurities (including degradation products)

- Lead: < 2 mg/kq
- Total aerobic mesophile flora: < 10,000/g
- Coliforms: < 10 CFU/g
- *Staphylococcus aureus:* None in a 1 g sample
- *Salmonella:* None in a 25 g sample
- *Escherichia coli:* None in a 25 g sample
- Moulds: < 50 CFU/g
- Yeasts: < 10^2 CFU/g

4.3. Analytical methods

The following assay method for the determination of total polysaccharides in yeast extracts containing mannoproteins is proposed:

- Preparation of mannose standard solution (0.1mg/ml): Accurately weigh 100 mg of mannose, dissolve in deionized water and make up to 100 ml in a volumetric flask. Pipette 5 mL of solution into a 50 ml volumetric flask and make up to volume with deionized water (0.1 mg/ml).
- Preparation of sample solution (15 mg/l): Accurately weigh 150 mg (W) of sample, dissolve in deionized water and make up to 100 ml in a volumetric flask. Pipette 1 ml of solution into a 100 ml volumetric flask and make up to volume with deionized water (15 mg/l).

- Procedure:
    - Add 200 µl of phenol solution and 1 ml of concentrated sulfuric acid to 200 µl of the sample solution and mix immediately.
    - Prepare a reference solution by adding 200 µl of phenol solution and 1 ml of concentrated sulphuric acid to 200 µl of mannose solution and mix immediately.
    - Heat both solutions to 100 ° in a water bath for 5, remove tubes and quickly cool to 0° in ice. Take out the tubes from ice and allow tubes to reach room temperature. Then measure the absorbance values at 490 nm in a spectrophotometer against a blank solution prepared similarly by omitting the standard and calculate the total polysaccharides using the 100 mg/l mannose solution as a reference solution.

Calculate the amount of total polysaccharides according to:

$$Total\ polysaccharides = \frac{A_{Sample} \times 10^7}{A_{Std} \times W \times (100 - \%M)}$$

Where:

- Total polysaccharides are given in %w/w on dried basis
- A_{Sample} is the absorbance of the sample solution
- A_{Std} is the absorbance of the standard solution (0.1 mg/ml)
- W is the weight of sample, mg
- %M is the loss on drying,

The following assay method for determination of % Mannose in total polysaccharides in yeast extracts containing mannoproteins is proposed:

Instrumentation and reagents:

- Spectrophotometer: 340 nm
- Stop-watch
- Triethanolamine hydrochloride ($C_6H_5NO_3 \cdot HCl$): : >99 % pure
- Magnesium sulfate ($MgSO_4 \cdot 7H_2O$): AR grade
- Sodium hydroxide: AR grade
- Disodium nicotinamide adenine dinucleotide phosphate: AR grade
- Adenosine-5'-triphosphate (ATP): AR grade
- Sodium hydrogen carbonate: AR grade
- Hexokinase solution: 2 mg of protein/ml or 280 U/ml
- Glucose-6-phosphate(G-6-P)-dehydrogenase solution: 1 mg of protein/ml
- Phosphoglucose-isomerase (PGI) solution: 2 mg of protein/ml or 700 U/ml
- Phosphomannose isomerase solution: 616 U/ml
- Sulfuric acid: 5 M
- Potassium hydroxide: 10 M

Buffer solution (0.3 M triethanolamine, 0.004 M Mg^{2+}, pH 7.6): Dissolve 11.2 g of triethanolamine hydrochloride and 0.2 g magnesium sulfate in 150 ml deionized water, adjust the pH 7.6 with about 4 ml of 5 mol/l sodium hydroxide solution and make up to 200 ml.

Nicotinamide adenine dinucleotide phosphate (NADP) solution (10 mg/m ml, about 0.12M): Dissolve 50 mg disodium nicotinamide adenine dinucleotide phosphate in 5 ml of deionized water.

Adenosine-5'-triphosphate (ATP) solution (50 mg/ml, about 0.08 M): Dissolve 250 mg disodium adenosine-5'-triphosphate and 250 mg sodium hydrogen carbonate in 5 ml of deionized water.

Hexokinase/glucose-6-phosphate(G-6-P)-dehydrogenase solution: Mix 0.5 ml hexokinase solution with 0.5 ml G-6-P-dehydrogenase solution.

Preparation of sample solution (5 mg/ml):
Accurately weigh 500 mg (W) of sample, dissolve in 100 ml of deionized water. Place 100 µl of the sample solution in airtight sealed tubes and add 1 ml of 5M sulphuric acid solution. Cap the tubes, heat at 100° in a water bath for 30 min, remove tubes and quickly cool to 0° in ice. Take out the tubes from ice and allow tubes to reach room temperature. Neutralise the acid by adding 1 ml of 10 M potassium hydroxide solution to each tube.

Procedure:
Set the spectrophotometer at 340 nm wavelength. Using matched cells, zero the instrument (according to the manufacturer's instructions),

Prepare the reference cell with 1 cm path length with 2.50ml buffer solution (@20°), 0.10 ml NADP solution, 0.10 ml ATP solution and 0.20 ml deionised water

Prepare the sample cell like the reference cell by replacing the deionised water with 0.20 ml of sample solution

Start the timer and mix the solution in the cell. Add 0.02 ml of G-6-P-dehydrogenase solution to both cells after three minutes and mix. Add 0.02 ml of PGI Solution to both cells after 17 min and mix. Read the absorbance of the solution in reference as well as sample cells, after 10 min. After two more minutes, read the absorbance (A_1) of the solution to ensure that the reaction has stopped (indicated by no increase in absorbance).

Add 0.02 ml each of phosphomannose isomerase solution (616 U/ml) and mix. Read the absorbance after 30 min. Check absorbance (A_2) after two more minutes to ensure that the reaction has stopped (indicated by no increase in absorbance).

Note: The time needed for the completion of enzyme activity may vary from one batch to another. The above value is given only for guidance and it is recommended that it be determined for each batch.

Calculation:
Calculate the differences in absorbance between A_1 and A_2 for the reference cell (ΔA_T) and the sample cell (ΔA_D), and then obtain $\Delta A_M = \Delta A_D - \Delta A_T$

Calculate mannose %w/w (on dried basis) by the following expression:

$$Mannose = \frac{0.423 \times \Delta A_M \times 10^6 \times 21}{W \times (100 - \%M)}$$

Where:
- Mannose is expressed as % w/w on dried basis
- W is the weight of sample, mg
- %M is the loss on drying, %

Calculate the % Mannose in total polysaccharides as follows:

$$\%\ \text{Mannose in total polysaccharides} = \frac{\%\text{Mannose on dried basis} \times 100}{\%\ Polysaccharides\ on\ dried\ basis}$$

All other methods in the specifications for Yeast extracts containing mannoproteins are based on standard methods, published in the Combined Compendium of Food Additive Specifications FAO JECFA Monographs 1, Vol 4 (JECFA, 2006).

4.4. Rationale for proposed specifications

The identity assay for Yeast extracts from mannoproteins is intended to define the identity of the products of commerce as verified by visual inspection and solubility.
The purity of the product of commerce is established by determination of loss on drying, specific rotation, total ash, lead, and presence of microbiological contaminants.

The specifications are consistent with the specification for yeast mannoproteins in the OIV International Oenological Codex (OIV Codex), OIV Resolution Oeno 26/2004. This specification indicates that Yeast extracts containing mannoproteins can be used for tartaric and/or protein stabilization of wine.

5. Functional use

5.1. Technological function

Yeast mannoproteins contribute to the chemical stabilization of wine by preventing crystallization of tartrate salts (potassium bitartrate crystallization) (Feuillat et al. 1998, Gerbaud et al. 1996; Moine et al. 1997) and protein haze (Dupin et al. 2000; Gonzalez-Ramos et al. 2008; Gonzalez-Ramos and Gonzalez 2006; Gonzalez-Ramos et al. 2009, Waters et al. 1994). Accordingly, mannoproteins falls into a class of colloids termed "protective colloids", other examples of which include Gum Arabic and β-glucan, derived from *Botrytis cinerea* infection of grapes (Ribereau-Gayon et al. 2006). Protective colloids function by coating the site of crystallization or aggregation. Mechanisms for this process have been postulated but not yet elucidated.

Wine clarity refers to the absence or presence of suspended particles or sediments in wine and consumers usually reject wine bottles containing crystalline and cloudy precipitates (Ferreira et al. 2004, thereby reducing their commercial value (Bayly and Berg 1967, Hsu and Heatherbell 1987, Waters et al. 1991, Waters et al. 1992, Dupin et al. 2000, Lomolino and Curioni 2007), despite the fact that most do not affect the sensory characteristics of the wine (Ferreira et al. 2004, Lomolino and Curioni 2007).

5.1.1 Tartrate stability

Tartrate crystals (potassium hydrogen tartrate and calcium tartrate) develop naturally in wine and are the major cause of sediment in bottled wines. Accordingly, before the delivery for domestic or international trade, particularly white wine and sparkling wines have to be stabilized against tartrate salt precipitation, which is from a natural grape source. The concentration of tartaric acid itself in the wine, alcohol, colloids, calcium, and potassium, the pH value of the wine, the duration and temperature of storage, and the surface (roughness) of the storage container all impact on tartrate salt precipitation in wine (Pilone and Berg 1965). Tartaric acid predominantly precipitates as the potassium salt (potassium bitartrate) followed by calcium tartrate. Mannoproteins inhibit the crystallization of potassium bitartrate.

Mannoproteins are naturally released from yeast during the fermentation process and are able to reduce tartrate crystallization, such that barrel-aging white wines on yeast lees for several months often provides sufficient tartrate stability to overcome the need for further stabilization. The macromolecules responsible for inhibiting crystal formation have been shown to be glycosylated proteins of the yeast cell wall released by enzymatic degradation of the yeast cell wall (Llauberes et al. 1987, Dupin et al. 2000).

The addition of a purified yeast cell wall preparation exploits the ability of these mannoproteins to act as protective colloids. While the exact mechanism of this process is unknown, protective colloids are thought to act by coating the site of crystallization or aggregation and hindering access to nearby particles.

5.1.2 Protein stability

Wines contain varying amounts of different nitrogenous substances, amongst which are proteins.

These wine proteins do not contribute significantly to the nutritive value of wines since their concentration varies typically from 15-300 mg/L (Ferreira et al. 2002, Waters et al. 2005). The majority of the wine proteins derive from the grape pulp and include chitinases, thaumatin-like proteins and osmotins (Monteiro et al. 2001, Waters et al. 1998), which are particularly stable under winemaking conditions (low pH value and proteolysis enzymes), therefore passing selectively into the wine (Ferreira et al., 2000). The slow denaturation of residual amount of unstable wine proteins, possibly resulting from unfavourable storage conditions leads to protein aggregation and flocculation into a hazy suspension, which results in the appearance of a haze or deposit in the bottled wine. For example, it has been shown that that all the major wine protein fractions are present in wine hazes and all have been shown to be heat unstable (Waters 1991, Waters and Høj 1999, Waters et al. 1990, Waters et al. 1991, Waters et al. 1992).

Yeast mannoproteins have been shown to possess haze-protective properties, while also positively impacting on the sensorial properties of the product. While such mannoproteins are released into the wine during the wine making process, the amounts are often low and therefore of limited oenological significance. Mannoproteins do not prevent haze-inducing proteins in wine from precipitating but compete to complex with other compounds, reducing the size of haze particles to the threshold level of human detection (Waters et al. 1993, Dupin et al. 2000). The yeast mannoprotein preparation is added to the wine after fining, just before the final stage of filtration prior to bottling. The correct dose is determined by preliminary testing of each wine, with the recommended dosage being the lowest concentration at which no crystallization appears plus 50 mg/l. Addition of excess mannoproteins can reduce the stabilizing effect. The dose of the yeast extracts containing mannoproteins to be added depends on factors such as the amount of tartrate and mannoproteins naturally present in the wine, and may typically range between 200 and 400 mg/l.

5.2. Food categories and use levels

Levels of Yeast extracts containing mannoproteins used in food or expected to be used in food based on technological function and the range of foods are as follows.

Food categories: Wine, sparkling wine and fortified wine.
The amount of yeast extracts containing mannoproteins to be added to wine will range between 50–400 mg/l as determined by the winemaker, with a recommended dose of 200 mg/l and a maximum recommended dosage of 400 mg/l depending on the amount of naturally occurring mannoproteins and tartrate in the wine.

6. Reactions and fate in foods

Yeast extracts containing mannoproteins are stable for two years in a sealed container < 12 °. As they may decompose in excessive temperatures they are recommended to be stored at 4 – 12 °.

7. References

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