

FAO SPECIFICATIONS AND EVALUATIONS FOR AGRICULTURAL PESTICIDES

AZADIRACTIN¹

The chemical name for azadirachtin A is: dimethyl [2a*R*-[2a α ,3 β ,4 β (1a*R**,2*S**,3a*S**,6a*S**,7*S**,7a*S**),4a β ,5 α ,7a*S**,8 β (*E*),10 β ,10a α ,10b β]]-10-(acetyloxy)octahydro-3,5-dihydroxy-4-methyl-8-[(2-methyl-1-oxo-2-butenyl)oxy]-4-(3a,6a,7,7a)-tetrahydro-6a-hydroxy-7a-methyl-2,7-methanofuro[2,3-*b*]oxireno[*e*]oxepin-1a(2*H*)-yl)-1*H*,7*H*-naphtho-[1,8-*bc*:4,4a-*c*]difuran-5,10a(8*H*)-dicarboxylate

2006



Food and Agriculture Organization
of the United Nations

¹ "Azadirachtin" is the collective term applied to a large group of insecticidally-active limonoid compounds, extracted from seeds of the neem tree (*Azadirachta indica* A. Juss.), in which the most active and abundant single compound is known as azadirachtin A. Azadirachtin, *sensu lato*, is neither completely defined nor quantifiable and so azadirachtin A is used as a lead or marker compound for the purposes of identification and quantification.

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DISCLAIMER¹

FAO specifications are developed with the basic objective of promoting, as far as practicable, the manufacture, distribution and use of pesticides that meet basic quality requirements.

Compliance with the specifications does not constitute an endorsement or warranty of the fitness of a particular pesticide for a particular purpose, including its suitability for the control of any given pest, or its suitability for use in a particular area. Owing to the complexity of the problems involved, the suitability of pesticides for a particular purpose and the content of the labelling instructions must be decided at the national or provincial level.

Furthermore, pesticides which are manufactured to comply with these specifications are not exempted from any safety regulation or other legal or administrative provision applicable to their manufacture, sale, transportation, storage, handling, preparation and/or use.

FAO disclaims any and all liability for any injury, death, loss, damage or other prejudice of any kind that may arise as a result of, or in connection with, the manufacture, sale, transportation, storage, handling, preparation and/or use of pesticides which are found, or are claimed, to have been manufactured to comply with these specifications.

Additionally, FAO wishes to alert users to the fact that improper storage, handling, preparation and/or use of pesticides can result in either a lowering or complete loss of safety and/or efficacy.

FAO is not responsible, and does not accept any liability, for the testing of pesticides for compliance with the specifications, nor for any methods recommended and/or used for testing compliance. As a result, FAO does not in any way warrant or represent that any pesticide claimed to comply with a FAO specification actually does so.

¹ This disclaimer applies to all specifications published by FAO.

INTRODUCTION

FAO establishes and publishes specifications* for technical material and related formulations of agricultural pesticides, with the objective that these specifications may be used to provide an international point of reference against which products can be judged either for regulatory purposes or in commercial dealings.

Since 1999 the development of FAO specifications follows the **New Procedure**, described in the 5th edition of the “Manual on the development and use of FAO specifications for plant protection products” (FAO Plant Production and Protection Page No. 149). This **New Procedure** follows a formal and transparent evaluation process. It describes the minimum data package, the procedure and evaluation applied by FAO and the Experts of the FAO/WHO Joint Meeting on Pesticide Specifications (JMPS). [Note: prior to 2002, the Experts were of the FAO Panel of Experts on Pesticide Specifications, Registration Requirements, Application Standards and Prior Informed Consent, which now forms part of the JMPS, rather than the JMPS.]

FAO Specifications now only apply to products for which the technical materials have been evaluated. Consequently from the year 2000 onwards the publication of FAO specifications under the **New Procedure** has changed. Every specification consists now of two parts namely the specifications and the evaluation report(s):

PART ONE: The Specification of the technical material and the related formulations of the plant protection product in accordance with chapter 4, 5 and 6 of the 5th edition of the “Manual on the development and use of FAO specifications for plant protection products”.

PART Two: The Evaluation Report(s) of the plant protection product reflecting the evaluation of the data package carried out by FAO and the JMPS. The data are to be provided by the manufacturer(s) according to the requirements of Appendix A, annex 1 or 2 of the “Manual on the development and use of FAO specifications for plant protection products” and supported by other information sources. The Evaluation Report includes the name(s) of the manufacturer(s) whose technical material has been evaluated. Evaluation reports on specifications developed subsequently to the original set of specifications are added in a chronological order to this report.

FAO specifications under the **New Procedure** do not necessarily apply to nominally similar products of other manufacturer(s), nor to those where the active ingredient is produced by other routes of manufacture. FAO has the possibility to extend the scope of the specifications to similar products but only when the JMPS has been satisfied that the additional products are equivalent to that which formed the basis of the reference specification.

Specifications bear the date (month and year) of publication of the current version. Dates of publication of the earlier versions, if any, are identified in a footnote. Evaluations bear the date (year) of the meeting at which the recommendations were made by the JMPS.

* NOTE: PUBLICATIONS ARE AVAILABLE ON THE INTERNET AT
(<http://www.fao.org/pest-and-pesticide-management/en/>)

PART ONE

SPECIFICATIONS

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AZADIRACHTIN

INFORMATION

ISO common name

None.

For the purposes of FAO specifications, “azadirachtin” is the collective term applied to a large group of insecticidally-active limonoid compounds, extracted from seeds of the neem tree (*Azadirachta indica* A. Juss.), in which the most active and abundant single compound is known as azadirachtin A. Azadirachtin, *sensu lato*, is neither completely defined nor quantifiable and so azadirachtin A is used as a lead or marker compound for the purposes of identification and quantification. The proportions of components in azadirachtin are variable.

Synonyms

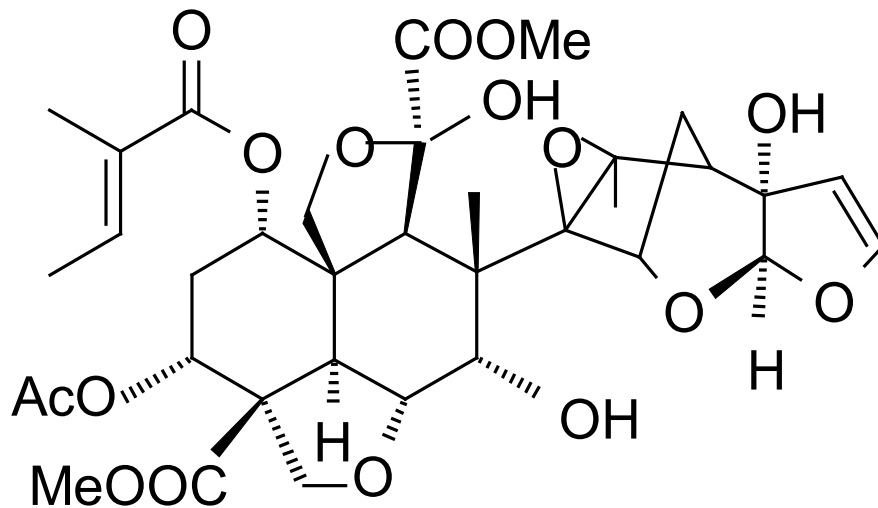
Azad

Chemical names of azadirachtin A

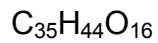
IUPAC 1*H*,7*H*-naphtho[1,8-*bc*:4,4*a-c'*]difuran-5,10*a*(8*H*)-dicarboxylic acid, 10-(acetyloxy)octahydro-3,5-dihydroxy-4-methyl-8-[(2-methyl-1-oxo-2-butenyl)oxy]-4-(3*a*,6*a*,7,7*a*,tetrahydro-6*a*-hydroxy-7*a*-methyl-2,7-methanofuro[2,3-*b*]oxireno[*e*]oxepin-1*a*(2*H*)-yl)-dimethylester, [2*aR*-[2*α*,3*β*,4*β*(1*aR*^{*},2*S*^{*},3*aS*^{*},6*aS*^{*},7*S*^{*},7*aS*^{*}),4*aβ*,5*α*,7*aS*^{*},8*β*(*E*),10*β*,10*α*,10*bβ*]]

CA dimethyl [2*aR*-[2*α*,3*β*,4*β*(1*aR*^{*},2*S*^{*},3*aS*^{*},6*aS*^{*},7*S*^{*},7*aS*^{*}),4*aβ*,5*α*,7*aS*^{*},8*β*(*E*),10*β*,10*α*,10*bβ*]]-10-(acetyloxy)octahydro-3,5-dihydroxy-4-methyl-8-[(2-methyl-1-oxo-2-butenyl)oxy]-4-(3*a*,6*a*,7,7*a*)-tetrahydro-6*a*-hydroxy-7*a*-methyl-2,7-methanofuro[2,3-*b*]oxireno[*e*]oxepin-1*a*(2*H*)-yl)-1*H*,7*H*-naphtho-[1,8-*bc*:4,4*a-c'*]difuran-5,10*a*(8*H*)-dicarboxylate

Structural formula of azadirachtin A



Empirical formula of azadirachtin A



Relative molecular mass of azadirachtin A

720.7

CAS Registry number of azadirachtin A

11141-17-6

CIPAC number of azadirachtin A

627

Identity tests

HPLC retention time; TLC R_f value.

AZADIRACHTIN TECHNICAL CONCENTRATE

FAO specification 627/TK (May 2006*)

This specification, which is PART ONE of this publication, is based on an evaluation of data submitted by the manufacturers whose names are listed in the evaluation report (627/2003). It should be applicable to relevant products of these manufacturers but it is not an endorsement of those products, nor a guarantee that they comply with the specifications. The specification may not be appropriate for the products of other manufacturers. The evaluation report (627/2003) as PART TWO forms an integral part of this publication.

1 Description

The material shall consist of azadirachtin A, together with related extracted chemicals which may variously support and/or stabilize the activity of azadirachtin A, and others which may be considered as impurities. The material shall be a brownish to yellow powder, with a characteristic garlic-like odour, and shall be free from visible extraneous matter and added modifying agents except for the diluent.

2 Active ingredient

2.1 Identity tests (627/TK/M/2, CIPAC Handbook, Note 1)

The azadirachtin A shall comply with an identity test and, where the identity remains in doubt, shall comply with at least one additional test.

2.2 Azadirachtin A content (627/TK/M/3, CIPAC Handbook, Note 1)

The azadirachtin A content shall be declared and, when determined, the average measured content shall not differ from that declared by more than the following tolerance.

Declared content of azadirachtin A, in g/kg	Tolerance
Above 250 up to 500	± 15% of the declared content
<i>Note:</i> the range includes the upper limit.	

3 Relevant impurities

3.1 Aflatoxins (sum of aflatoxins B₁, B₂, G₁ and G₂) (Note 2)

Maximum: 0.00003% (300 µg/kg) of the azadirachtin A found under 2.2.

* Specifications may be revised and/or additional evaluations may be undertaken. Ensure the use of current versions by checking at: <http://www.fao.org/pest-and-pesticide-management/en/>

Note 1 Methods for the identification and determination of azadirachtin A content were adopted by CIPAC in 2005 but are not yet published in a Handbook. Prior to publication of the Handbook, copies of the methods may be obtained through the CIPAC website, <http://www.cipac.org>.

Note 2 The analytical method for determination of aflatoxins is available from the Pesticide Management Group of the FAO Plant Protection Service or can be [downloaded here](#).

AZADIRACHTIN EMULSIFIABLE CONCENTRATE

FAO specification 627/EC (May 2006*)

This specification, which is PART ONE of this publication, is based on an evaluation of data submitted by the manufacturers whose names are listed in the evaluation report (627/2003). It should be applicable to relevant products of these manufacturers but it is not an endorsement of those products, nor a guarantee that they comply with the specifications. The specification may not be appropriate for the products of other manufacturers. The evaluation report (627/2003) as PART TWO forms an integral part of this publication.

1 Description

The material shall consist of technical azadirachtin, complying with the requirements of FAO specification 627/TK (May 2006), dissolved in suitable solvents, together with any other necessary formulants. It shall be in the form of a stable homogeneous liquid, free from visible suspended matter and sediment, to be applied as an emulsion after dilution in water.

2 Active ingredient

2.1 Identity tests (627/EC/M/2, CIPAC Handbook, Note 1)

The azadirachtin A shall comply with an identity test and, where the identity remains in doubt, shall comply with at least one additional test.

2.2 Azadirachtin A content (627/EC/M/3, CIPAC Handbook, Note 1)

The azadirachtin A content shall be declared (g/kg) and, when determined, the average measured content shall not differ from that declared by more than the following tolerance:

Declared content, g/kg	Tolerance
up to 25	± 15% of the declared content
Note: the upper limit is included in the range	

3 Relevant impurities

3.1 Aflatoxins (sum of B₁, B₂, G₁ and G₂) (Note 2)

Maximum: 0.00003% (300 µg/kg) of the azadirachtin A found under 2.2.

* Specifications may be revised and/or additional evaluations may be undertaken. Ensure the use of current versions by checking at: <http://www.fao.org/pest-and-pesticide-management/en/>

4 Physical properties

4.1 pH range (MT 75.3, CIPAC Handbook J, p.131, 2000)

The pH of an aqueous dispersion shall be 5.0 to 7.0.

4.2 Emulsion stability and re-emulsification (MT 36.3, CIPAC Handbook K, p.137, 2003) (Note 3)

The formulation, when diluted at $30 \pm 2^\circ\text{C}$ with CIPAC Standard Waters A and D, shall comply with the following:

Time after dilution	Limits of stability
0 h	Initial emulsification complete
0.5 h	"Cream" maximum: none
2.0 h	"Cream" maximum: none Free oil maximum: none
24 h	Re-emulsification complete
24.5 h	"Cream" maximum: none Free oil maximum: none.
Note: tests at 24 h are required only where the results at 2 h are in doubt.	

4.3 Persistent foam (MT 47.2, CIPAC Handbook F, p.152, 1995) (Note 4)

Maximum: none after 1 min.

5 Storage stability

5.1 Stability at 0°C (MT 39.3, CIPAC Handbook J, p.126, 2000)

After storage at $0 \pm 2^\circ\text{C}$ for 7 days, the volume of solid and/or liquid which separates shall not be more than 0.3 ml.

5.2 Stability at elevated temperature (MT 46.3, CIPAC Handbook J, p.128, 2000)

After storage at $54 \pm 2^\circ\text{C}$ for 14 days, the determined average active ingredient content must not be lower than 75% relative to the determined average content found before storage (Note 5) and the formulation shall continue to comply with the clauses for:

- pH range (4.1);
- emulsion stability and re-emulsification (4.2).

Note 1 Methods for the identification and determination of azadirachtin A content were adopted by CIPAC in 2005 but are not yet published in a Handbook. Prior to publication of the Handbook, copies of the methods may be obtained through the CIPAC website, <http://www.cipac.org>

Note 2 The analytical method for determination of aflatoxins is available from the Pesticide Management Group of the FAO Plant Protection Service or can be [downloaded here](#).

Note 3 This test will normally only be carried out after the heat stability test, 5.2.

Note 4 The mass of sample to be used in the test should correspond to the highest rate of use recommended by the supplier.

Note 5 Samples of the formulation taken before and after the storage stability test should be analyzed concurrently after the test in order to reduce the analytical error.

PART TWO

EVALUATION REPORTS

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2003	
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AZADIRACHTIN

FAO/WHO EVALUATION REPORT 627/2003

Recommendation

The Meeting recommended that the specifications for azadirachtin TK and EC, jointly proposed by Trifolio-M (Germany) and EID Parry (India), should be adopted by FAO.

Appraisal

The Meeting considered data on azadirachtin, provided by Trifolio-M (Germany) and EID Parry (India), in support of proposed new FAO specifications for TK and EC. The submission was a joint one, with both companies using azadirachtin prepared, as NeemAza[®] TK, in a special process by EID Parry (India) and therefore it was not necessary to consider equivalence. The data submitted were in accordance with the requirements of the FAO/WHO Manual.

Azadirachtin has not been evaluated by the FAO/WHO JMPR or IPCS. It was reviewed by the German authorities (BBA/BGVV) in 1998. Neem extracts and azadirachtin are not under patent but the specially purified azadirachtin TK known as NeemAza[®] is under patent in various countries until 2012.

Azadirachtin is a botanical pesticide, extracted from seeds (and other plant parts) of the neem tree (*Azadirachta indica* A.Juss., Meliaceae), a naturally occurring tree species from the Indo-Malaysia area which is also grown in other tropical and subtropical areas of Asia, Africa, South America and Australia. The tree is also used for timber and fuel and seed or leaf extracts are used for various medicinal and pest control purposes. As with most naturally-produced materials, the proportions of components present in neem seeds vary considerably. Neem extracts are produced widely in tropical countries, using a wide range of processes, and therefore the nature of neem extracts can be extremely variable.

Neem extracts contain "azadirachtin", a convenient collective term for the active ingredient that consists of a complex group of insecticidally active tetraterpene limonoid compounds. Azadirachtin is not wholly defined and therefore cannot be isolated as an entity, nor can the technical grade be expected to have the high level of purity normally expected for a synthetic compound. The Meeting recognized that the use by the proposers of well-controlled raw materials, together with carefully-controlled extraction and purification processes, leads to a particularly well-characterized and relatively uniform azadirachtin TK. However, the Meeting also recognized that even the purest and most uniform azadirachtin TK is not comparable with the TK of a synthetic pesticide and that it should therefore be approached differently.

Azadirachtin A is the most abundant and most insecticidally active compound of azadirachtin. However, azadirachtin A is labile when isolated as a pure chemical and it also has the potential to induce the development of insect resistance much more rapidly than it does as part of the azadirachtin mixture. Thus it is both undesirable and unnecessary to produce a pesticide based on pure azadirachtin A (Schmutterer 2002, Rembold 2004, Isman *et al.*, 1990). For these reasons, and

because “pure azadirachtin” cannot exist, most of the data presented in this evaluation refer to azadirachtin TK (NeemAzal[®]). Given the complexity and variability of azadirachtin TKs, and the relatively high potency and abundance of azadirachtin A in azadirachtin, the Meeting agreed that azadirachtin A is a suitable marker for identification and quantification of azadirachtin in the TK and formulations.

Neem preparations are produced by two main processes: solvent extraction of ripe seeds and pressing of seeds to extract neem oil. Neem seeds contains about 40% oil and have the highest concentration of azadirachtin in the plant (Isman *et al.*, 1990). Neem oil, as a fraction, is an even more complex and less well-defined mixture than azadirachtin and it was not considered in this evaluation. Solvent extracts of neem seeds may be subjected to further purification and, together with careful selection of the seeds used, can be used to produce a rather uniform product of relatively high azadirachtin content, with a correspondingly low content of unwanted materials such as oil, carbohydrates, protein and water. The azadirachtin TK (NeemAzal) produced by Trifolio-M (Germany) and EID Parry (India) is in this category, containing >25% azadirachtin A.

The Meeting was provided with commercially confidential information on the manufacturing process for azadirachtin in the form of NeemAzal[®] (TK) and batch analysis data were provided for all identified components ≥ 1 g/kg and on proposed relevant impurities < 1 g/kg. Mass balances were considered acceptable for a botanical pesticide (34.6-47.5%), the remainder apparently consisting mainly of unidentified natural product impurities. The commercially confidential information and toxicological/eco-toxicological data were confirmed as being identical to those submitted for registration of NeemAzal[®] products in Germany.

Mycotoxins can arise from fungal infection of many types of seed. Contamination with *Aspergillus spp.* and other fungi is particularly likely if neem seeds fall to the ground before collection and extraction but, even with the greatest care in harvesting the seeds, it is practically impossible to produce extracts which contain no detectable aflatoxins. The Meeting therefore agreed with the proposers that aflatoxins B₁, B₂, G₁, and G₂ should be considered as relevant impurities in azadirachtin and that the limit for the sum of these should be 0.00003% (300 $\mu\text{g}/\text{kg}$) of the azadirachtin A, which corresponds to a manufacturing limit of approximately 0.00001% (100 $\mu\text{g}/\text{kg}$) in NeemAzal[®] TK. The proposers stated that *Fusarium spp.* do not grow on neem seeds and that mycotoxins other than aflatoxins are not likely to occur in azadirachtin.

The Meeting asked if information was available on (i) the identity of the degradation products derived from azadirachtin and/or azadirachtin A and (ii) the toxicity of the TK after partial or complete degradation of its azadirachtin A content. The Proposers stated that the toxicological studies had been conducted with azadirachtin stored for periods covering the recommended storage period of the products sold and thus any additional toxicity of the breakdown products would have been manifested in the results. The Meeting was provided with the published data referenced in Table 1, below. The observations of Barrek *et al.* (2004) on the degradation of neem products were reported by the manufacturer to be consistent with observations made by the companies. The Meeting therefore agreed that there was no evidence to suggest that other impurities should be considered relevant.

The Meeting noted that the nature of azadirachtin and neem products makes it virtually impossible to determine equivalence with the purity and impurity profile of

the azadirachtin TK produced by Trifolio-M and EID Parry (India) (NeemAza[®]) and that therefore equivalence would have to be determined, in future, primarily on the basis of the toxicology/ecotoxicology profiles and the identity and levels of relevant impurities.

Azadirachtin TK (NeemAza[®]) is generally of low acute toxicity but is mildly irritating to the eyes and is apparently a dermal sensitizer, though it is possible that these responses may have been due to mechanical effects of the powder. It is of low sub-acute and chronic toxicity, shows no teratogenicity, carcinogenicity or mutagenicity and is of low toxicity to aquatic and terrestrial wildlife. Azadirachtin, in any form, has not been evaluated by IPCS or the FAO/WHO JMPR and there is no WHO hazard classification. In the opinion of WHO/PCS, the hazard data presented in support of the proposed specifications were satisfactory and sufficient.

The azadirachtin in azadirachtin TK and EC must be identified and quantified as azadirachtin A. The analytical method for determination of azadirachtin A was collaboratively studied under the auspices of CIPAC and adopted with provisional status in 2005. It is important to recognise that isolated and purified azadirachtin A is a labile compound and the purity of reference standard materials may be suspect. The Meeting noted that great care to ensure the use of a good quality standard of azadirachtin A is therefore required, to ensure that analysis of azadirachtin TK and EC produces meaningful results.

The method for determination of aflatoxins B₁, B₂, G₁ and G₂ was satisfactorily peer-validated.

The proposed specifications for azadirachtin TK and EC were in accordance with the requirements of the manual (FAO/WHO 2002).

**SUPPORTING INFORMATION
FOR
EVALUATION REPORT 627/2003**

Uses

Azadirachtin is broad-spectrum insect growth regulator, used on plants and against animal ectoparasites for the control of insects in many orders, such as Orthoptera, Heteroptera, Homoptera, Thysanoptera (to a limited extent), Hymenoptera, Coleoptera, Lepidoptera and Diptera. It usually acts via ingestion but shows contact toxicity towards soft-bodied pests such as aphids, mites, etc. It is applied as a diluted EC when pests first appear, either as a crop canopy spray or as a soil drench, and then about weekly thereafter.

Identity

ISO common name

None.

For the purposes of FAO specifications, "azadirachtin" is the collective term applied to a large group of insecticidally-active limonoid compounds, extracted from seeds of the neem tree (*Azadirachta indica* A. Juss.), in which the most active and abundant single compound is known as azadirachtin A. Azadirachtin, *sensu lato*, is neither completely defined nor quantifiable and so azadirachtin A is used as a lead or marker compound for the purposes of identification and quantification. Among the many lesser components present in azadirachtin are azadirachtins B, D to I, and K¹, azadirachtinin, nimbin, desacetyl nimbin, salannin and desacetyl salannin, but the proportions of all components in azadirachtin are variable.

Synonyms

Azad.

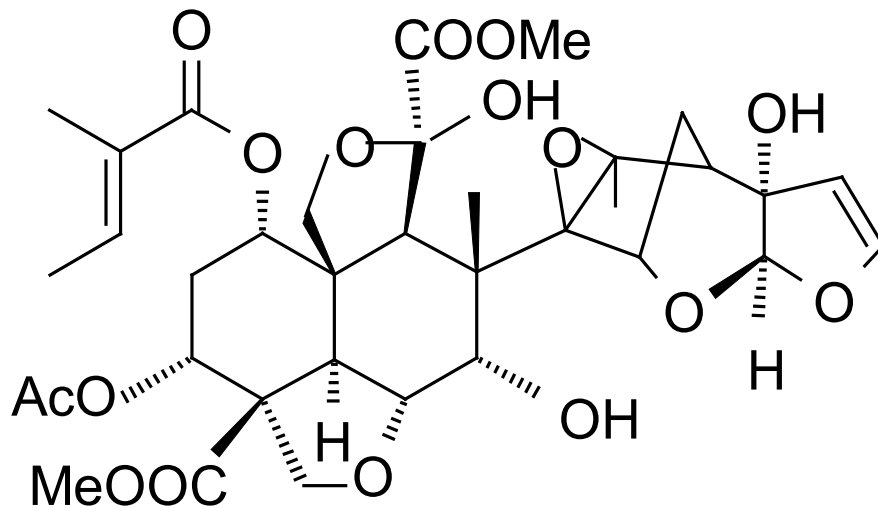
Chemical names of azadirachtin A

IUPAC 1*H*,7*H*-naphtho[1,8-*bc*:4,4*a-c'*]difuran-5,10*a*(8*H*)-dicarboxylic acid, 10-(acetyloxy)octahydro-3,5-dihydroxy-4-methyl-8-[(2-methyl-1-oxo-2-butenyl)oxy]-4-(3*a*,6*a*,7,7*a*,tetrahydro-6*a*-hydroxy-7*a*-methyl-2,7-methanofuro[2,3-*b*]oxireno[*e*]oxepin-1*a*(2*H*)-yl)-dimethylester, [2*aR*-[2*a* α ,3 β ,4 β (1*aR*^{*},2*S*^{*},3*aS*^{*},6*aS*^{*},7*S*^{*},7*aS*^{*}),4*a* β ,5 α ,7*aS*^{*},8 β (*E*),10 β ,=10 α ,10*b* β]]

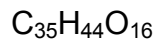
CA dimethyl [2*aR*-[2*a* α ,3 β ,4 β (1*aR*^{*},2*S*^{*},3*aS*^{*},6*aS*^{*},7*S*^{*},7*aS*^{*}),4*a* β ,5 α ,7*aS*^{*},8 β (*E*),10 β ,10 α ,10*b* β]]-10-(acetyloxy)octahydro-3,5-dihydroxy-4-methyl-8-[(2-methyl-1-oxo-2-butenyl)oxy]-4-(3*a*,6*a*,7,7*a*)-tetrahydro-6*a*-hydroxy-7*a*-methyl-2,7-methanofuro[2,3-*b*]oxireno[*e*]oxepin-1*a*(2*H*)-yl)-1*H*,7*H*-naphtho-[1,8-*bc*:4,4*a-c'*]difuran-5,10*a*(8*H*)-dicarboxylate

¹ Azadirachtin B and azadirachtin H are usually the most abundant minor azadirachtins present.

Structural formula of azadirachtin A



Empirical formula of azadirachtin A



Relative molecular mass of azadirachtin A

720.7

CAS Registry number of azadirachtin A

11141-17-6

CIPAC number of azadirachtin A

627

Identity tests

HPLC retention time; TLC R_f value.

Physical and chemical properties

Table 1. Physico-chemical properties of azadirachtin A and azadirachtin TK (NeemAzal[®])

Characteristic	Value	Purity, %	Method	Reference
Vapour pressure	3.6 x 10 ⁻¹³ Pa at room temperature	100% azadirachtin A	By extrapolation	Not given
Melting point and temperature of decomposition	Melting point: (TK: NeemAzal [®]): liquifies partially at 120°C, decomposes at above 200°C Melting point: 161 ± 1°C	TK, 30% azadirachtin A 96% azadirachtin A	EC directive 92/69/EEC, Annex part A, method A.1 OECD 102	955040827 A Not given
Solubility in water	In mixtures containing TK >200 g/l (>60 g/l azadirachtin A) at 21 ± 2°C at pH 7: 10.7g/l dissolved and azadirachtin A corresponded to 2.9 g/l.	TK, 30% azadirachtin A	In-house method, as standard methods are not applicable	LP 95.9
Octanol:water partition coefficient, log P K _{ow} values at 20.5°C at pH 7	Azadirachtin A: 0.99 ± 0.05 Azadirachtin B: 1.29 ± 0.06 Azadirachtin H: 0.68 ± 0.06 NeemAzal (TK): ca. 1	Not stated TK, 30% azadirachtin A	OECD 107 Estimated from above entries	TM 1195.13 TM 1195.13
Hydrolysis characteristics	Half-life of azadirachtin A in aqueous NeemAzal [®] solutions: 10.7 d at 30°C at pH 4 4.8 d at 30°C at pH 7 0.96 d at 30°C at pH 8 Azadirachtin A is most stable in mildly acidic aqueous solutions between pH 4 and 6 at room temperature. Unstable in mildly alkaline and strongly acidic solutions Azadirachtin A is very labile when exposed to moisture	TK, 30% azadirachtin A "pure" (>95%) "pure" (>95%)	OECD 111	TM 1195.15 Jarvis <i>et al.</i> 1998; 95 50 40 827 B Sundaram & Curry 1994; Sundaram <i>et al.</i> 1995

Table 1. Physico-chemical properties of azadirachtin A and azadirachtin TK (NeemAzal[®])

Characteristic	Value	Purity, %	Method	Reference
Photolysis characteristics	NeemAzal [®] has a quantum yield of 5.55×10^{-4} in purified water (pH 5.9; 18°C) Estimated half-life in water under central European conditions: 1.8 month (January); 5.5 days (July) The tigloyl moiety of azadirachtin A is likely to undergo photo-oxidation, and the ester linkages tend to undergo hydrolysis and cleavage of the strained 3- and 5-membered epoxide rings.	TK, 30% azadirachtin A >95%	ABIWAS	95 50 40 827 B Sundaram & Curry 1994
Dissociation characteristics	Does not dissociate.	-	-	-

Table 2. Chemical composition and properties of technical azadirachtin (TK)

Manufacturing process, maximum limits for impurities ≥ 1 g/kg, 5 batch analysis data	Confidential information supplied and held on file by FAO. Mass balances were 34.6-47.5%, the remainder being of unidentified impurities.
Declared minimum azadirachtin A content	250 g/kg.
Relevant impurities ≥ 1 g/kg and maximum limits for them	None.
Relevant impurities < 1 g/kg and maximum limits for them	Sum of aflatoxins B ₁ , B ₂ , G ₁ and G ₂ , less than 300 $\mu\text{g/kg}$ of the azadirachtin A.
Stabilizers or other additives and maximum limits for them	None.
Melting temperature range of TK	liquifies partially at 120°C, decomposes above 200°C.

Hazard summary

Azadirachtin has not been evaluated by the FAO/WHO JMPR or IPCS and there is no WHO hazard classification.

Formulations

The main formulation type is EC. Azadirachtin formulations containing NeemAzal[®] TK are registered and sold in many countries throughout the world. Azadirachtin is not co-formulated with other pesticides.

Methods of analysis and testing

The analytical method for determination of azadirachtin A¹ content in TK and EC was validated by collaborative study and adopted by CIPAC with provisional status in 2005. Azadirachtin A is determined by HPLC, using UV detection at 214 nm and external standardization.

The method for determination of the relevant impurities, aflatoxins B₁, B₂, G₁, and G₂ in the TK and EC, is based on a method which is widely-used in food analysis. It involves extraction with methanol/water, clean-up on an immunoaffinity column, HPLC with post-column derivatization and fluorescence detection. The method was peer-validated and found to give acceptable results from analysis of samples fortified with aflatoxins individually in the range 10-70 µg/kg on a whole product, not an azadirachtin A basis (azadirachtin A content not stated). Aflatoxin-free samples of azadirachtin cannot be produced but, allowing for the aflatoxins already present in the TK (9 µg/kg), recoveries at 10-70 µg/kg were in the range 84-118%, with RSDs in the range 0-10% (n=3 for each combination of laboratory, aflatoxin and concentration). However, most recoveries were close to 100% and RSDs were generally low. Recoveries from the EC, fortified at 0.3-3 µg/kg, were in the range 65-108%, with RSDs in the range 0-15%. No other signals were observed in chromatograms and therefore the specificity, accuracy and precision were considered to be acceptable.

Test methods for determination of physico-chemical properties of technical azadirachtin were OECD, EU or US-EPA, while those for the EC formulation were CIPAC, as indicated in the specifications.

Physical properties

The physical properties and the limits proposed for the EC formulation complied with the requirements of the Manual (FAO/WHO 2002).

Containers and packaging

Containers should not be prone to redox reactions and should be airtight. Thus containers should be of glass, polyethylene, polypropylene, or steel internally coated with polyethylene and/or polypropylene.

Expression of active ingredient

The active ingredient is azadirachtin, *sensu lato*, which cannot be identified or measured as such. It is therefore identified, measured and expressed as azadirachtin A, in g/kg. Although this under-estimates the total amount present, it forms the only practicable means for determination and expression of the active ingredient.

¹ Determination of azadirachtin A, which is normally the most active and abundant component of azadirachtin, under-estimates the amount of azadirachtin present but it is the best available marker compound. Determination of all azadirachtin components is impracticable.

ANNEX 1

HAZARD SUMMARY PROVIDED BY THE PROPOSER

Note: The proposers provided written confirmation that the toxicological and ecotoxicological data included in the following summary were derived from azadirachtin/Neemazal® TK having impurity profiles similar to those referred to in Table 2, above.

Table A. Toxicology profile of azadirachtin TK (NeemAzal[®]), based on acute toxicity, irritation and sensitization

Species	Test	Duration and conditions	Result	References
Rat (m,f)	oral	15 d (single dose), EPA guideline 152-10	LD ₅₀ >5000 mg/kg bw	EIP 6/950799/AC
Mouse (m,f)	oral	14 d, FIPPAT, India, standard protocol	LD ₅₀ >3365 mg/kg bw	Unnumbered A; 1749
Rat (m,f)	dermal	15 d, EPA guideline 152-11	LD ₅₀ >2000 mg/kg bw	EIP 7/950800/AC
Rat (m,f)	inhalation	4 hr inhalation (observation 14 d), EPA guideline 152-12	LC ₅₀ >720 mg/m ³	EIP 5/951566
Rabbit, white (m,f)	skin irritation	4 d, EPA guideline 152-14	Non-irritant (0.5 g/animal)	EIP 8/950822/SE
Rabbit, white (m,f)	eye irritation	7 d, EPA guideline 152-13	Slight (70 mg/eye)	EIP 9/950823/SE
Guinea pig (f)	skin sensitization	3 d, EPA guideline 152-15	Slight sensitization	EIP 10/950818/SS

Table B. Toxicology profile of azadirachtin TK (NeemAzal[®]), based on repeated administration (sub-acute to chronic)

Species	Test	Duration and conditions	Result	References
Rat (m,f)	Oral	90 d, EPA guideline 152-153	NOEL: 100 ppm in feed NOAEL = 10 mg/kg bw/d LOEL = 400 ppm	EIP 4/963100
Rat (m,f)	Feeding, carcinogenicity	105 wk, Gaitonde committee (India) guidelines No. 6.3.0.C.4	Non-carcinogenic	7291
Rat (m,f)	Feeding, 2-generation reproduction	105 wk, Gaitonde committee (India) guidelines No. 6.3.0.C.4	NOEL: 750 mg/kg bw (highest dose)	4826
Rat (f)	Teratogenicity	105 wk, Gaitonde committee (India) guidelines No. 6.3.0.C.4	Not teratogenic NOEL: 500 mg/kg bw	4824
Rat (f)	Developmental toxicity	20 d, EPA guideline 152-23	Not teratogenic NOAEL: 50 mg/kg bw	EIP 2/952493

The sub-acute to chronic oral toxicity of azadirachtin/NeemAzal[®] TK is relatively low. Azadirachtin was not carcinogenic in rats after administration via the diet, and did not lead to any malformations in rats and their offspring.

Table C. Mutagenicity profile of azadirachtin TK (NeemAza[®]) based on *in vitro* and *in vivo* tests.

Species	Test	Conditions	Result	References
<i>Salmonella typhimurium</i>	Point mutation, Ames test	Dose range: 5, 1.5, 0.5, 0.15 or 0.05 mg/plate, EPA FIFRA Guideline 152-17	Not mutagenic	EIP 11/950642
Chinese hamster ovary (CHO) cell line (HGPRT locus)	Point mutation, HO/HGPRT test	Dose range (µg/ml ethanol: 25, 50, 100, 200, 400, 800, 1000, 1250), EPA FIFRA Guideline 152-17	Not mutagenic	7291
CD-1 Mouse bone marrow cells	Chromosome aberration, micronucleus test, <i>in vivo</i>	Oral administration, dose range: 1250, 2500, 5000 mg/kg bw, EPA Subdiv. M Guideline 152-17	Not mutagenic	EIP 13/952782

The genotoxic potential of azadirachtin/ NeemAza[®] TK was tested covering the endpoints gene mutation and chromosome damage. *In vivo* studies performed with CD-1 mice gave no indication of chromosome aberration. Azadirachtin/NeemAza[®] technical was thus found to be devoid of mutagenic activity on the basis of the studies performed.

Table D. Ecotoxicology profile of azadirachtin TK (NeemAza[®])

Species	Test	Duration and conditions	Result	References
<i>Scenedesmus subspicatus</i> (green alga)	Effect on growth, static water	72 h, static water, 22°C; pH 7.6–8.5; OECD (201)	EC ₅₀ (biomass) = 530 mg/l EC ₅₀ (growth rate) = 996 mg/l NOEC (biomass) <13.5 mg/l NOEC (growth rate) <13.5 mg/l	TRF-001/4-30
<i>Daphnia magna</i> (water flea)	Acute toxicity	24/48 h, static water; OECD (202) Part I	EC ₅₀ (24 h) = 107.55 mg/l EC ₅₀ (48 h) = 23.63 mg/l	TRF-001/4-21
<i>Daphnia magna</i> (water flea)	Chronic toxicity	21 d, semi-static; OECD guideline 202 Part II	NOEC = 2.5 mg/l (highest test concentration)	TRF-001/4-21
<i>Danio rerio</i> (zebra fish)	Acute toxicity (early life stage test)	37 d; OECD (210)	NOEC = 6.4 mg/l	TRF-001/4-60
<i>Danio rerio</i> (zebra fish)	Reproductive toxicity (full life cycle)	134 d; OECD (210)	NOEC (lethal) = 6.4 mg/l NOEC (sub-lethal) = 6.4 mg/l	TRF-001/4-60
<i>Colinus virginianus</i> (bobwhite quail)	Acute oral toxicity	14 d; EPA Subdiv. E 71-1	NOEL = 4000 mg/kg bw (highest dose)	EIP 21/960383
<i>Colinus virginianus</i> (bobwhite quail)	Dietary toxicity	8 d; EPA Subdiv. E 71-2	NOEL = 5200 mg/kg diet (highest dose)	EIP 22/960382

Azadirachtin/ NeemAza[®] TK is of low toxicity to aquatic and terrestrial organisms including fish, aquatic invertebrates and birds.

ANNEX 2. REFERENCES

Proposer's document number or other reference	Year and title of report or publication details
1749	1993. Acute oral toxicity of Neemazal technical in mice.
4824	1998. Effect of NeemAzal technical 27.3% on reproductive processes: segment II teratological study in wistar rats.
4826	2000. Evaluation of toxicity of NeemAzal technical to reproductive process in wistar rats - segment IV - toxicity to two generation reproductive process.
7291	1997. NeemAzal technical - mammalian cell mutation assay.
7291	2000. Long-term carcinogenicity study of NeemAzal technical in wistar rats.
95 50 40 827 B	1995. Direct phototransformation study (quantum yield) in purified water, OECD draft test guideline, part A, "direct phototransformation" NeemAzal.
955040827 A	1995. Melting point, EC Directive 92/69/EEC, A.1., NeemAzal.
Barrek <i>et al.</i> 2004	Barrek S., Paise O. and Grenier-Loustalot M.F., 2004. Analysis of neem oils by LC-MS and degradation kinetics of azadirachtin-A in a controlled environment. <i>Anal. Bioanal. Chem.</i> , 378 , 753-763.
EIP 10/950818/SS	1997. NeemAzal technical - skin sensitisation in the guinea-pig.
EIP 11/950642	1997. NeemAzal technical bacterial mutation assay.
EIP 13/952782	1997. NeemAzal technical mouse micronucleus test.
EIP 2/952493	1997. A study of developmental toxicity in rats (gavage administration).
EIP 21/960383	1996. NeemAzal technical: acute oral toxicity (ld 50) to bob-white quail.
EIP 22/960382	1996. NeemAzal technical: dietary toxicity (ld 50) to the bob-white quail.
EIP 4/963100	1997. NeemAzal technical toxicity study in rats by dietary administration for 13 weeks.
EIP 5/951566	1997. NeemAzal technical acute inhalation toxicity in rats 4-hour exposure.
EIP 6/950799/AC	1997. NeemAzal technical acute oral toxicity to the rat.
EIP 7/950800/AC	1997. NeemAzal technical acute dermal toxicity to the rat.
EIP 8/950822/SE	1996. NeemAzal technical skin irritation to the rabbit.
EIP 9/950823/SE	1996. NeemAzal technical eye irritation to the rabbit.
FAO/WHO 2002	Manual on development and use of FAO and WHO specifications for pesticides, 1 st edition. FAO and WHO, 2002, Rome.
Isman <i>et al.</i> 1990	Isman M.B., Koul O., Luczynski A. and Kaminski J., 1990. Insecticidal and antifeedant bioactivities of neem oils and their relationship to azadirachtin content. <i>J Agric. Food Chem.</i> , 38 , 1406-1411.
Jarvis <i>et al.</i> 1998	Jarvis A.P.; Johnson S. and Morgan D., 1998. Stability of the natural insecticide in aqueous and organic solvents. <i>Pesticide Science</i> 53 , 217-222.
LP 95.9	1995. Löslichkeitsverhalten von neemazal in wasser.
Pesticide Manual	Tomlin C.D.S., Ed., 2000. The Pesticide Manual, 12 th Edition, British Crop Protection Council, UK.
Rembold 2004	Rembold H., 2004. Der Neembbaum: Quelle für eine neue Strategie im Pflanzenschutz? <i>Entomologie heute</i> , 16 , 235-243.
Schmutterer 2002	Schmutterer H., Ed. 2002. The neem tree. Neem Foundation, Mumbai, India.
Sundaram & Curry 1994	Sundaram K.M.S and Curry J., 1994. Initial deposits and persistence of Azadirachtin in fir and oak foliage after spray application of 'Margosan-O' formulation. <i>Pesticide Science</i> , 41 , 129-138.
Sundaram <i>et al.</i> , 1995	Sundaram, K.M.S., Sloane L., and Curry J., 1995. Kinetics of azadirachtin hydrolysis in model aquatic systems by high-performance liquid chromatography. <i>J Liquid Chromatography</i> 18 : 363-376.
TM 1195.13	1995. Verteilungskoeffizienten verschiedener Inhaltsstoffe vonNeemAzal in n-octanol-wasser.
TM 1195.15	1996. Hydrolysestabilität von NeemAzal.

Proposer's document number or other reference	Year and title of report or publication details
TRF-001/4-21	1999. Effects of NeemAzal on <i>Daphnia magna</i> , acute immobilization test and reproduction test.
TRF-001/4-30	2002. Algal growth inhibition test effects of NeemAzal on the growth of <i>Scenedesmus subspicatus</i> .
TRF-001/4-60	1999. Effects of neemazal on fish: full life cycle test with the zebra fish, <i>Danio rerio</i> , test period: early life stage test (until day 37).
TRF-001/4-60	2000. Effects of NeemAzal on fish: full life cycle test with the zebra fish, <i>Danio rerio</i> .
Unnumbered A	1993. Acute oral toxicity of NeemAzal technical in mice.

Determination of Aflatoxins in NeemAzal and NeemAzal-T/S

HPLC-Method

compiled by:
Trifolio-M GmbH
Sonnenstraße 22
35633 Lahnau, Germany

General Information about Aflatoxins

Neem seed kernels are the source of a natural insecticide named Azadirachtin. These kernels may be infected by *Aspergillus* spp. during collection and storage.

The concentrations of aflatoxins in NeemAzal technical are analysed routinely during the production process. Also each batch of the formulated product NeemAzal-T/S is analysed to comply with the maximum residue limit (MRL) for foodstuffs, which is 4 µg/kg for the sum of aflatoxins B₁, B₂, G₁ and G₂.

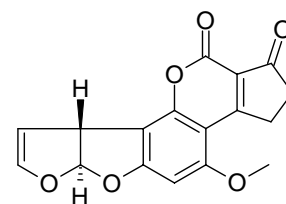
The method used is based on DIN EN 14123 and was peer validated.

Aflatoxin B₁

CAS-No.: 1162-65-8

Structure:

Molecular formula: C₁₇H₁₂O₆

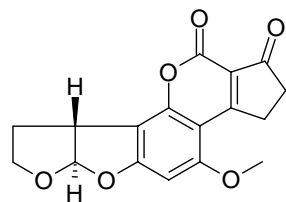


Aflatoxin B₂

CAS-No.: 7220-81-7

Structure:

Molecular formula: C₁₇H₁₄O₆

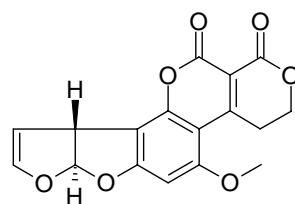


Aflatoxin G₁

CAS-No.: 1165-39-5

Structure:

Molecular formula: C₁₇H₁₂O₇

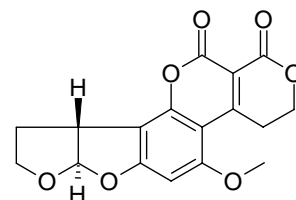


Aflatoxin G₂

CAS-No.: 7241-98-7

Structure:

Molecular formula: C₁₇H₁₄O₇



1. Aflatoxins B₁, B₂, G₁ and G₂ (in NeemAzal technical)

Sampling. Take at least a 50 g sample of NeemAzal technical.

OUTLINE OF METHOD.

Aflatoxins are determined by RP-HPLC using post-column derivatisation and fluorescence detection.

REAGENTS

General

Use only reagents of recognized analytical grade unless otherwise stated.

Reference material - Aflatoxin stock solutions of known content

Acetonitrile - HPLC grade

Methanol - HPLC grade

Water - HPLC grade

Toluene

Potassium bromide

Nitric acid, HNO₃ = 4 mol/l

Dilution solvent for aflatoxin standard solutions

Toluene + acetonitrile, 98 + 2 (v/v)

Phosphate buffered saline (PBS)

Dissolve 0.2 g KCl, 0.2 g KH₂PO₄, 2.92 g Na₂HPO₄·12 H₂O and 8.0 g NaCl in 0.9 l water and adjust to pH 7.4 with HCl (0.1 mol/l) or NaOH (0.1 mol/l) as appropriate. Make up to 1 l with water.

Immunoaffinity column. Contains antibodies raised against aflatoxins B₁, B₂, G₁ and G₂.

Mobile phase

Water + acetonitrile + methanol, 6 + 2 + 3 (v/v) Add 120 mg KBr and 350 µl HNO₃ per litre of mobile phase. Degas solution prior to use.

Extraction solvent

Methanol + water, 8 + 2 (v/v)

Tween 20[®] - 10% in water

Calibration stock solutions

Prepare a stock solution of each aflatoxin (B₁, B₂, G₁ and G₂) at a concentration of 10 µg/ml each in the dilution solvent. Since the aflatoxins are photolabile, wrap the flasks tightly with aluminium foil and store them below 4°C.

Prepare a mixed aflatoxin stock solution containing 1000 ng/ml of aflatoxins B₁ and G₁ and 200 ng/ml of aflatoxins B₂ and G₂ by appropriate dilution (with dilution solvent) of the single stock solutions. Pipette exactly 2 ml of this solution into a 20 ml volumetric flask and fill to the mark with the dilution solvent. Shake well and keep the solutions also tightly wrapped in aluminium foil and store them below 4°C.

Calibration standard solutions

Pipette volumes of the mixed aflatoxin stock solution into 10 ml volumetric flasks as given below:

Standard solution	Volume of diluted mixed stock solution [μ l]	Mass concentration of standard solution [ng/ml]			
		B ₁	B ₂	G ₁	G ₂
1	10	0.1	0.02	0.1	0.02
2	50	0.5	0.10	0.5	0.10
3	100	1.0	0.20	1.0	0.20
4	150	1.5	0.30	1.5	0.30
5	200	2.0	0.40	2.0	0.40
6	250	2.5	0.50	2.5	0.50
7	300	3.0	0.60	3.0	0.60

Evaporate the toluene + acetonitrile solutions just to dryness under a stream of nitrogen at room temperature. To each flask add 5 ml methanol. After dissolving the aflatoxins, fill to the mark with water and shake well.

Spiking solution

Prepare a spiking solution by pipetting volumetrically 2 ml of the mixed aflatoxin stock solution (containing 1000 ng/ml of aflatoxins B₁ and G₁, and 200 ng/ml of aflatoxins B₂, and G₂) into a 10-ml calibrated volumetric flask. Evaporate the toluene/acetonitrile solution just to dryness under a stream of nitrogen at room temperature. Dilute to the mark with methanol and shake well. The aflatoxin concentrations of this spiking solution is 200 ng/ml of B₁ and G₁, and 40 ng/ml of B₂, and G₂. Wrap the flask tightly in aluminium foil and store it at less than 4°C. Before use, do not open the flask until the contents have reached room temperature to avoid incorporation of water by condensation.

APPARATUS

General

Wash all glassware coming into contact with aqueous solutions of aflatoxins with acid solution prior to use, e.g. with sulphuric acid (2 mol/l) for several hours. Rinse well with water to remove traces of acid and check the absence of acid with pH paper.

Volumetric glassware (flasks and pipettes). Class A.

Reservoir. 10 ml with luer tip connector for immunoaffinity column (IAC).

High performance liquid chromatograph equipped with a fluorescence detector with a wavelength of 360 nm excitation filter and a wavelength 420 nm cut-off emission filter; sample injector; a valve with a 200- μ l loop is recommended.

Integrator or other data system.

Column. any RP-C18 column with an appropriate guard column (with the same packing material as the column). Flow and retention times vary due to column dimension.

Balance. 0.01 mg accuracy

Post-column derivatisation system. Kobra Cell[®] - System for derivatisation with electrochemically-generated bromine.

Calibrated microlitre syringes or microlitre pipettes. 10 to 500 μ l

Manifold

Disposable filter units (optional). Prior to usage, verification that no aflatoxin losses occur during filtration is necessary (recovery testing).

PROCEDURES

(a) *Chromatographic conditions*

<i>Column temperature</i>	room temperature
<i>Flow rate</i>	depending on column
<i>Wavelength</i>	360 nm excitation filter and above 420 nm cut-off emission filter, or equivalent
<i>Injection volume</i>	200 μ l
<i>Retention times</i>	order of elution: G ₂ , G ₁ , B ₂ , B ₁ . Retention times depend on the column.

(b) *Linearity check.* Check the linearity of the detector response by using the mixed aflatoxin standard solutions as described in the previous Section on Reagents. These solutions cover the range of 0.1 ng/ml to 3.0 ng/ml for B₁ and G₁ and also the range of 0.02 ng/ml to 0.6 ng/ml for B₂ and G₂.

If aflatoxin contents in the sample are outside the calibration range, prepare diluted or concentrated sample solutions with aflatoxin contents appropriate for the established calibration curve.

(c) Preparation of the sample solution.

Weigh (to the nearest 0.1 g) 5 g of NeemAzal into a 100 ml volumetric flask and fill up with the extraction solvent to the mark. Shake thoroughly by hand. Take 5 ml of the extract and dilute it with 20 ml of Tween 20[®] solution. Add the diluted sample extract to a 10-ml reservoir connected to the conditioned immunoaffinity column and proceed as described below.

Immunoaffinity cleanup.

Follow different conditioning procedures according to the manufacturer's instruction.

Connect the column to a manifold and attach a 10-ml reservoir to it. Take 9 ml of the sample solution and transfer it to the reservoir and let it pass through the column at a flow rate of approximately 3 ml/min. Do not exceed a flow rate of maximum 5 ml/min. Wash the column three times with 5-ml portions of water and dry it by applying little vacuum for 5 to 10 seconds or passing air through the column by means of a syringe.

Elute the aflatoxins in a two-step procedure by applying 0.75 ml methanol to the column and letting it pass through by gravity. Collect the eluate in a 3-ml volumetric flask and repeat the elution into the same flask with another portion of 0.75 ml after 1 minute. Collect most of the methanol by passing air through the column. Make up with water and shake well. If a clear solution is obtained, use directly for HPLC analysis, but otherwise use a disposable filter prior to injection.

(d) HPLC determination. Inject 200 µl of each sample solution in duplicate. The aflatoxins are separated by RP-phase at room temperature. The recommended flow rate is 1.0 ml/min for a column of inner diameter of 4.6 mm and may be adjusted according to the column dimension used. The aflatoxins elute in the order G2, G1, B2 and B1 and should be base-line resolved (typical chromatograms are enclosed).

(e) Post column derivatisation

When using a KOBRA cell[®] (electrochemically-generated bromine), follow the instructions for installing the cell as supplied by the manufacturers, and operate with a flow rate of 1.0 ml/min for the mobile phase and a current of 100 µA.

(f) *Spiking procedure*

For recovery determinations, carry out the spiking procedure using the methanol spiking solution of aflatoxin standards. The spiking level must be within the calibration range (preferably mean value). Take care that not more than 2 ml of the spiking solution are added and that the subsequent evaporation takes place in the dark and should last 30 min to 2 h.

(g) *Calculation.* Calculate the concentration of aflatoxins in ng/g from the established calibration curve using linear regression.

$$\text{Content of aflatoxin} = \frac{C_{Afla} \cdot V_e \cdot V_{final}}{m_S \cdot V_{iac}} \text{ ng/g}$$

where:

C_{Afla} = concentration of aflatoxin in the injection solution calculated from linear regression, in ng/ml

V_e = volume of the extraction solvent in ml ($V_e = 100$ ml)

V_{final} = final volume after elution from IAC in ml (3 ml)

m_S = mass of the sample taken for analysis in g ($m_S = 5$ g for NeemAzal)

V_{iac} = volume of the sample extract taken for IAC in ml (9 ml)

(h) *Recovery.* The recovery (R) is calculated using the following equation:

$$R(\%) = \frac{R_{fortified}}{F} \cdot 100\%$$

$R_{fortified}$ = residues measured in the fortified specimen, in $\mu\text{g/kg}$

F = fortification level, in $\mu\text{g/kg}$

2. Aflatoxins B₁, B₂, G₁ and G₂ (in NeemAzal-T/S)

Sampling. Take at least a 50 ml sample of NeemAzal-T/S.

Follow details as for aflatoxins in NeemAzal technical except for the following:

(c) *Preparation of the sample solution.*

Weigh (to the nearest 0.1 g) 20 g of NeemAzal-T/S into a 100 ml volumetric flask and make up with the extraction solvent. Proceed as described for NeemAzal technical.

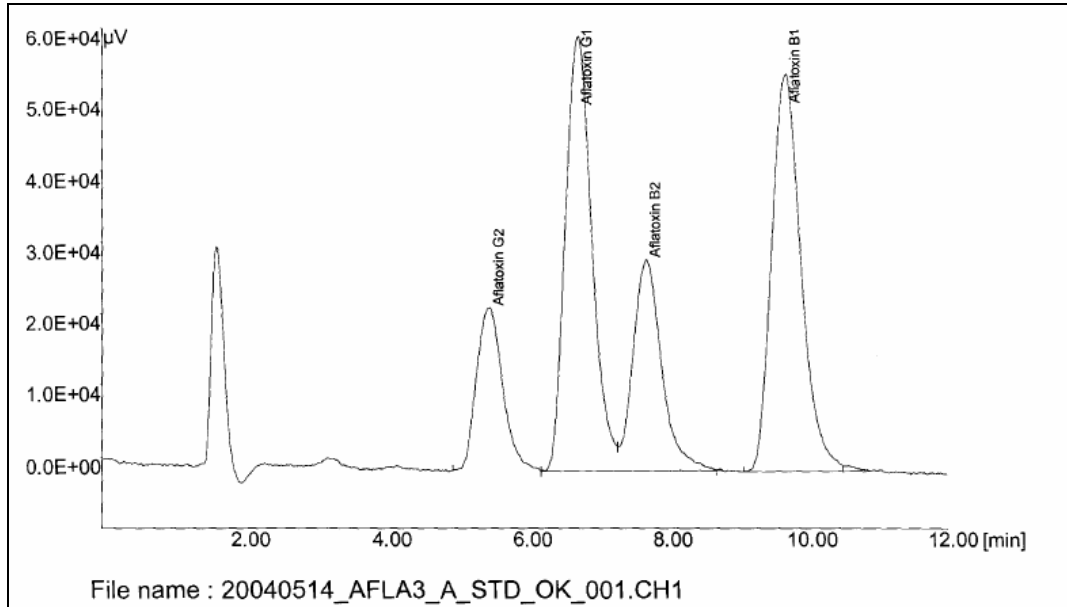
Representative chromatograms

Fig. 1: Chromatogram of a multiple aflatoxin reference standard (1 ppb).

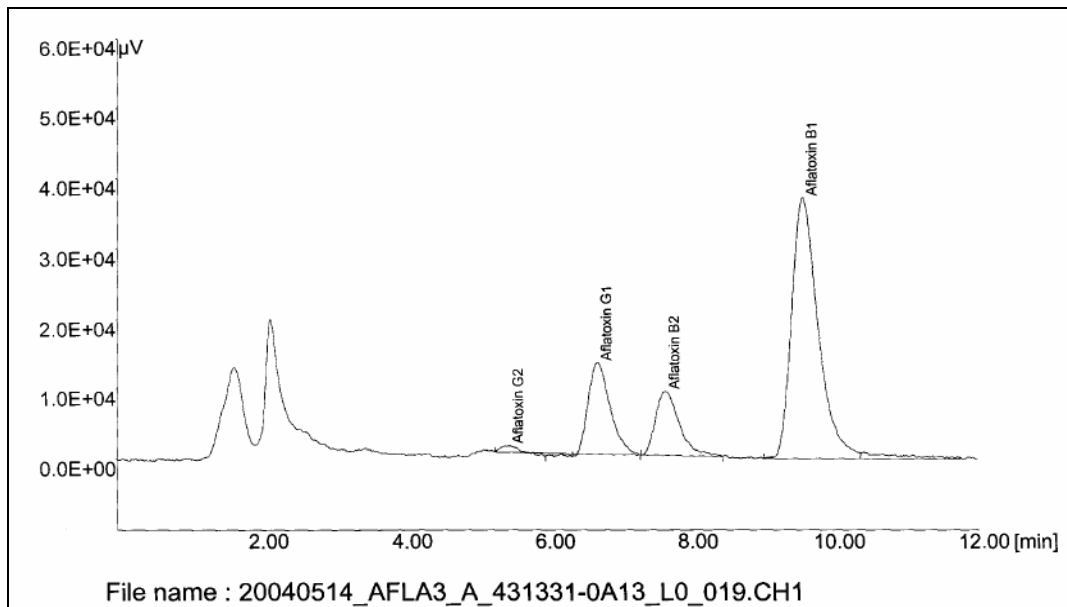


Fig. 2: Chromatogram of a sample extract of NeemAzal technical (TK)

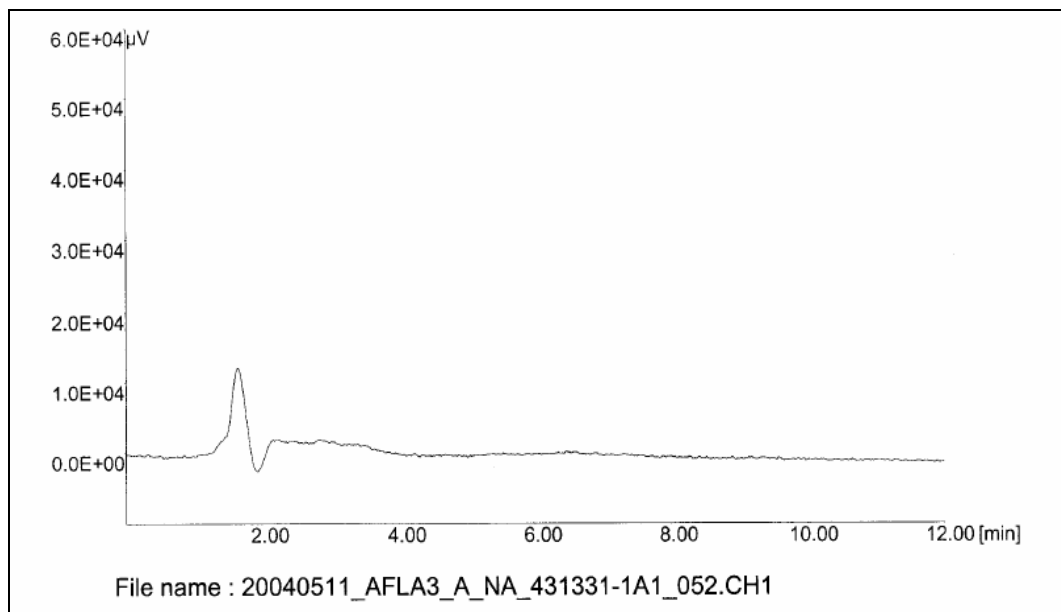


Fig. 3: Chromatogram of a sample extract of NeemAzal-T/S (EC)