



SLOVENSKI STANDARD

oSIST prEN 17521:2020

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Živila - Določevanje toksinov "Alternaria" v paradižniku, pšenici in sončničnih semenih s čiščenjem SPE in HPLC-MS/MS

Foodstuffs - Determination of Alternaria toxins in tomato, wheat and sunflower seeds by SPE clean-up and HPLC-MS/MS

Lebensmittel - Bestimmung von Alternariatoxinen in Tomaten, Weizen und Sonnenblumenkernen mit Flüssigchromatographie und Tandem-Massenspektrometrie

Produits alimentaires - Détermination de la teneur en toxines d'Alternaria dans la tomate, le blé et les graines de tournesol purification par SPE et CLHP-SM/SM

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67.050

Splošne preskusne in
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General methods of tests and
analysis for food products

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EUROPEAN STANDARD
NORME EUROPÉENNE
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prEN 17521

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English Version

Foodstuffs - Determination of Alternaria toxins in tomato, wheat and sunflower seeds by SPE clean-up and HPLC-MS/MS

Produits alimentaires - Détermination de la teneur en toxines d'*Alternaria* dans la tomate, le blé et les graines de tournesol par chromatographie liquide couplée à la spectrométrie de masse en tandem

Lebensmittel - Bestimmung von Alternariatoxinen in Tomaten, Weizen und Sonnenblumenkernen mit Flüssigchromatographie und Tandem-Massenspektrometrie

This draft European Standard is submitted to CEN members for enquiry. It has been drawn up by the Technical Committee CEN/TC 275.

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Recipients of this draft are invited to submit, with their comments, notification of any relevant patent rights of which they are aware and to provide supporting documentation.

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EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

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European foreword

This document (prEN 17521:2020) has been prepared by Technical Committee CEN/TC 275 “Food analysis – Horizontal methods”, the secretariat of which is held by DIN.

This document is currently submitted to the CEN Enquiry.

This document has been prepared under a mandate given to CEN by the European Commission and the European Free Trade Association.

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Introduction

Alternaria species, the most prevalent being *A. alternata*, produce more than 70 secondary metabolites, but only a few of them have been structurally identified and reported as mycotoxins [1]. *Alternaria* fungi are common plant pests in cereals, oilseeds, fruits and vegetables, among other foods, and the presence of *Alternaria* toxins in these commodities has been widely reported [1]. These toxins do not only contaminate harvests but they can also spoil foods in the fridge-temperatures.

Among these *Alternaria* toxins altenuene (ALT), alternariol (AOH), alternariol monomethyl ether (AME), tentoxin (TEN) and tenuazonic acid (TEA) are the ones of major concern. ALT, AOH, AME are dibenzo- α -pyrones, TEN is a cyclic tetrapeptide and TEA is a tetramic acid derivative. ALT and TEA have shown high acute toxicity *in vitro* and in animal experiments. AME and AOH are not very acutely toxic; however, they have been described to induce genotoxic and mutagenic effects [1] to [3].

These toxins generally appear in tomato products, cereals and oilseeds (e.g. sunflower seeds), therefore they are the focus of this document.

WARNING 1 — Suitable precaution and protection measures need to be taken when carrying out working steps with harmful chemicals. The latest version of the hazardous substances ordinance, Regulation (EC) No 1907/2006 [4] should be taken into account as well as appropriate national statements.

WARNING 2 — The use of this document can involve hazardous materials, operations and equipment. This document does not purport to address all the safety problems associated with its use. It is the responsibility of the user of this document to establish appropriate safety and health practices and determine the compatibility with regulatory limitations prior to use.

WARNING 3 — Some *Alternaria* toxins exhibit genotoxic and mutagenic effects.

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1 Scope

This document specifies a procedure for the determination of five *Alternaria* toxins in wheat, tomato puree and sunflower seeds by high-performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (MS/MS).

The method has been validated with both naturally contaminated and spiked samples of wheat, tomato puree and sunflower seeds.

Validation levels for altenuene (ALT) ranged from 2,18 µg/kg to 13,8 µg/kg.

Validation levels for alternariol (AOH) ranged from 1,82 µg/kg to 46,7 µg/kg.

Validation levels for alternariol monomethyl ether (AME) ranged from 1,29 µg/kg to 47,2 µg/kg.

Validation levels for tentoxin (TEN) ranged from 5,29 µg/kg to 218 µg/kg.

Validation levels for tenuazonic acid (TEA) ranged from 41,8 µg/kg to 1 618 µg/kg.

Limits of quantification of 1 µg/kg for ALT (except in wheat and sunflower seeds), AOH and AME; 5 µg/kg for TEN and 10 µg/kg for TEA or lower are achievable using this method.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN ISO 3696, *Water for analytical laboratory use - Specification and test methods (ISO 3696)*

3 Terms and definitions

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No terms and definitions are listed in this document.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

- IEC Electropedia: available at <http://www.electropedia.org/>
- ISO Online browsing platform: available at <http://www.iso.org/obp>

4 Principle

A test portion of the sample spiked with the isotopically-labelled internal standards is extracted with a methanol, water and acetic acid solution. The sample/extraction solvent mixture is centrifuged and an aliquot of the supernatant is collected. The extract is diluted with an equal volume of 1 % aqueous acetic acid solution, and concentrated on a polymeric solid-phase extraction (SPE) adsorbent. The extract is eluted from the SPE column with a methanol and ethyl acetate solution. The eluate is then evaporated, reconstituted, filtered through a polytetrafluoroethylene (PTFE) syringe filter and subsequently analysed by HPLC-MS/MS.

5 Reagents

Use only reagents of recognized analytical grade and water complying with grade 1 of EN ISO 3696, unless otherwise specified. Solutions shall be of quality for LC-MS analysis, unless otherwise specified. Commercially available solutions with equivalent properties to those listed may be used.

- 5.1 **Nitrogen compressed gas**, purity equivalent to 99,99 % or better.
- 5.2 **Water (H₂O)**, HPLC grade.
- 5.3 **Water (H₂O)**, LC-MS grade.
- 5.4 **Methanol**, analytical grade.
- 5.5 **Methanol**, LC-MS grade.
- 5.6 **Ethyl acetate**, analytical grade or higher.
- 5.7 **Ammonium hydroxide (NH₄OH)**, LC-MS grade, volume fraction $\varphi(\text{NH}_4\text{OH}) = 25 \%$.
- 5.8 **Ammonium hydroxide (NH₄OH)**, $\varphi(\text{NH}_4\text{OH}) = 2,5 \%$.

Add 1 ml of ammonium hydroxide 25 % (5.7) to a 10 ml volumetric flask containing approximately 5 ml of water (5.3) and fill up to the mark with water (5.3).

- 5.9 **Acetic acid**, $\varphi \geq 99,7 \%$.

- 5.10 **Ammonium acetate (CH₃COONH₄)**, LC-MS grade.

- 5.11 **Ammonium acetate solution (CH₃COONH₄)**, molar concentration $c = 1 \text{ mol/l}$.

Dissolve 77,08 g of ammonium acetate (5.10) in 1 l water (5.3).

- 5.12 **Extraction mixture**, methanol + water + acetic acid (85+14+1, v+v+v) mixture.

Mix 850 ml of methanol (5.4) with 140 ml of water (5.2) and 10 ml of acetic acid (5.9).

- 5.13 **Aqueous acetic acid solution**, acetic acid + water (1+99, v+v), $\varphi = 1 \%$.

Mix 10 ml of acetic acid (5.9) with 990 ml of water (5.2) and homogenize well.

- 5.14 **Elution solution**, methanol + ethyl acetate (75+25, v+v).

Mix 750 ml of methanol (5.4) with 250 ml of ethyl acetate (5.6) and homogenize well.

- 5.15 **HPLC mobile phase A**, 5 mmol/l ammonium acetate buffer at pH approximately 8,0.

Mix 5 ml of ammonium acetate solution (5.11) and approximately 200 μl of ammonium hydroxide 2,5 % (5.8) with 900 ml of water (5.3). Adjust the volume with water (5.3) to 1 l and homogenize well.

Check the pH of the mobile phase A. The pH shall be between 7,95 and 8,05. Adjust with ammonium hydroxide 2,5 % (5.8) to be within that range.

- 5.16 **HPLC mobile phase B, methanol (5.5).**

- 5.17 **Alternaria toxins' standards**, e.g. crystalline, as a film or as a reference material.

- 5.17.1 **Altenuene**, at least 96 % purity (m/m).

- 5.17.2 **Alternariol**, at least 96 % purity (m/m).

- 5.17.3 **Alternariol monomethyl ether**, at least 96 % purity (m/m).

5.17.4 Tentoxin, at least 96 % purity (m/m).

5.17.5 Tenuazonic acid, at least 96 % purity (m/m).

NOTE Laboratories can seek for an higher purity, if available on the market.

5.18 Isotopically-labelled internal standards, e.g. crystalline or as a standard solution.

5.18.1 Altenuene isotopically-labelled internal standard (ALT-ISTD), e.g. ALT-(methoxy-d3, methyl-d3) (ALT-d6).

5.18.2 Alternariol isotopically-labelled internal standard (AOH-ISTD), e.g. AOH-(methyl-d3) (AOH-d3).

5.18.3 Alternariol-monomethyl ether isotopically-labelled internal standard (AME-ISTD), e.g. AME-(1-methyl-d3) (AME-d3).

5.18.4 Tentoxin isotopically-labelled internal standard (TEN-ISTD), e.g. TEN-d3.

5.18.5 Tenuazonic acid isotopically-labelled internal standard (TEA-ISTD), e.g. TEA-(acetyl-¹³C₂) (TEA ¹³C₂), mixture of diastereomers in methanol.

NOTE Laboratories can seek for isotopically-labelled internal standards with a high degree of deuteration or ¹³C-enrichment.

WARNING – Protective clothing, gloves and safety goggles should be worn at all times, and all standard and sample preparation stages should be carried out in a fume hood.

5.19 Stock solutions of ALT, AOH, AME, TEN and TEA, e.g. at a mass concentration $\rho = 100 \mu\text{g/ml}$.

In case of crystalline powders, weigh in e.g. 5 mg of ALT (5.17.1), AOH (5.17.2), AME (5.17.3), TEN (5.17.4) and TEA (5.17.5) to the nearest 0.1 mg into individual 50 ml volumetric flasks and fill up to the mark with methanol (5.5). Homogenize vigorously.

In case of dried-down films, reconstitute the standard in the vial according to the certificate of each individual standard.

The stock solutions are stable for at least six months if stored at $\leq 18 \text{ }^\circ\text{C}$.

NOTE For the purpose of photometrically verifying the concentration of gravimetrically prepared standards, molar extinction coefficients are available in the literature for AOH [5], AME [5], ALT [6], TEN [7] and TEA [8].

5.20 Standard solution 1.

Prepare a methanolic standard mixture that contains ALT (5.17.1), AOH (5.17.2), AME (5.17.3) in 500 ng/ml concentration, TEN (5.17.4) in 2 500 ng/ml concentration and TEA (5.17.5) in 5000 ng/ml concentration.

For that purpose, transfer e.g. 25 μl of the stock solution of ALT, AOH and AME (5.19) into a 5 ml volumetric flask. Transfer e.g. 125 μl of stock solution of TEN (5.19) and e.g. 250 μl of stock solution of TEA (5.19) into the same 5 ml volumetric flask. Fill up to the calibration mark with methanol (5.5). Homogenize vigorously. The standard solution 1 is stable for at least six months if stored at $\leq 18 \text{ }^\circ\text{C}$.

NOTE The exact volumes to be measured to prepare the standard solution 1 (5.20) are derived from the exact concentration of the stock solutions (5.19).

5.21 Standard solution 2.

Prepare a methanolic standard mixture that contains ALT (5.17.1), AOH (5.17.2), AME (5.17.3) in 100 ng/ml concentration, TEN (5.17.4) in 500 ng/ml concentration and TEA (5.17.5) in 1 000 ng/ml concentration. Standard solution 2 is prepared by diluting standard solution 1 (5.20).

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Transfer 1 000 µl of the standard solution 1 (5.20) into a 5 ml volumetric flask. Fill up to the calibration mark with methanol (5.5). Homogenize vigorously. Standard solution 2 is stable for at least six months if stored at ≤ 18 °C.

5.22 Stock solutions of ALT-ISTD, AOH-ISTD, AME-ISTD, TEN-ISTD and TEA-ISTD, e.g. at a mass concentration $\rho = 750 \mu\text{g/ml}$.

Prepare the stock solutions, e.g. dissolve 1 mg of crystalline powders of ALT-ISTD (5.18.1), AOH-ISTD (5.18.2), AME-ISTD (5.18.3), TEN-ISTD (5.18.4) and TEA-ISTD (5.18.5) in individual vials with 1500 µl of methanol (5.5). Homogenize vigorously. The stock solutions are stable for at least six months if stored at ≤ 18 °C. TEA-ISTD might be supplied already as a methanolic solution with approximately the same concentration.

5.23 Internal standard solution 1.

Prepare a methanolic standard mixture that contains AOH-ISTD (5.18.2), AME-ISTD (5.18.3) and TEN-ISTD (5.18.4) in 5 µg/ml concentration, ALT-ISTD (5.18.1) in 10 µg/ml concentration and TEA-ISTD (5.18.5) in 25 µg/ml concentration.

For that purpose, transfer e.g. 33,3 µl of the stock solutions of AOH-ISTD, AME-ISTD and TEN-ISTD (5.22) into a 5 ml volumetric flask. Transfer e.g. 66,7 µl of stock solution of ALT-ISTD (5.22) and e.g. 166,7 µl of stock solution of TEA-ISTD (5.22) into the same 5 ml volumetric flask. Fill up to the calibration mark with methanol (5.5). Homogenize vigorously. The internal standard solution 1 is stable for at least six months if stored at ≤ 18 °C.

5.24 Internal standard solution 2.

Prepare a methanolic standard mixture that contains AOH-ISTD (5.18.2), AME-ISTD (5.18.3) and TEN-ISTD (5.18.4) in 500 ng/ml concentration, ALT-ISTD (5.18.1) in 1 000 ng/ml concentration and TEA-ISTD (5.18.5) in 2 500 ng/ml concentration. Internal standard solution 2 is prepared by diluting internal standard solution 1 (5.23).

Transfer 1 000 µl of the internal standard solution 1 (5.23) into a 10 ml volumetric flask. Fill up to the calibration mark with methanol (5.5). Homogenize vigorously. The internal standard solution 2 is stable for at least six months if stored at ≤ 18 °C.

5.25 Polysorbate 20 (C₅₈H₁₁₄O₂₆) (Tween®20¹), analytical grade.

5.26 Polysorbate 20 (C₅₈H₁₁₄O₂₆) solution, 2 % (v+v) in water.

Pipette 2 ml of polysorbate 20 (5.25) into a 100 ml volumetric flask and fill up to the mark with water (5.2). Homogenize well. This solution (polysorbate 20 + water, 2+98, v+v) can be used for three months if stored at approximately 4 °C.

5.27 Calibration solutions.

Add different volumes of standard solution (5.20 resp. 5.21) and the internal standard solution 2 (5.24) to five HPLC vials (6.18) e.g. as listed in Table 1. Add the methanol (5.5) volumes specified in Table 1 and mix gently. Add 600 µl of HPLC mobile phase A (5.15), cap the vial and mix it (6.15) for 20 s.

Prepare a sixth vial (blank) without standard solutions and without internal standard solution and use it as an instrumental blank.

¹ Tween®20 is a trade name of a polysorbate 20-type nonionic surfactant available from different suppliers. This information is given for the convenience of users of this European standard and does not constitute an endorsement by CEN of this product. Equivalent products may be used if they can be shown to lead to the same results.

Table 1 — Preparation of calibration solutions

					ALT	AOH	AME	TEN	TEA
Calibration solution	Standard solution 1 (5.20)	Standard solution 2 (5.21)	Internal standard solution 2 (5.24)	Methanol (5.5)	Equivalent concentration				
	μl	μl	μl	μl	$\mu\text{g/kg}$				
1		10	50	340	1	1	1	5	10
2		50	50	300	5	5	5	25	50
3		100	50	250	10	10	10	50	100
4	50	–	50	300	25	25	25	125	250
5	200	–	50	150	100	100	100	500	1000
Blank	–	–	–	400	–	–	–	–	–

6 Apparatus and equipment

Usual laboratory glassware and equipment and, in particular, the following:

6.1 pH meter.

6.2 Polypropylene (PP) centrifuge tube, (50 ml) with scale on it.

6.3 Laboratory balance, accuracy of 0,01 g.

6.4 Analytical balance, accuracy of 0,01 mg.

6.5 Adjustable mechanical vertical or horizontal shaker.

6.6 High speed blending device (e.g. Ultra-turrax^{®2}).

6.7 Centrifuge, with temperature control and capable of generating a relative centrifugal force of approximately 3 200 *g*.

6.8 Graduated volumetric pipettes, 10 ml capacity.

6.9 Displacement pipettes, e.g. 10 μl , 20 μl , 100 μl , 250 μl and 1 000 μl capacity, with appropriate tips.

6.10 Solid-phase extraction (SPE) column, with hydrophilic modified styrene polymer with 6 ml reservoir capacity, 200 mg adsorbent mass and, 100 μm particle size or smaller.

NOTE Phenomenex Strata-XL², with 6 ml reservoir capacity, 200 mg adsorbent mass and 100 μm particle size have shown to meet these specifications.

6.11 PP reservoirs (approximately 25 ml), fit to SPE columns (6.10).

² Ultra-turrax and Strata-XL are used as examples, available commercially from IKA and Phenomenex respectively. This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of the products named. Equivalent products may be used if they can be shown to lead to the same results.

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6.12 Polytetrafluoroethylene (PTFE) syringe filter, 0,2 µm pore size and e.g. 13 mm or 15 mm of diameter.

6.13 Syringe with needle, 1 ml.

6.14 Vacuum manifold, for SPE clean-up, with taps.

6.15 Mixer, with high shear rate.

6.16 Sample concentrator, with temperature control and gas supply.

6.17 Glass receiving tubes, for sample elution and evaporation.

6.18 Silanized glass HPLC vials, approximately 1,5 ml capacity and crimp caps or equivalent.

6.19 Beakers, 250 ml capacity.

6.20 Volumetric flasks, 5 ml, 10 ml, 50 ml, 100 ml and 1 l capacity.

6.21 HPLC-MS/MS system, with the following components:

6.21.1 HPLC pump, capable of maintaining a binary gradient at flow rates appropriate for the analytical column in use with sufficient accuracy.

6.21.2 Degasser, optional, for degassing HPLC mobile phases.

6.21.3 Injection system, capable of injecting an appropriate volume of injection solution with sufficient accuracy.

6.21.4 HPLC reversed phase column.

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A suitable column and appropriate HPLC conditions with sufficient retention capacity of the first eluting analyte. A capacity factor of 3 ($k' \geq 3,0$) and a minimum plate number for any of the analytes ($N \geq 2\ 000$) has shown to provide chromatograms as shown in Annex A.

Examples of suitable columns and gradients are reported in Annex B.

6.21.5 Pre-column, with the same stationary phase material as the analytical column (6.21.4).

6.21.6 Column oven, capable of maintaining a constant temperature.

6.21.7 Triple stage mass spectrometer.

Triple quadrupole, ion trap or quadrupole linear ion trap instrument, amongst others, equipped with an electrospray ionization (ESI) interface and operated in multiple reaction monitoring (MRM) mode. Any ionization mode (typically negative, or positive) giving sufficient yield may be employed.

6.21.8 Computer-based instrument control and data evaluation system.