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BSI Standards Publication

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

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National foreword

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The UK participation in its preparation was entrusted to Technical Committee AW/275, Food analysis - Horizontal methods.

A list of organizations represented on this committee can be obtained on request to its committee manager.

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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 purification par SPE et CLHP-SM/SM

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

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European foreword

This document (EN 17521:2021) has been prepared by Technical Committee CEN/TC 275 "Food analysis – Horizontal methods", the secretariat of which is held by DIN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by February 2022, and conflicting national standards shall be withdrawn at the latest by February 2022.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. CEN shall not be held responsible for identifying any or all such patent rights.

This document has been prepared under a Standardization Request 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 at refrigerator 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 - 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 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 – 1,4 µg/kg and 1,2 µg/kg, respectively), 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

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 https://www.electropedia.org/
- ISO Online browsing platform: available at https://www.iso.org/obp

4 Principle

A test portion of the sample spiked with the isotopically-labelled internal standards is extracted with a mixture of methanol, water and acetic acid. 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 % (φ) 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.

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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 φ = 99,99 % or better.

5.2 Water (H_2O), HPLC grade.

5.3 Water (H_2O), LC-MS grade.

5.4 Methanol (CH_3OH), analytical grade.

5.5 Methanol (CH_3OH), LC-MS grade.

5.6 Ethyl acetate ($CH_3COOC_2H_5$), analytical grade or higher.

5.7 Ammonium hydroxide (NH_4OH), LC-MS grade, mass fraction $w(NH_4OH)$ = 25 %.

5.8 Ammonium hydroxide (NH_4OH), $w(NH_4OH)$ = 2,3 %.

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 (CH_3COOH), $w \geq$ 99,7 %.

5.10 Ammonium acetate (CH_3COONH_4), LC-MS grade.

5.11 Ammonium acetate solution (CH_3COONH_4), molar concentration c = 1 mol/l.

Dissolve 77,08 g of ammonium acetate (5.10) in 1 l of 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, $V + V$), φ = 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 2,3 % (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 2,3 % (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 w = 96 % purity.

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5.17.2 Alternariol, at least w = 96 % purity.

5.17.3 Alternariol monomethyl ether, at least w = 96 % purity.

5.17.4 Tentoxin, at least w = 96 % purity.

5.17.5 Tenuazonic acid, at least w = 96 % purity.

Laboratories can seek for a 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-d_3, methyl-d_3) (ALT-d_6).

5.18.2 Alternariol isotopically-labelled internal standard (AOH-ISTD), e.g. AOH-(methyl-d_3) (AOH-d_3).

5.18.3 Alternariol monomethyl ether isotopically-labelled internal standard (AME-ISTD), e.g. AME-(1-methyl-d_3) (AME-d_3).

5.18.4 Tentoxin isotopically-labelled internal standard (TEN-ISTD), e.g. TEN-d_3.

5.18.5 Tenuazonic acid isotopically-labelled internal standard (TEA-ISTD), e.g. TEA-(acetyl-$^{13}C_2$) (TEA-$^{13}C_2$), mixture of diastereomers in methanol.

Laboratories can seek for isotopically-labelled internal standards with a high degree of deuteration or ^{13}C-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 ρ = 100 µg/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 ≤ −18 °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 5 000 ng/ml concentration.

For that purpose, transfer e.g. 25 µl of the stock solutions 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 mark with methanol (5.5). Homogenize vigorously. The standard solution 1 is stable for at least six months if stored at ≤ −18 °C.

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

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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).

Transfer 1 000 µl of the standard solution 1 (5.20) into a 5 ml volumetric flask. Fill up to the 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 ρ = 750 µg/ml.

Prepare the stock solutions, e.g. dissolve 1,12 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 1 500 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 µl of the stock solutions of AOH-ISTD, AME-ISTD and TEN-ISTD (5.22) into a 5 ml volumetric flask. Transfer e.g. 67 µl of stock solution of ALT-ISTD (5.22) and e.g. 167 µl of stock solution of TEA-ISTD (5.22) into the same 5 ml volumetric flask. Fill up to the 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 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 ($C_{58}H_{114}O_{26}$) (Tween®20[1]), analytical grade.

5.26 Polysorbate 20 ($C_{58}H_{114}O_{26}$) solution, φ = 2 % 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 can be used for three months if stored at approximately 4 °C.

[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 document 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.

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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 approximately 20 s.

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

Table 1 — Preparation of calibration solutions

					ALT	AOH	AME	TEN	TEA
Calibration solution	**Standard solution 1 (5.20)** µl	**Standard solution 2 (5.21)** µl	**Internal standard solution 2 (5.24)** µl	**Methanol (5.5)** µl	**Equivalent concentration** µg/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.

[2] Ultra-turrax and Strata-XL are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by CEN of these products. Equivalent products may be used if they can be shown to lead to the same results.

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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-XL[2], 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).

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 test solution with sufficient accuracy.

6.21.4 HPLC reversed phase column.

A suitable column and appropriate HPLC conditions providing sufficient retention capacity of the first eluting analyte. Acceptable chromatograms, as shown in Annex A, can be achieved using a column with a minimum capacity factor of 3 ($k' \geq 3{,}0$) and a minimum plate number of 2 000 ($N \geq 2\,000$) for any of the analytes.

Examples of suitable columns and analytical conditions are reported in Annex A and Annex B.

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

6.21.6 Column oven, capable of maintaining a constant temperature.

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