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BSI Standards Publication

Foodstuffs – Determination of ergot alkaloids in cereals and cereal products by dSPE clean-up and HPLC-MS/MS

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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 ergot alkaloids in cereals and cereal products by dSPE clean-up and HPLC-MS/MS

Produits alimentaires - Dosage des alcaloïdes de l'ergot dans les céréales et les produits céréaliers par purification par dSPE et CL-SM/SM

Lebensmittel - Bestimmung von Ergotalkaloiden in Getreiden und Getreideerzeugnissen mit dSPE-Reinigung und LC-MS/MS

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European foreword

This document (EN 17425:2021) 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of an identical text or by endorsement, at the latest by October 2021, and conflicting national standards shall be withdrawn at the latest by October 2021.

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Introduction

Ergot alkaloids are a group of mycotoxins produced by several species of *Claviceps* fungi growing on cereals and forage grass. These toxins are a risk for consumers as they can enter the food chain. All ergot alkaloids share a common structure, the ergoline system, and are divided into several classes, based on the presence of functional groups. The chiral carbon atom C-8 is responsible for the epimerization.

The isomers of each of these compounds are nominally known as 'ines' and the 'inines'. And besides, ergocryptine and ergocryptinine can both occur as α- and β-forms.

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 [3], 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 applicability of regulatory limitations prior to use.

WARNING 3 — Ergot alkaloids can cause vasoconstrictive, neurotoxic, reproductive and developmental adverse effects, and can be acutely and chronically toxic [4].

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1 Scope

This document describes a method for the determination of the sum of six ergot alkaloids (ergocornine, ergometrine, ergocristine, ergotamine, ergosine and ergocryptine) and their -inine epimer pairs by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) after clean-up by dispersive solid phase extraction (dSPE).

The method has been validated in the range 13,2 µg/kg to 168 µg/kg for the sum of the twelve ergot alkaloids, in rye flour, rye bread and cereal products (breakfast cereal, infant breakfast cereal, and crispbread) that contained rye as an ingredient, as well as seeded wholemeal flour and a barley and rye flour mixture.

Method performance was satisfactory in the range 24,1 µg/kg to 168 µg/kg, however at lower concentrations RSD_R values were greater than 44 %, and HorRat values exceeded 2,0, indicating the method may not be fully suitable at concentrations below 24 µg/kg for sum of ergot alkaloids, although it is suitable for screening at these concentrations.

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 http://www.electropedia.org/

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

4 Principle

Ergot alkaloids are extracted from cereals and cereal-based foods with a buffer at pH 8,9 and cleaned up with a dispersive solid phase material prior to filtering. Ergot alkaloids are detected and quantified by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

5 Reagents

Use only reagents of recognized analytical grade and water complying with grade 1 of EN ISO 3696, unless otherwise specified. Solvents shall be of quality for LC analysis, unless otherwise specified.

NOTE Ergometrine and ergotamine are listed as Category 1 scheduled substances in Regulation (EC) No 273/2004 [5] on drug precursors. It is a requirement to have an appropriate licence in order to purchase and store these compounds (and their related -inine epimers).

5.1 Ergocornine, e.g. crystalline, as a film or as certified standard solution.

5.2 Ergocorninine, e.g. crystalline, as a film or as certified standard solution.

5.3 Ergocristine, e.g. crystalline, as a film or as certified standard solution.

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5.4 Ergocristinine, e.g. crystalline, as a film or as certified standard solution.

5.5 α-Ergocryptine, e.g. crystalline, as a film or as certified standard solution.

5.6 α-Ergocryptinine, e.g. crystalline, as a film or as certified standard solution.

5.7 Ergometrine (maleate), e.g. crystalline, as a film or as certified standard solution.

5.8 Ergometrinine, e.g. crystalline, as a film or as certified standard solution.

5.9 Ergosine, e.g. crystalline, as a film or as certified standard solution.

5.10 Ergosinine, e.g. crystalline, as a film or as certified standard solution.

5.11 Ergotamine (tartrate), e.g. crystalline, as a film or as certified standard solution.

5.12 Ergotaminine, e.g. crystalline, as a film or as certified standard solution.

5.13 Acetonitrile, LC-MS grade.

5.14 Methanol, LC grade.

5.15 Water, suitable for LC-MS/MS.

5.16 Solid phase extraction material, primary secondary amine (PSA) bulk sorbent, 40 µm, 10 g; e.g. Bondesil ™, [1].

5.17 Sodium hydroxide (NaOH), analytical reagent grade.

5.18 Sodium hydroxide solution, molar concentration c(NaOH) = 1,0 mol/l.

Dissolve 4 g NaOH (5.17) in water (5.15) to a final volume of 100 ml.

5.19 Hydrochloric acid (HCl), analytical reagent grade, volume fraction φ(HCl) = 37 % (acidimetric), density: 1,18 g/cm^3 (20 °C).

5.20 Hydrochloric acid solution, molar concentration c(HCl) = 1,0 mol/l.

Dilute 8,3 ml HCl (5.19) in water (5.15) to a final volume of 100 ml.

5.21 Ammonium carbonate ($(NH_4)_2CO_3$), LC-MS grade.

5.22 Ammonium carbonate solution, mass concentration $\rho((NH_4)_2CO_3)$ = 200 mg/l; pH = 8,9 ± 0,3.

Weigh 200 mg of ammonium carbonate (5.21) to the nearest 1 mg and transfer into a 1 l glass flask. Add 500 ml of water (5.15). Shake the flask vigorously to ensure all solid has been dissolved.

After dissolution adjust the pH 8,9 ± 0,3 with sodium hydroxide solution (5.18) or hydrochloric acid solution (5.20) as appropriate, then fill up to the mark with water (5.15).

This solution can be stored at room temperature for 3 days.

[1] Bondesil ™ is a trade name of a product commercially available from various suppliers. 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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5.23 Extraction solvent.

Mix acetonitrile (5.13) and ammonium carbonate solution (5.22) (84 + 16, *V* + *V*). Shake vigorously.

5.24 Individual stock solutions, ρ = 100 µg/ml, in acetonitrile.

Follow any specific manufacturers' instructions to re-dissolve the films of individual ergot alkaloids (5.1 to 5.12). If crystalline material is used, weigh 5 mg to the nearest 0,2 mg, of each of the solid standards (5.1 to 5.12) individually into glass weighing boats and transfer quantitatively into individual 50 ml volumetric flasks, then fill up to the mark with acetonitrile (5.13). For ergometrine (5.7) and ergometrinine (5.8) a mixture of acetonitrile (5.13) and water (5.15) (90 + 10, *V* + *V*) is recommended to ensure complete dissolution. Shake vigorously.

5.25 Mixed standard solution, ρ = 0,5 µg/ml.

Using a pipette (6.5), transfer 50 µl of each of the individual stock solutions (5.24) into a 10 ml volumetric flask and make up to volume with acetonitrile (5.13).

When using an individual stock solution with a mass concentration other than ρ = 100 µg/ml calculate the appropriate volume required to prepare the 0,5 µg/ml standard solution mixture.

5.26 Calibration solutions.

Prepare e.g. the following calibration solutions as outlined in Table 1. Dispense volumes of mixed standard solution (5.25) into volumetric flasks and fill up to the mark with acetonitrile (5.13).

Table 1 — Examples of suitable calibration solutions

Calibration solution	Mixed standard solution (5.25) µl	Final volume ml	Mass concentration of alkaloids ng/ml	Equivalent to mass fraction of alkaloids µg/kg
1	10	50	0,1	0,5
2	20	50	0,2	1
3	10	5	1	5
4	20	5	2	10
5	40	5	4	20
6	100	5	10	50

6 Apparatus and equipment

Usual laboratory apparatus and, in particular, the following. Unless otherwise stated volumetric glassware shall be of grade 'A' quality. Ergot alkaloids are sensitive to epimerization by light and amber glass shall be used where possible.

6.1 Laboratory balance, accuracy of 0,01 g.

6.2 Analytical balance, accuracy of 0,1 mg.

6.3 Single or multiple grinding mill.

6.4 Extraction flasks with cap, of sufficient volume to contain 50 ml extraction solvent and 10 g sample, e.g. 100 ml.

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6.5 Pipette, adjustable, e.g. 25 µl, 50 µl, 250 µl, 1 000 µl, suitable for organic solvents, with disposable tips.

6.6 Laboratory shaker, for solvent extraction with suitable 100 ml flasks.

6.7 Folded filter paper, diameter 12,5 cm, hardened, grade 54.

6.8 Vials with caps, 40 ml amber vials.

6.9 Vials with caps, 4,0 ml amber vials.

6.10 Plastic Luer-lock syringe, 1 ml.

6.11 Polytetrafluoroethylene (PTFE) plastic syringe filters, 13 mm × 0,22 µm.

6.12 Vials with caps, 2,0 ml amber vials.

6.13 Autosampler vials suitable for LC-MS/MS analysis, e.g. 200 µl.

6.14 LC-MS/MS system with the following components:

6.14.1 LC pump, capable of delivering a binary gradient at flow rates appropriate for the analytical column in use with sufficient accuracy.

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

6.14.3 LC column, capable of retaining the ergot alkaloids, preferably with a retention factor of at least two. The column shall be suitable for use with a mobile phase at pH > 7.

6.14.4 Column filter, in-line filter suitable for the LC column used (6.14.3).

6.14.5 Column oven, capable of maintaining a constant temperature.

6.14.6 Tandem mass spectrometer (MS/MS), capable of ionization of the ergot alkaloids (resulting in positive ions) and selected reaction monitoring (SRM) with a sufficiently wide dynamic range.

Any ionization source providing sufficient yield may be used.

6.14.7 Data evaluation system.

7 Procedure

7.1 Preparation of the test sample

Grind and homogenize the sample with a grinding mill with a mesh or sieve size of 0,5 mm or smaller (6.3) before analysis.

Depending on the starting material (ground or unground material), it is advisable to first grind the sample through a sieve of 1 mm to prevent excessive heat formation during milling, which could lead to partial decomposition of the analytes. Then grind the sample through a sieve of 0,5 mm.

Mix samples well before taking a test portion for analysis. Store the samples at room temperature.

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7.2 Extraction of ergot alkaloids

7.2.1 Precautions

Ergot alkaloids are sensitive to epimerization by light and amber glass shall be used where possible. Analysis should take place immediately after extraction. Only if absolutely necessary, extracts may be stored overnight at 4 °C.

7.2.2 Test sample

Weigh 10 g of the sample to the nearest 0,05 g into a flask (6.4).

A larger test sample size may be used. In that case the amount of extraction solvent shall be adjusted accordingly (7.2.4).

7.2.3 Spiking procedure

Add 200 µl of the mixed standard solution (5.25) to 10 g ± 0,05 g of a 'blank' sample. Allow the spiked samples to dry. Extract the samples as described in 7.2.4. If a larger test sample size has been used adjust the spiking volume accordingly.

7.2.4 Sample extraction

Add 50 ml of the extraction solvent (5.23) to the flask (6.4) using a measuring cylinder.

Place the sample flasks in the laboratory shaker (6.6). Shake the samples for approximately 30 min at a moderate speed.

Whilst the samples are shaking, prepare sufficient glass funnels containing folded filter paper (6.7) ready to filter the samples into 40 ml amber vials (6.8).

When the samples have finished shaking, shake each flask individually by hand for approximately 10 s prior to pouring through funnels and filter paper (6.7) into 40 ml amber vials (6.8).

7.2.5 Clean-up

Transfer 1 ml of sample filtrate into a 4 ml amber vial (6.9) containing 50 mg ± 5 mg of the solid phase extraction material (5.16).

Shake each tightly sealed sample vial with a laboratory shaker (6.6) at high speed for approximately 45 s.

Using a plastic Luer-lock syringe (6.10), take up as much as possible of the sample. Fit a 13 mm PTFE 0,22 µm syringe filter (6.11) and holding the syringe vertically, allow any solid phase material to rest on the bottom of the syringe. Push the liquid through the filter into a 2 ml amber vial (6.12).

8 LC-MS/MS analysis

8.1 General

Optimize analytical parameters (i.e. selection of masses of precursor and product ions, cone voltages and collision energies) by infusion and injection of standard solutions of individual ergot alkaloids. Use a tandem mass spectrometer or equivalent in positive electrospray ionization (ESI^+) mode. Set the acquisition mode to multiple reaction monitoring (MRM), monitoring at least two product ions [6]. Examples are given in Table A.3.

Satisfactory separation of ergot alkaloid epimers can be achieved using a mobile phase with a pH > 7. The use of a mobile phase with pH > 7, requires an analytical column that contains a stationary phase that is resistant to high pH.

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Examples of measurement conditions and transitions are given in Annex A. Examples of typical chromatograms are shown in Annex B.

8.2 Batch composition and analytical sequence

An example is as follows: The first injection of every run sequence (following any test or priming injections) is usually an aliquot of sample extraction solvent (5.23). Then inject the calibration solutions, followed by an extraction solvent to check for possible carry over. Subsequently inject the sample test solutions, inject one calibration solution at periodic intervals, e.g. one calibration solution for every 5 to 10 sample test solutions injected. At the end of the batch, re-inject the calibration solutions.

8.3 Identification

Identify each ergot alkaloid by comparing the retention times of the calibration solutions with that of the sample test solution. Identify the analyte on the basis of at least two mass transitions. In addition the retention times (peaks in both mass traces) and the area ratio of the two peaks shall match that of the standard substance [6].

Under the conditions given (Annex A) it is possible that a naturally contaminated sample shows separate α- and β-ergocryptine and α- and β-ergocryptinine peaks in the chromatogram. As long as standards for β-ergocryptine and β-ergocryptinine are not available, use standards for α-ergocryptine and α-ergocryptinine to quantify both the α- and β-ergocryptine and the α- and β-ergocryptinine respectively. Report a sum for α- and β-ergocryptine and α- and β-ergocryptinine. The separation of the α- and β- pairs is not critical for the application of the method as results are reported as total ergocryptine and total ergocryptinine.

8.4 Calibration

For each ergot alkaloid, plot the peak areas of the quantifier ion (y-axis) of all individual calibration solutions (5.26, calibration solutions 1 to 6) against the corresponding mass fraction (µg/kg) (x-axis). The quantifier is the transition which overall gives the best S/N (signal/noise) ratio. Construct a calibration curve using (possibly weighted) regression with all individual data points obtained, compute the slope and possible intercept of each of the calibration curves.

9 Calculation

9.1 Calculation of individual ergot alkaloid concentration

Using the calibration graph for each analyte (8.4), calculate the mass fraction w of each ergot alkaloid in the sample, expressed in µg/kg, according to Formula (1):

$$w = \frac{A - b}{a} \tag{1}$$

where

A is the peak area from the chromatogram of the test solution;

a is the value of the slope of the linear function;

b is the value where the calibration function intercepts the y-axis.

Rate after recovery: Calculate the spike recovery RR for each analyte, expressed in %, according to Formula (2):

$$RR = \frac{w_{\mathrm{s}} - w_{\mathrm{u}}}{w_{\mathrm{L}}} \times 100 \tag{2}$$

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w_s is the measured mass fraction of the spiked sample, in µg/kg;

w_u is the mass fraction of the unspiked sample, in µg/kg;

w_L is the mass fraction added to the spiked sample, in µg/kg (here: 10 µg/kg).

If required, calculate the recovery corrected mass fraction for each analyte, w_c in µg/kg; using Formula (3):

$$w_c = \frac{w \times 100}{RR} \tag{3}$$

9.2 Calculation of ergot alkaloid sum concentration

To obtain the sum value of ergot alkaloids in the test sample add the mass fraction of each individual ergot alkaloid obtained from the calibration graphs. Where an analyte result is less than the limit of quantitation (LOQ) [7] of the method, use a value of zero for that analyte in the calculation of the sum concentration.

10 Precision

10.1 General

Details of the interlaboratory test of the precision of the method are summarized in Annex C. The values derived from the interlaboratory test may not be applicable to analyte concentration ranges and matrices other than given in Annex C.

10.2 Repeatability

The absolute difference between two single test results found on identical test material by one operator using the same apparatus within the shortest feasible time interval will exceed the repeatability limit *r* in Table 2 in not more than 5 % of the cases.

10.3 Reproducibility

The absolute difference between two single test results found on identical test material reported by two laboratories will exceed the reproducibility limit *R* in Table 2 in not more than 5 % of the cases.

Table 2 — Validation data for sum (12) ergot alkaloids

Sample	$\bar{x}$ µg/kg	*r* µg/kg	*R* µg/kg
rye flour	150	44,9	84,0
rye flour	168	82,4	111
breakfast cereal	72,0	58,0	74,7
infant breakfast cereal	18,7	16,4	26,5
crispbread	13,2	10,1	18,2
rye bread	110	15,6	86,6
seeded wholemeal flour	24,1	5,94	14,7
barley and rye flour	73,5	90,4	90,4

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11 Test report

The test report should comply with EN ISO/IEC 17025 and shall contain at least the following data:

a) all information necessary for the identification of the sample (kind of sample, origin of sample, designation);

b) a reference to this document;

c) the date and type of sampling procedure (if known);

d) the date of receipt;

e) the date of test;

f) the test results and the units in which they have been expressed;

g) any particular points observed in the course of the test;

h) any operations not specified in the method or regarded as optional, which might have affected the results.

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