SLOVENSKI STANDARD
oSIST prEN 17256:2018

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Krma: metode vzorčenja in analize - Določevanje alkaloidov rženih rožičkov (ergot) in tropanskih alkaloidov v sestavinah krme in krmni mešanici z LC-MS

Animal feeding stuffs: Methods of sampling and analysis - Determination of ergot and tropane alkaloids in feed materials and compound feed by LC-MS

Futtermittel - Probenahme- und Untersuchungsverfahren - Bestimmung der Alkaloide des Mutterkorns und der Tropanalkaloiden in Einzelfuttermitteln und Mischfuttermitteln mittels LC-MS

Aliments des animaux : Méthodes d'échantillonnage et d'analyse - Détermination des teneurs en alcaloïdes de l□ergot et alcaloïdes tropaniques dans les matières premières pour aliments et les aliments composés pour animaux, par CL□ SM

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65.120	Krmila	Animal feeding stuffs

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Animal feeding stuffs: Methods of sampling and analysis - Determination of ergot alkaloids and tropane alkaloids in feed materials and compound feeds by LC-MS/MS

Aliments des animaux: Méthodes d'échantillonnage et d'analyse - Détermination de la teneur en alcaloïdes de l'ergot et en alcaloïdes tropaniques dans les matières premières et les aliments composés par CL-SM/SM

Futtermittel: Probenahme- und Untersuchungsverfahren - Bestimmung der Alkaloide des Mutterkorns und der Tropanalkaloiden in Einzelfuttermitteln und Mischfuttermitteln mittels LC-MS/MS

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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 17256:2018) has been prepared by Technical Committee CEN/TC 327 “Animal feeding stuffs: Methods of sampling and analysis”, the secretariat of which is held by NEN.

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.

Introduction

Ergot alkaloids are mycotoxins produced by species of the genus *Claviceps*. In Europe, *Claviceps purpurea* is the most widespread fungal species. The fungus may infest plant species of the Poaceae family (true grasses), producing dark coloured bodies, called sclerotia or (rye) ergot. Economically important cereal grains that may be infected by *C. purpurea* are rye, wheat, tritcale, barley, millet and oats. The sclerotia contain a suit of ergot alkaloids, of which twelve have been recognized as major components: ergocornine, ergocorninine, ergocristine, ergocristinine, α-ergocryptine, α-ergocryptinine, ergometrine, ergometrinine, ergosine, ergosinine, ergotamine and ergotaminine.

Tropane alkaloids are plant toxins produced by several species within the family of Solanaceae (nightshades). The most relevant are *Datura* (thornapple), *Hyoscyamus* (henbane) and *Atropa* (belladonna, deadly nightshade) species. Seeds and other plant parts contain substantial amounts of atropine (hyoscyamine) and scopolamine, which are the most important toxic principles. Datura, *Hyoscyamus* and *Atropa* species can be present as weeds in arable fields and may be co-harvested, resulting in contaminated feed grains and feed products.

WARNING — The use of this protocol involves hazardous materials, operations and equipment. It is advised to prevent inhalation and skin contact with the standards, reagents and other solutions used, and wear a lab coat and use where necessary a fume hood, safety glasses and safety gloves.

This protocol does not purport to address all the safety problems associated with its use. It is the responsibility of the user of this protocol to establish appropriate safety and health protection measures and to ensure that regulatory and legal requirements are complied with.

1 Scope

This document describes a method for the determination of individual ergot alkaloids and tropane alkaloids in unprocessed cereals and cereal-based compound feeds by high performance liquid chromatography with tandem mass spectrometry (LC-MS/MS).

This method has been successfully validated by collaborative trial in the following matrices: rye, barley, wheat, complete feed for bovine, porcine and poultry. Validation in buckwheat produced acceptable results, but the relative standard reproducibility was higher for most analytes in comparison with the other matrices. This may be related to the matrix. The validated range of the method is approximately 10 µg/kg to 250 µg/kg for individual alkaloids. Determination of concentrations above 250 µg/kg is possible by applying a higher spiking level and dilution of the sample extract, but this has not been validated in the collaborative trial.

The method is applicable for the determination, by means of one-point standard addition to the sample, of ergocornine in the tested range of 12 µg/kg to 221 µg/kg, ergocorninine in the tested range of 9 µg/kg to 196 µg/kg, ergocristine in the tested range of 14 µg/kg to 312 µg/kg, ergocristinine in the tested range of 12 µg/kg to 258 µg/kg, α-ergocryptine in the tested range of 10 µg/kg to 184 µg/kg, α-ergocryptinine in the tested range of 8 µg/kg to 171 µg/kg, ergometrine in the tested range of 12 µg/kg to 174 µg/kg, ergometrinine in the tested range of 3 µg/kg to 172 µg/kg, ergosine in the tested range of 12 µg/kg to 226 µg/kg, ergosinine in the tested range of 9 µg/kg to 273 µg/kg, ergotamine in the tested range of 11 µg/kg to 443 µg/kg, ergotaminine in the tested range of 10 µg/kg to 273 µg/kg, atropine in the tested range of 16 µg/kg to 252 µg/kg and scopolamine in the tested range of 15 µg/kg to 246 µg/kg.

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 3696:1995, *Water for analytical laboratory use - Specification and test methods (ISO 3696:1987)*

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 http://www.iso.org/obp

4 Principle

The alkaloids are extracted by mixing 4 g of a homogenized, finely ground, sample with 40 ml methanol/water 60/40 (v/v) containing 0,4 % formic acid. The mixture is shaken for 30 min. After centrifugation, a portion of the supernatant is further purified by passing it through a 30 kD ultrafilter. The filtrate is transferred to a vial and it is analysed with a liquid chromatography – tandem mass spectrometry (LC-MS/MS) system. A reverse-phase column in combination with an aqueous mobile phase with a pH > 7 and an organic modifier is used to separate the analytes. Quantification is performed by one-point standard addition to the sample.

5 Reagents

5.1 Analytical standards

Analytical standards should have a demonstrated purity of at least 90 %, preferably of 95 % or higher.

NOTE 1 Ergotamine and ergometrine are listed as category I drug precursors and for these compounds an official licence would be required and special procedures for storage and management would need to be followed (EC 273/2004) [1].
Atropine is the racemic mixture of L-(-)-hyoscyamine and D-(+)-hyoscyamine. In this method the enantiomers of hyoscyamine are not separated. Both enantiomers produce identical fragmentation spectra. In this method either a standard of atropine or hyoscyamine can be used.

β-Ergocryptine and β-ergocryptinine are not included in the scope of the method because no analytical standards of sufficient purity and quality are currently available from commercial providers. It should be noted that β-ergocryptine and β-ergocryptinine do naturally occur in sclerotia of *Claviceps* species. See under Clause 8 for more information on the analysis of these alkaloids.

NOTE 2 Isotopically labelled analogues of atropine and scopolamine are available from commercial providers. Optionally, these isotopically labelled analogues can be used as internal standards, provided that the mass increment in the molecule by the isotope labels is at least 3. Isotopically labelled analogues are currently not available for any of the ergot alkaloids.

5.1.1 Ergocornine

5.1.2 Ergocorninine

5.1.3 Ergocristine

5.1.4 Ergocristinine

5.1.5 α-Ergocryptine

5.1.6 α-Ergocryptinine

5.1.7 Ergometrine (maleate)

5.1.8 Ergometrinine

5.1.9 Ergosine

5.1.10 Ergosinine

5.1.11 Ergotamine (tartrate)

5.1.12 Ergotaminine

5.1.13 Atropine or hyoscyamine

5.1.14 Scopolamine (hydrochloride)

5.2 Chemicals

5.2.1 Acetonitrile, LC-MS or HPLC quality

5.2.2 Methanol, LC-MS or HPLC quality

5.2.3 Formic acid, 98 – 100 %, p.a. quality

5.2.4 Ammonium carbonate, anhydrous

5.2.5 Ammonia, 25 %

5.2.6 Water

Water of LC-MS grade, double-distilled or water of grade 1 as defined in EN ISO 3696:1995.

5.3 Standard solutions

Accurately weigh (6.1) between 5 and 6 mg of each standard (5.1.1 – 5.1.14) into a separate amber-coloured glass bottle of 60 ml (6.12). Add a volume of acetonitrile (5.2.1) to produce a solution with a concentration of 100 µg/ml. Take into account the weight, the purity and the appearance form of the standard.

Many ergot alkaloid standards are commercially available only in small amounts (5 mg or less). Preferably a standard should be prepared using a quantity of at least 5 mg. However, when the standard is only available in a quantity of 5 mg or less, a smaller quantity can be weighed in, provided an accurate weight measurement can be guaranteed. In principle this is preferred above flushing the contents of the container with several volumes of solvent to dissolve and collect the material. Nevertheless, some ergot standards may only be available as dried down standards that need to be reconstituted in solvent.

Stock solutions are stable for 6 months below -18 °C. However, ergot alkaloid standards are sensitive to light and may epimerise rapidly in the presence of acid or base. Standard solutions should be kept in amber coloured glass bottles and they should be stored at a temperature below -18 °C. Acetonitrile is the preferred solvent because the rate of epimerisation is lowest in this solvent.

Before use, stock solutions and mixed standard solutions that have been stored in the freezer, may need to be vortexed (6.7) or sonicated (6.8) to ensure the complete dissolution of the analytes.

To further reduce the impact of epimerisation, mixed standard stock solutions containing the corresponding epimeric pairs may be prepared.

5.3.1 Ergocornine stock solution (100 µg/ml)

5.3.2 Ergocorninine stock solution (100 µg/ml)

5.3.3 Ergocristine stock solution (100 µg/ml)

5.3.4 Ergocristinine stock solution (100 µg/ml)

5.3.5 α-Ergocryptine stock solution (100 µg/ml)

5.3.6 α-Ergocryptinine stock solution (100 µg/ml)

5.3.7 Ergometrine stock solution (50 µg/ml)

NOTE Ergometrine may be difficult to dissolve (particularly when present as the maleate salt form). The solution can be sonicated (6.8) for up to 30 min or placed in a water bath of 60 °C for up to 30 min. Sonication is preferred above warming in a water bath. Alternatively, a small amount of water (10 %) can be added to the solvent to facilitate solvation of the salt form.

5.3.8 Ergometrinine stock solution (100 µg/ml)

5.3.9 Ergosine stock solution (100 µg/ml)

5.3.10 Ergosinine stock solution (100 µg/ml)

5.3.11 Ergotamine stock solution (100 µg/ml)

5.3.12 Ergotaminine stock solution (100 µg/ml)

5.3.13 Atropine stock solution (100 µg/ml)

NOTE Instead of atropine, hyoscyamine can be used.

5.3.14 Scopolamine stock solution (100 µg/ml)

5.3.15 Mixed standard solution (5 µg/ml)

Pipette (6.10) 1 ml of each stock solution 5.3.1 – 5.3.7 and 5.3.9 – 5.3.14 (100 µg/ml) and 2 ml of stock solution 5.3.8 (50 µg/ml) in a calibrated volumetric flask of 20 ml (6.11) and make up the volume with acetonitrile (5.2.1) and mix. Transfer the contents to an amber coloured glass bottle of 30 ml (6.12). The solution can be used for 12 months when stored in the dark at below −18 °C.

NOTE It is advised to divide the prepared mixed standard solution in 4 portions of 5 ml and store these in separate amber coloured glass bottles of 10 ml (6.12).

5.3.16 Mixed standard solution (1 000 ng/ml)

Pipette (6.10) 4 ml of the mixed standard solution (5 µg/ml) (5.3.15) in a calibrated volumetric flask of 20 ml (6.11) and make up the volume with acetonitrile (5.2.1) and mix. Transfer the contents to an amber coloured glass bottle of 30 ml (6.12). The solution can be used for 12 months when stored in the dark at below −18 °C.

NOTE It is advised to prepare a new mixed standard solution every 3 months, by using a new, unused, portion of the mixed standard solution 5.3.15.

5.3.17 Mixed standard solution (200 ng/ml)

Pipette (6.10) 4 ml of the mixed standard solution (1000 ng/ml) (5.3.16) in a calibrated volumetric flask of 20 ml (6.11) and make up the volume with acetonitrile (5.2.1) and mix. Transfer the contents to an amber coloured glass bottle of 30 ml (6.12). The solution can be used for 12 months when stored in the dark at below −18 °C.

NOTE It is advised to prepare a new mixed standard solution every 3 months, from a freshly prepared mixed standard solution 5.3.16.

5.3.18 Mixed standard solution (50 ng/ml)

Pipette (6.10) 5 ml of the mixed standard solution (200 ng/ml) (5.3.17) in a calibrated volumetric flask of 20 ml (6.11) and make up the volume with acetonitrile (5.2.1) and mix. Transfer the contents to an amber coloured glass bottle of 30 ml (6.12). The solution can be used for 12 months when stored in the dark at below −18 °C.

NOTE It is advised to prepare a new mixed standard solution every 3 months, from a freshly prepared mixed standard solution 5.3.17.

5.3.19 Working standard solution (10 ng/ml)

Dilute the mixed standard solution 1000 ng/ml (5.3.16) with extraction solvent (5.4.1) in a 1 to 100 ratio by volume. Prepare a fresh solution every new day of analysis. Store at +4 °C.

5.4 Reagent solutions

5.4.1 Extraction solvent 0,4 % formic acid in methanol/water (60:40) (v/v)

Mix 600 ml methanol (5.2.2), 400 ml water (5.2.6) and 4 ml formic acid (5.2.3) in a glass bottle of 1000 ml. This solution is stored at room temperature and can be used for 3 months.

5.4.2 LC-MS/MS mobile phase A: 10 mM ammonium carbonate, pH 10,0

Weigh 0,96 g of ammonium carbonate (5.2.4) and dissolve in 1 000 ml water (5.2.6) in a glass bottle of 1 000 ml. Add with a positive displacement pipette (6.10) 25 % ammonia (5.2.5) to adjust the pH to 10,0 ± 0,1 using a pH meter (6.9). This solution is stored at room temperature and can be used for 1 month.

A mobile phase A consisting of 10 mM ammonium carbonate pH 9,0 has been shown to work equally well. Weigh 0,96 g of ammonium carbonate and dissolve in 1000 ml water (5.2.6) in a glass bottle of 1000 ml. If necessary, adjust the pH to 9,0 ± 0,1 with formic acid (5.2.3) or ammonia (5.2.5) using a pH meter (6.14). This solution is stored at room temperature and can be used for 1 month.

A mobile phase A consisting of a solution of 6 mM ammonia in water has been shown to work equally well. Mix 500 µL ammonia 25 % (5.2.5) with 1000 ml water (5.2.6) in a glass bottle of 1 000 ml. This solution is stored at room temperature and can be used for 1 week.

6 Apparatus

Usual laboratory equipment and, in particular, the following items.

6.1 Analytical balance, with a mass resolution of 0,1 mg or better

6.2 Laboratory balance, with a mass resolution of 0,1 g or better

6.3 Mill

Single or multiple mills capable of grinding test materials to particle sizes of ≤ 500 µm.

6.4 Mixer

Capable of sufficiently homogenizing the ground test materials.

6.5 Vertical or horizontal shaker, adjustable

6.6 Centrifuge

Suitable for 50 ml centrifuge tubes (6.13) and ultra-filters (6.15) and capable of generating a relative centrifugal force (rcf) of 3 000 g.