SLOVENSKI STANDARD
SIST EN 17256:2019

01-december-2019

Krma: metode vzorčenja in analize - Določevanje alkaloidov rožička in tropanskih alkaloidov v sestavinah krme in krmni mešanici z LC-MS/MS

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

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

Ta slovenski standard je istoveten z: **EN 17256:2019**

ICS:

65.120	Krmila	Animal feeding stuffs

SIST EN 17256:2019 **en,fr,de**

EUROPEAN STANDARD

NORME EUROPÉENNE

EUROPÄISCHE NORM

EN 17256

October 2019

ICS 65.120; 71.040.40

English Version

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

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Contents

Page

European foreword 3
Introduction 4
1 Scope 5
2 Normative references 5
3 Terms and definitions 6
4 Principle 6
5 Reagents 6
6 Apparatus 10
7 Procedure 11
8 Analysis 13
9 Results 14
10 Precision 15
11 Test report 17
Annex A (informative) Precision data 18
Annex B (informative) Example of LC-MS/MS conditions 34
Annex C (informative) Example LC-MS/MS chromatograms of ergot alkaloids and tropane alkaloids in a sample of complementary bovine feed 36
Bibliography 38

European foreword

This document (EN 17256:2019) 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 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of an identical text or by endorsement, at the latest by March 2020, and conflicting national standards shall be withdrawn at the latest by March 2020.

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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, triticale, 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, ergocryptine, ergocryptinine, ergometrine, ergometrinine, ergosine, ergosinine, ergotamine and ergotaminine. Ergocryptine and ergocryptinine occur as a mixture of α- and β-isomers.

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.

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 document 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.

This document 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;
- the sum of α- and β-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;
- 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 https://www.iso.org/obp

4 Principle

The alkaloids are extracted by mixing 4 g of a homogenized, finely ground, sample with 40 ml of 0,4 % formic acid in methanol:water (60:40). The mixture is shaken for 30 min. After centrifugation, a portion of the supernatant is further purified by passing it through a 30 kDa 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

WARNING: Mycotoxins may be highly hazardous to health. Certain mycotoxins have carcinogenic, mutagenic, toxic, teratogenic and immunotoxic effects. Inhalation or dermal exposure to mycotoxins may occur at workplaces. Depending on the level of exposure, both acute and chronic effects are possible. The tropane alkaloids atropine and scopolamine are acutely toxic, may be fatal if swallowed or if inhaled. In addition, scopolamine may be fatal if in contact with skin.

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.

NOTE 2 β-Ergocryptine and β-ergocryptinine are currently not available from commercial providers as analytical standards of sufficient purity and quality. In this document α-ergocryptinine is used for the determination of the sum of α- and β-ergocryptinine. For determination of β-ergocryptine, α-ergocryptine should be used, but this has not been validated in the interlaboratory study. See under Clause 8 for more information on the analysis of these alkaloids.

NOTE 3 Isotopically labelled analogues of ergometrine, ergometrinine, 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.

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 to 100 %

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 mg and 6 mg of each standard (5.1.1 to 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 can 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 to 5.3.7 and 5.3.9 to 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 The prepared mixed standard solution can be divided in 4 portions of 5 ml and stored 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 A new mixed standard solution can be prepared 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 (1 000 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 A new mixed standard solution can be prepared 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 A new mixed standard solution can be prepared 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 1 000 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)

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 1 000 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) ammonia 25 % (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 1 000 ml water (5.2.6) in a glass bottle of 1 000 ml. If necessary, adjust the pH to 9,0 ± 0,1 with formic acid (5.2.3) or ammonia 25 % (5.2.5) 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 a solution of 6 mM ammonia in water has been shown to work equally well. Mix 500 µl ammonia 25 % (5.2.5) with 1 000 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 (6.14) and capable of generating a relative centrifugal force (rcf) of 3 000 *g*.

6.7 Minishaker or vortex mixer

6.8 Ultrasonic bath

6.9 pH meter

6.10 Pipettors

Adjustable, suitable for organic solvents, properly calibrated, with appropriate tips.

6.11 Volumetric flasks, calibrated, 20 ml

6.12 Amber coloured glass bottle of 10, 30 and 60 ml size with screw cap

6.13 Centrifuge tube, polypropylene, 50 ml with screw cap

6.14 Ultrafilter (centrifugal filter unit) with a cut off of 30 kDa

The centrifugal filter unit shall be compatible with 60 % methanol and 0,4 % formic acid solution. The membrane should be made of regenerated cellulose. The ultrafilter should have a capacity of 4 ml and the membrane should have an active surface of ≥ 300 mm^2.

6.15 HPLC vial, glass or polypropylene, 2 ml

6.16 HPLC system consisting of

6.16.1 Autosampler, thermostated

Capable of maintaining a temperature of 10 °C± 1 °C.

6.16.2 Binary pump system

Capable of delivering a binary gradient at flow rates appropriate for the analytical column in use with sufficient accuracy.

6.16.3 Column oven, thermostated

Capable of maintaining a temperature of least 50 °C ± 1 °C.

6.16.4 Analytical column

Containing high pH-resistant cross-linked C18 reversed phase packing material, capable of the base-line separation of analytes with identical molecular mass.

6.16.5 Pre-column, optional

With the same stationary phase material as the analytical column and with appropriate dimensions.

6.17 Tandem mass spectrometer

Capable of performing multiple selected reaction monitoring in positive mode, with a sufficiently wide dynamic range and capable of unit mass separation and equipped with a computer based data processing system. Any ionization source giving sufficient yield may be employed.

7 Procedure

7.1 General

Animal feed is a complex matrix containing a wide range of ingredients in varying amounts. For this reason it is not possible, due to variable and sample-dependent matrix effects (suppression/enhancement), to conduct quantification of the analytes in a sample by a direct comparison with a set of calibration samples. Each feed sample is quantified by means of standard addition to the sample prior to extraction. It is not necessary to correct the results for recovery.

Depending on the sensitivity and linear range of the mass spectrometric instrument it may be necessary, to dilute the sample extracts (7.4, 7.5, 7.6) by an appropriate factor with extraction solvent (5.4.1), taking into account the sum of the expected concentration of the analyte in the sample and the added concentration, to keep the response of the detector within the dynamic range of the mass spectrometer. Alternatively, the injection volume of the sample can be reduced, within the calibrated range of the injection system.

7.2 Sample pre-treatment

Laboratory samples should be taken and prepared in accordance with European legislation [2] where applicable or, in any other case with EN ISO 6498.

In order to obtain a homogeneous sample it is essential that the sample is finely ground (≤0,5 mm) and homogenized with a grinding mill (6.3). Depending on the starting material (ground or unground material), it may 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. Next, the sample is ground through a sieve of 0,5 mm. The samples are stored at room temperature.

7.3 Test sample amount

The amount of homogenized test sample examined is 4,0 g ± 0,1 g.

A larger test sample size may be used by the laboratory in order to improve the representativeness of the sample. The amount of extraction solvent shall in that case be adjusted accordingly (7.4). The use of a larger test sample size should not be used as a solution for grinding the sample through a sieve larger than 0,5 mm.