SLOVENSKI STANDARD
SIST EN 17504:2022

01-junij-2022

Krma: metode vzorčenja in analize - Ugotavljanje gosipola v bombažnem semenu in krmi z LC-MS/MS

Animal feeding stuffs: Methods of sampling and analysis - Determination of gossypol in cotton seed and feeding stuff by LC-MS/MS

Futtermittel: Probenahme- und Untersuchungsverfahren - Bestimmung von Gossypol in Baumwollsamen und Futtermitteln mittels LC-MS/MS

Aliments des animaux - Méthodes d'échantillonnage et d'analyse - Dosage du gossypol dans les graines de coton et les aliments pour animaux par CL-SM/SM

Ta slovenski standard je istoveten z: **EN 17504:2022**

ICS:

65.120	Krmila	Animal feeding stuffs

SIST EN 17504:2022 **en,fr,de**

EUROPEAN STANDARD

NORME EUROPÉENNE

EUROPÄISCHE NORM

EN 17504

March 2022

ICS 65.120

English Version

Animal feeding stuffs: Methods of sampling and analysis - Determination of gossypol in cotton seed and feeding stuff by LC-MS/MS

Aliments des animaux - Méthodes d'échantillonnage et d'analyse - Dosage du gossypol dans les graines de coton et les aliments pour animaux par CL-SM/SM

Futtermittel: Probenahme- und Untersuchungsverfahren - Bestimmung von Gossypol in Baumwollsamen und Futtermitteln mittels LC-MS/MS

This European Standard was approved by CEN on 10 January 2022.

CEN members are bound to comply with the CEN/CENELEC Internal Regulations which stipulate the conditions for giving this European Standard the status of a national standard without any alteration. Up-to-date lists and bibliographical references concerning such national standards may be obtained on application to the CEN-CENELEC Management Centre or to any CEN member.

This European Standard exists in three official versions (English, French, German). A version in any other language made by translation under the responsibility of a CEN member into its own language and notified to the CEN-CENELEC Management Centre has the same status as the official versions.

CEN members are the national standards bodies of Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Republic of North Macedonia, Romania, Serbia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and United Kingdom.

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

CEN-CENELEC Management Centre: Rue de la Science 23, B-1040 Brussels

Ref. No. EN 17504:2022 E

Contents

Page

European foreword

This document (EN 17504:2022) 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 September 2022, and conflicting national standards shall be withdrawn at the latest by September 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.

Any feedback and questions on this document should be directed to the users’ national standards body. A complete listing of these bodies can be found on the CEN website.

According to the CEN-CENELEC Internal Regulations, the national standards organisations of the following countries are bound to implement this European Standard: Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Republic of North Macedonia, Romania, Serbia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and the United Kingdom.

Introduction

Gossypol is a polyphenolic plant toxin produced by species of the genus *Gossypium* (cotton plant). The plants are primarily grown for fibre and oil production. The seeds and processed seed materials are also used as animal feeding stuffs. Gossypol is present in the seeds in two forms: free gossypol and bound gossypol (mostly to proteins). The measured content of free gossypol depends on the method of extraction and the specificity of the subsequent method of analysis used ([1], [2]). Consequently, this method might not be directly comparable to existing standards based on spectrophotometric measurement (e.g. [3], [4]).

WARNING — 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 specifies a method for the determination of free gossypol, extractable by acidified acetonitrile/water, in cottonseed, cottonseed products and compound feeds by liquid chromatography with tandem mass spectrometry (LC-MS/MS).

The method described in this document has been successfully validated in the range of 69 mg/kg to 5 950 mg/kg by collaborative trial in the following matrices: cottonseed, cottonseed products (cake/meal, hulls) and compound feeds for bovine, porcine and poultry.

NOTE It is possible to reach quantification limits of approximatively 5 mg/kg in compound feeds. The method might be applicable at lower and at higher concentrations than the concentration range validated in the collaborative trial. However, this needs to be assessed by in-house validation.

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:

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

— IEC Electropedia: available at https://www.electropedia.org/

4 Principle

Free gossypol is extracted by mixing an homogenized sample of 0,4 g of cottonseed or 1,0 g of cottonseed product or compound feed with 40 ml of 0,1 % phosphoric acid in acetonitrile:water (80:20) (V:V). The mixture is shaken for 1 h. After centrifugation a portion of the supernatant is diluted with extraction solvent in a vial. The final extract is analysed by reversed phase liquid chromatography with tandem mass spectrometry (LC-MS/MS). Quantification of gossypol in cottonseed and cottonseed products is based on multi-level calibration using standards in extraction solvent. Quantification of gossypol in compound feeds is based on multi-level calibration using standards in extraction solvent and corrected for the apparent recovery.

5 Reagents

WARNING — Gossypol can be hazardous to health. It has a strong toxic effect on the reproductive organs. Depending on the level of exposure, both acute and chronic effects are possible.

5.1 **Gossypol,** ≥ 95 %, as gossypol base

Gossypol is a racemic mixture of (+)- and (-)-gossypol. In this method the enantiomers of gossypol are not separated. A standard containing a mixture of both enantiomers may be used.

Gossypol is also available in the form of a 1:1 complex with acetic acid. In this method either a standard of gossypol or gossypol-acetic acid may be used.

NOTE Gossypol is sensitive to light.

5.2 Acetonitrile, LC-MS or HPLC quality

5.3 Phosphoric acid 85 % w/w, d = 1,71 g/cm^3, p.a. quality

5.4 Formic acid, ≥ 98 %, p.a. quality

5.5 Water

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

5.6 Extraction solvent: 0,1 % phosphoric acid in acetonitrile:water (80:20)

Mix 800 ml acetonitrile (5.2) and 200 ml water (5.5) in a bottle of 1 000 ml. Add 1,0 ml phosphoric acid (5.3) and mix. This solution is stored at room temperature and may be used for 1 month.

5.7 Mobile phase A: 0,5 % formic acid in acetonitrile:water (5:95)

Mix 50 ml acetonitrile (5.2) and 950 ml water (5.5) in a bottle of 1 000 ml. Add 5 ml formic acid (5.4) and mix. This solution is stored at room temperature and may be used for 1 month.

5.8 Mobile phase B: 0,5 % formic acid in acetonitrile:water (95:5)

Mix 950 ml acetonitrile (5.2) and 50 ml water (5.5) in a bottle of 1 000 ml. Add 5 ml formic acid (5.4) and mix. This solution is stored at room temperature and may be used for 1 month.

5.9 Gossypol stock solution, 1 mg/ml

Weigh (6.1) 20 mg gossypol (5.1) and transfer into a 20 ml amber volumetric flask. Take into account the mass, the purity and the chemical form of the standard. Add extraction solvent (5.6), dissolve by mixing and make up to the mark with extraction solvent (5.6). Transfer to an amber glass bottle (6.7) and store the stock solution at < −18 °C. Under these conditions the solution may be used for 6 months.

5.10 Gossypol standard solution, 10 µg/ml

Transfer 100 µl of gossypol stock solution (5.9) into a 10 ml amber volumetric flask (6.7) using a pipette. Fill up to the mark with extraction solvent (5.6) and mix. Transfer to an amber glass bottle (6.7). Store the standard solution at < −18 °C. Under these conditions the solution can be used for 2 months. Use this standard solution for preparation of calibration curves.

5.11 Gossypol standard solution, 1 µg/ml

Transfer 1,00 ml of gossypol standard solution (5.10) into a 10 ml amber volumetric flask (6.7) using a pipette. Fill up to the mark with extraction solvent (5.6) and mix. Transfer to an amber glass bottle (6.7). Store the standard solution at < −18 °C. Under these conditions the solution can be used for 2 months. Use this standard solution for preparation of calibration curves.

5.12 Calibration standards

Prepare calibration standards using the standard solutions (5.10) and (5.11) and extraction solvent (5.6) according to Table 1 (See NOTE). Pipette directly in HPLC vials (6.8).

NOTE The number of calibration points required depends on the concentrations expected in the samples (7.4) and the dynamic range of the mass spectrometer. It is advised to use all calibration standards, but at least calibration standards Cal 1, 2, 4 to 6 and 8.

Table 1 — Preparation of calibration standards

	Concentration µg/ml	Standard solution of 1 µg/ml (5.11) µl	Standard solution of 10 µg/ml (5.10) µl	Extraction solvent (5.6) µl
Cal 1	0,00	0	0	1 000
Cal 2	0,025	25	0	975
Cal 3	0,05	50	0	950
Cal 4	0,10	100	0	900
Cal 5	0,25	0	25	975
Cal 6	0,50	0	50	950
Cal 7	0,75	0	75	925
Cal 8	1,00	0	100	900

6 Apparatus

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

6.1 Analytical balance, with an accuracy of 0,1 mg or better

6.2 Laboratory balance, with an accuracy of 0,01 g or better

6.3 Pipettors, adjustable, suitable for organic solvents, properly calibrated, with appropriate tips

6.4 Filter disc units, polytetrafluoroethylene (PTFE) with a pore size of 0,45 µm or less

6.5 Centrifuge tubes, polypropylene, 50 ml with screw cap

6.6 Centrifuge, suitable for 50 ml centrifuge tubes (6.5)

6.7 Glass bottles and volumetric flasks, amber coloured, different sizes

6.8 HPLC autosampler vials, amber glass 1,5 ml with caps

6.9 Minishaker or vortex mixer

6.10 Ultrasonic bath

6.11 Vertical or horizontal shaker, adjustable

6.12 Laboratory mill

6.13 HPLC system, consisting of:

6.13.1 Autosampler, thermostated

As an example, an autosampler capable of maintaining a temperature of 10 °C ± 1 °C is suitable.

6.13.2 Binary pump system

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

6.13.3 Column oven, thermostated

As an example, a column oven capable of maintaining a temperature of 40 °C ± 1 °C is suitable.

6.13.4 Analytical column

Containing C18 reversed phase packing material, capable of the base-line separation of the analyte from compounds with identical molecular mass.

6.13.5 Pre-column, optional

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

6.14 Tandem mass spectrometer

Capable of performing multiple selected reaction monitoring in negative ionization 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. Sample and instrument dependent matrix effects (suppression/enhancement) can occur that may affect the quantification of the analyte in the sample (see NOTE). To correct for these matrix effects a recovery sample (7.4.3) is included.

Depending on the sensitivity and linear range of the mass spectrometric instrument, it can be necessary to dilute the sample extracts (7.4) by an appropriate factor with extraction solvent (5.6), taking into account the expected concentration of the analyte in the sample, to keep the response of the detector within the dynamic range of the mass spectrometer. Alternatively, the injection volume of the sample may be reduced, within the calibrated range of the injection system.

NOTE At the conditions described in this document, matrix effects (ion suppression or enhancement) were not significant for cottonseed and cottonseed products. For compound feed matrix effects were observed.

7.2 Sample pre-treatment

Laboratory samples should be taken and prepared in accordance with European legislation [3] where applicable or, in any other case with EN ISO 6498.

Homogenize samples in a laboratory mill (6.12) to < 1 mm.

Cottonseeds can contain high amounts of gossypol. Carefully clean all parts of the grinder between samples to avoid significant carry-over. When cottonseed samples are analysed together with cottonseed products or compound feed materials, the cottonseed products and the compound feed samples should be ground prior to the cottonseed samples.

7.3 Test portion

7.3.1 Cottonseed

The amount of homogenized cottonseed material examined is 0,4 g ± 0,01 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).

7.3.2 Cottonseed products and compound feed

The amount of homogenized cottonseed product or compound feed material examined is 1,0 g ± 0,05 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).

7.4 Extraction

7.4.1 Cottonseed

Weigh (6.2) a test portion of 0,4 g ± 0,01 g homogenized sample (7.3) into a centrifuge tube of 50 ml (6.5). Add 40,0 ml of extraction solvent (5.6) and mix for 15 s using a vortex mixer (6.9). Place the tube in an ultrasonic bath (6.10) for 5 min and subsequently shake the tube for 1 h (6.11).

Centrifuge the tube for 5 min at 3 000 min^{-1} at room temperature (6.6). Transfer 10 µl of the extract into a HPLC vial (6.8) and add 990 µl extraction solvent (5.6) and mix (see NOTE).

NOTE Cottonseeds can contain high concentrations of gossypol (ranging from 2 000 mg/kg to 8 000 mg/kg). The expected gossypol concentration in undiluted cottonseed extracts is in the range of 20 µg/ml to 80 µg/ml. After dilution of the sample extract the expected concentration is in the range of 0,2 µg/ml to 0,8 µg/ml.

7.4.2 Cottonseed products and compound feed

Weigh (6.2) a test portion of 1,0 g ± 0,05 g homogenized sample (7.3) into a centrifuge tube of 50 ml (6.5). Add 40,0 ml of extraction solvent (5.6) and mix for 15 s using a vortex mixer (6.9). Place the tube in an ultrasonic bath (6.10) for 5 min and subsequently shake the tube for 1 h (6.11).

Centrifuge the tube for 5 min at 3 000 min^{-1} at room temperature (6.6). Transfer 200 µl of the extract (see NOTE 1) into a HPLC vial (6.8) and add 800 µl extraction solvent (5.6) and mix (see NOTE 2).

When a sample contains gossypol in a concentration that exceeds the range of the calibration curve, reanalysis is required. For this purpose, the sample extract is diluted with extraction solvent (5.6) in an appropriate ratio, to produce a concentration in the sample extract that is within the range of the calibration curve.

In case of turbid extracts, 1 ml of the extract may first be filtered through a 0,45 µm PTFE membrane filter (6.4) into a new HPLC vial. Take 200 µl of this extract to dilute with 800 µl extraction solvent.

NOTE 1 The expected range of gossypol in cottonseed products or compound feeds is from 10 mg/kg to 200 mg/kg. The gossypol concentration in undiluted extracts prepared from cottonseed products or compound feed is expected to be in the range of 0,25 µg/ml to 5 µg/ml. After dilution of the sample extract the concentration is expected to range from 0,05 µg/ml to 1 µg/ml.

NOTE 2 In specific products higher concentrations of gossypol can be present, in the range of 200 mg/kg to 1 000 mg/kg. For these products the gossypol concentration in undiluted extracts is expected to range from 5 µg/ml to 25 µg/ml and in the diluted extracts from 1 µg/ml to 5 µg/ml. By applying an additional fivefold dilution step, the gossypol concentration in the sample extracts is expected to range from 0,2 µg/ml to 1,0 µg/ml.