SLOVENSKI STANDARD
SIST EN 17270:2020

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Krma: metode vzorčenja in analize - Določevanje teobromina v sestavinah krme in krmnih mešanicah, vključno s pridobljenimi sestavinami iz kakava, s tekočinsko kromatografijo

Animal feeding stuffs: Methods of sampling and analysis - Determination of theobromine in feed materials and compound feed, including cocoa derived ingredients, by liquid chromatography

Futtermittel: Probenahme- und Untersuchungsverfahren - Bestimmung von Theobromin in Einzel- und Mischfuttermitteln, einschließlich aus Kakao gewonnenen Bestandteilen, mittels Flüssigchromatographie

Aliments des animaux : Méthodes d'échantillonnage et d'analyse — Détermination par chromatographie en phase liquide de la teneur en théobromine dans les matières premières destinées aux aliments des animaux et dans les aliments composés pour animaux, y compris les ingrédients issus du cacao

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Animal feeding stuffs: Methods of sampling and analysis - Determination of theobromine in feed materials and compound feed, including cocoa derived ingredients, by liquid chromatography

Aliments des animaux : Méthodes d'échantillonnage et d'analyse - Détermination par chromatographie en phase liquide de la teneur en théobromine dans les matières premières destinées aux aliments des animaux et dans les aliments composés pour animaux, y compris les ingrédients issus du cacao

Futtermittel: Probenahme- und Untersuchungsverfahren - Bestimmung von Theobromin in Einzelfuttermitteln, vor allem aus Kakao gewonnen sowie in Mischfuttermitteln mittels Flüssigchromatographie

This European Standard was approved by CEN on 28 July 2019.

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European foreword

This document (EN 17270:2019) 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of an identical text or by endorsement, at the latest by April 2020, and conflicting national standards shall be withdrawn at the latest by April 2020.

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

Theobromine is naturally present in the cacao tree and its seeds and consequently in cocoa product and by-products. Cocoa bean shells, cocoa bean meal, cocoa germs and discarded confectionery are used for feed purposes in Europe. Maximum levels of theobromine in feeding stuffs are controlled by EU regulations.

WARNING — the use of this protocol involves hazardous materials, operations and equipment. 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 health and safety practices and determine the compatibility with regulatory limitations prior to use.

1 Scope

This document specifies a test method for the determination of theobromine in feed material or compound feed in the working range 27 mg/kg to 307 mg/kg using liquid chromatography coupled to a UV detector (HPLC-UV) or in the working range 49 mg/kg to 307 mg/kg using liquid chromatography with tandem mass spectrometry (LC-MS/MS).

This method has been fully validated using complementary compound feed for adult dogs and complementary compound feed for horses.

This method is also considered applicable for determining theobromine in baking chocolate using either HPLC-UV or LC-MS/MS systems.

The working range can be extended provided the extended range is validated.

2 Normative references

There are no normative references in this document.

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

A test portion of finely ground and homogeneous material is defatted with hexane, an internal standard added and the theobromine extracted into ammonium acetate buffer. The extract is cleaned with the addition of Carrez reagents, filtered and the extract analysed by liquid chromatography with UV detection. Alternatively, the theobromine content can be determined by liquid chromatography tandem mass spectrometry (LC-MS/MS) providing it can be demonstrated that there is no interference from the sample matrix.

5 Reagents

Use only reagents of recognized analytical grade unless otherwise specified. Commercially available solutions with equivalent properties to those listed may be used. References to products or vendors are for information only and do not preclude the use of products of similar quality from alternative suppliers.

For reagents specific to the analysis of the extracts by HPLC-UV see Annex A. For reagents specific to the analysis of the extracts by LC-MS/MS see Annex B.

WARNING — Dispose of waste solvents according to applicable environmental rules and regulations.

5.1 Ammonium acetate, analytical reagent grade

5.2 Glacial acetic acid, 99,5 %

5.3 Acetic acid, 1 mol/l

Add 5,7 ml of glacial acetic acid (5.2) to 60 ml high-purity water (5.4) in a 100 ml volumetric flask, mix thoroughly and dilute to the mark with water (5.4). Mix the flask contents thoroughly again by inversion before use.

5.4 Water, LC-MS grade or of comparable purity, e.g. resistance of 18,2 MΩ cm or conductivity of 55 nS/cm at 20 °C

5.5 2,5 mol/l Ammonium acetate buffer, pH 5,5

Weigh 192,7 g ± 0,1 g ammonium acetate (5.1) into a 1 l beaker and dissolve in approximately 600 ml of high-purity water (5.4). Using a calibrated pH meter (6.13), adjust the pH of the buffer using initially glacial acetic acid (5.2) and then 1 mol/l acetic acid (5.3) until it is in the range of pH 5,4 to 5,6.

Quantitatively transfer the pH-adjusted buffer from the beaker to a 1 l volumetric flask and then dilute to volume with water (5.4). Mix thoroughly by inversion prior to use. This solution is stable for up to 1 month when stored at room temperature.

5.6 Hexane, reagent grade

5.7 Zinc acetate dihydrate, reagent grade

5.8 Potassium ferrocyanide trihydrate, reagent grade

5.9 Carrez reagent I

Weigh 219 g ± 1 g zinc acetate dihydrate (5.7) into a 1 l beaker, add 30 ml glacial acetic acid (5.2) and approximately 800 ml water (5.4). Mix thoroughly until dissolved, transfer to a 1 l volumetric flask and dilute to volume with water (5.4). Mix well before use. This solution is stable for up to 3 months when stored at room temperature.

5.10 Carrez reagent II

Weigh 106 g ± 1 g potassium ferrocyanide trihydrate (5.8) into a 1 l beaker and add approximately 800 ml water (5.4). Mix thoroughly until dissolved, transfer to a 1 l volumetric flask and dilute to volume with water (5.4). Mix well before use. This solution is stable for up to 3 months when stored at room temperature.

5.11 Theobromine, ≥ 98,5 %

5.12 Theobromine stock solution, 125 µg/ml

Weigh 62,5 mg ± 1 mg theobromine (5.11) into a 500 ml volumetric flask and add approximately 400 ml water (5.4). Place in an ultrasonic bath until the theobromine has completely dissolved then dilute to volume with water (5.4). Mix well before use. The exact weight of theobromine taken should be recorded and the concentration of the solution calculated. This solution is stable for up to 1 month when stored at 2 °C to 8 °C.

5.13 7-(β-Hydroxyethyl)theophylline, ≥ 98 %

5.14 7-(β-Hydroxyethyl)theophylline internal standard stock solution, 1 mg/ml

Weigh 100 mg ± 1 mg 7-(β-Hydroxyethyl)theophylline (5.13) into a 100 ml volumetric flask and add approximately 80 ml water (5.4). Shake well to dissolve then dilute to volume with water (5.4). Mix well before use. This solution is stable for up to 1 month when stored at 2 °C to 8 °C.

5.15 7-(β-Hydroxyethyl)theophylline internal standard solution, 100 µg/ml

Pipette 1 ml 7-(β-Hydroxyethyl)theophylline internal standard stock solution, 1 mg/ml (5.14) into a 10 ml volumetric flask and dilute to volume with water (5.4). Mix well before use. This solution is stable for up to 1 month when stored at 2 °C to 8 °C.

5.16 Calibration standards

Add by pipette 7-(β-Hydroxyethyl)theophylline solution, 100 µg/ml (5.15) and different volumes of theobromine stock solution, 125 µg/ml (5.12), into six 10 ml volumetric flasks such that six calibration standards across the calibration range are obtained. The solutions should be diluted to volume with water (5.4). These solutions are stable for up to 1 week when stored at room temperature.

Table 1 provides examples of volumes to be taken to obtain calibration standards at 0 µg/ml, 0,5 µg/ml, 1, 5 µg/ml, 10 µg/ml and 15 µg/ml theobromine. If the extraction procedure is followed, the calibration standards are equivalent to 0 mg/kg, 20 mg/kg, 40 mg/kg, 200 mg/kg, 400 mg/kg and 600 mg/kg in the sample. The exact concentration of the calibration standards should be calculated from the weight of theobromine used to prepare the stock standard solution (5.12).

Table 1 — Suggested Calibration Standards

Calibration Standard (nominal concentration µg/ml)	**0**	**0,5**	**1**	**5**	**10**	**15**
Volume (µl) of 125 µg/ml theobromine (5.12)	0	40	80	400	800	1200
Volume (µl) of 100 µg/ml 7-(β-Hydroxyethyl)theophylline (5.15)	200	200	200	200	200	200

5.17 Quality control material

It is recommended that a suitable quality control material be analysed in every batch, for example NIST SRM 2384 Baking chocolate, certified value for theobromine (11 600 ± 1 100) mg/kg. For NIST SRM 2384 a sample weight of 1,0 g ± 0,1 g should be used, 2 ml of 1 mg/ml 7-(β-Hydroxyethyl)theophylline internal standard stock solution (5.14) should be added and the extract should be diluted, for example, by a factor of 10 prior to analysis by HPLC-UV or LC-MS/MS.

6 Apparatus

Standard laboratory glassware and equipment including the following:

6.1 Mill, single mill or multiple mills, capable of comminuting test materials to particle sizes of < 500 µm

6.2 Sieve, 500 µm

6.3 Mixer, capable of sufficiently homogenizing the comminuted test materials

6.4 Conical polypropylene (PP) screw-cap centrifuge tubes, with screw cap, 50 ml or similar

6.5 Balance, with a mass resolution of 0,001 g or better

6.6 Centrifuge, capable of generating a relative centrifugal force (rcf) of 3000 *g*

6.7 Glass vials for use in the autosampler (usually approximately 1,5 ml capacity) and screw caps or equivalent

6.8 Nylon syringe filter, small internal volume, pore size: 0,45 µm

6.9 General purpose filter papers

6.10 Vortex mixer

6.11 Water bath, capable of maintaining 40 °C ± 5 °C.

6.12 Ultrasonic bath

6.13 Equipment for measuring pH, calibrated and sufficiently accurate for the purposes of this European Standard

7 Procedure

7.1 Sample preparation

It is important that the laboratory receives a sample which is truly representative and has not been damaged or altered during transport or storage. Laboratory samples should be taken and prepared in accordance with European legislation where applicable [1]. The laboratory sample should be finely ground and thoroughly mixed using a mill (6.1) and a mixer (6.3) or another process for which adequate homogenization has been demonstrated, before a test portion is taken for analysis.

It is important that the test portion is taken from a subsample which is sufficiently homogenous with a particle size of ≤ 500 µm. Care should be taken not to overheat the sample during this process.

7.2 Extraction

Weigh 2,5 g ± 0,1 g homogenized sample into a polypropylene screw-cap tube (6.4). Add 6 ml hexane (5.6) and mix thoroughly by vortex (6.10) then centrifuge at 2 700 *g* for 5 min (6.6). Discard the top hexane layer.

Repeat the hexane extraction two more times, each with 3 ml hexane.

Dry the sample with a stream of nitrogen to remove the last traces of hexane then add 0,2 ml of 1 mg/ml 7-(β-Hydroxyethyl)theophylline internal standard (5.14).

Add 25 ml 2,5 mol/l ammonium acetate, pH 5,5 (5.5), shake vigorously then place in a water bath (6.11) set at 40 °C ± 1 °C for (15 ± 2) min. Vortex to mix thoroughly ensuring that no solid material remains adhered to the bottom of the tube, then place in an ultrasonic bath (6.12) for (20 ± 2) min.

Transfer the sample and extract to a 100 ml volumetric flask, using water (5.4) to rinse the polypropylene tube.

Add 5 ml Carrez I (5.9) and mix well by hand. Add 5 ml Carrez II (5.10) and mix well by hand.

Dilute to volume with water (5.4) and mix well then filter through a general purpose filter paper (6.9). Pass an aliquot of the filtrate through a 0,45 µm syringe filter (6.8).

8 Chromatographic analysis

The sample extracts are analysed by HPLC-UV, an example of suitable conditions is given in Annex A. If the analyst does not have access to an HPLC-UV, the extracts can also be analysed by LC-MS/MS, see Annex B for example conditions. A combination of analytical column, mobile phase composition, gradient settings and injection volume should be such that it allows acceptable separation at the required levels to be achieved.

9 Calculation of results

The concentration of theobromine in the sample is calculated using the ratio of the area response for theobromine to the area response for 7-(β-Hydroxyethyl)theophylline. Plot the peak area ratios against the concentration of theobromine in the corresponding calibration standard and determine the slope and the intercept.

The concentration of theobromine in the injected solution can be calculated using the following equation:

$$C_\mathrm{e} = \frac{\left(\frac{A_1}{A_2}\right) - \mathrm{a}}{\mathrm{b}} \times \frac{c}{d} \qquad (1)$$

where

C_e	is the concentration of theobromine in the extract, in µg/ml;
A_1	is sample area response for theobromine;
A_2	is sample area response for 7-(β-Hydroxyethyl)theophylline;
a	is the intercept from the calibration graph;
b	is the slope from calibration graph;
c	is the concentration 7-(β-Hydroxyethyl)theophylline in initial 100 ml sample extract (see Note 1), in µg/ml;
d	is the concentration 7-(β-Hydroxyethyl)theophylline in calibration standard (see Note 2), in µg/ml.

NOTE 1 c = 2 when 0,2 ml of 7-(β-Hydroxyethyl)theophylline is added to the sample. c = 20 when 2 ml of 7-(β-Hydroxyethyl)theophylline is added to the sample.

NOTE 2 d = 2 when calibration standards are prepared as described in 5.16.

If the concentration of theobromine in the extract is outside the calibration range, the sample should be re-extracted and 2 ml 7-(β-Hydroxyethyl)theophylline should be added instead of 0,2 ml. The extract should be diluted by, for example, a factor of 10 prior to analysis by HPLC-UV or LC-MS/MS.