

SVENSK STANDARD

SS-EN 17270:2019

Djurfoder – Provtagnings- och analysmetoder – Bestämning av teobromin i foderråvaror och foderblandningar, inklusive kakao härledda ingredienser, genom vätskekromatografi – Kompletterande element

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



sis Svenska
Institutet för
Standarder

Språk: engelska/English

Utgåva: 1

This preview is downloaded from www.sis.se. Buy the entire standard via <https://www.sis.se/std-80017876>

Den här standarden kan hjälpa dig att effektivisera och kvalitetssäkra ditt arbete. SIS har fler tjänster att erbjuda dig för att underlätta tillämpningen av standarder i din verksamhet.

SIS Abonnemang

Snabb och enkel åtkomst till gällande standard med SIS Abonnemang, en prenumerationstjänst genom vilken din organisation får tillgång till all världens standarder, senaste uppdateringarna och där hela din organisation kan ta del av innehållet i prenumerationen.

Utbildning, event och publikationer

Vi erbjuder även utbildningar, rådgivning och event kring våra mest sålda standarder och frågor kopplade till utveckling av standarder. Vi ger också ut handböcker som underlättar ditt arbete med att använda en specifik standard.

Vill du delta i ett standardiseringsprojekt?

Genom att delta som expert i någon av SIS 300 tekniska kommittéer inom CEN (europeisk standardisering) och/eller ISO (internationell standardisering) har du möjlighet att påverka standardiseringsarbetet i frågor som är viktiga för din organisation. Välkommen att kontakta SIS för att få veta mer!

Kontakt

Skriv till kundservice@sis.se, besök [sis.se](https://www.sis.se) eller ring 08 - 555 523 10

© Copyright/Upphovsrätten till denna produkt tillhör Svenska institutet för standarder, Stockholm, Sverige. Upphovsrätten och användningen av denna produkt regleras i slutanvändarlicensen som återfinns på [sis.se/slutanvandarlicens](https://www.sis.se/slutanvandarlicens) och som du automatiskt blir bunden av när du använder produkten. För ordlista och förkortningar se [sis.se/ordlista](https://www.sis.se/ordlista).

© Copyright Svenska institutet för standarder, Stockholm, Sweden. All rights reserved. The copyright and use of this product is governed by the end-user licence agreement which you automatically will be bound to when using the product. You will find the licence at [sis.se/enduserlicenseagreement](https://www.sis.se/enduserlicenseagreement).

Upplysningar om sakinnehållet i standarden lämnas av Svenska institutet för standarder, telefon 08 - 555 520 00. Standarder kan beställas hos SIS som även lämnar allmänna upplysningar om svensk och utländsk standard.

Standarden är framtagen av kommittén för Djurfoder, SIS/TK 435/AG 02.

Har du synpunkter på innehållet i den här standarden, vill du delta i ett kommande revideringsarbete eller vara med och ta fram andra standarder inom området? Gå in på www.sis.se - där hittar du mer information.

Europastandarden EN 17270:2019 gäller som svensk standard. Detta dokument innehåller den officiella engelska versionen av EN 17270:2019.

The European Standard EN 17270:2019 has the status of a Swedish Standard. This document contains the official version of EN 17270:2019.

EUROPEAN STANDARD

EN 17270

NORME EUROPÉENNE

EUROPÄISCHE NORM

October 2019

ICS 65.120

English Version

**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.

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

SS-EN 17270:2019 (E)

Contents		Page
European foreword		3
Introduction		4
1	Scope	5
2	Normative references	5
3	Terms and definitions	5
4	Principle	5
5	Reagents	5
6	Apparatus	7
7	Procedure	8
8	Chromatographic analysis	9
9	Calculation of results	9
10	Accuracy	10
11	Test report	10
Annex A (informative) Example HPLC-UV conditions		11
Annex B (informative) Example LC-MS/MS conditions		14
Annex C (informative) Method performance data obtained during single laboratory validation		20
Annex D (informative) Method performance data obtained during collaborative trial		21
Bibliography		23

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.

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.

SS-EN 17270:2019 (E)

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

SS-EN 17270:2019 (E)

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 M Ω 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 \pm 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 \pm 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 \pm 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, \geq 98,5 %

5.12 Theobromine stock solution, 125 μ g/ml

Weigh 62,5 mg \pm 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, \geq 98 %

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

Weigh 100 mg \pm 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 $\mu\text{g}/\text{ml}$

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 $\mu\text{g}/\text{ml}$ (5.15) and different volumes of theobromine stock solution, 125 $\mu\text{g}/\text{ml}$ (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 $\mu\text{g}/\text{ml}$, 0,5 $\mu\text{g}/\text{ml}$, 1, 5 $\mu\text{g}/\text{ml}$, 10 $\mu\text{g}/\text{ml}$ and 15 $\mu\text{g}/\text{ml}$ 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 $\mu\text{g}/\text{ml}$)	0	0,5	1	5	10	15
Volume (μl) of 125 $\mu\text{g}/\text{ml}$ theobromine (5.12)	0	40	80	400	800	1200
Volume (μl) of 100 $\mu\text{g}/\text{ml}$ 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 \pm 1 100) mg/kg. For NIST SRM 2384 a sample weight of 1,0 g \pm 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*