**DRAFT FOR DEVELOPMENT**

**DD CEN ISO/TS 17764-1:2006**
*Incorporating amendment no. 1 (renumbers DD ISO/TS 17764-1:2002 as DD CEN ISO/TS 17764-1:2006)*

# Animal feeding stuffs — Determination of the content of fatty acids —

## Part 1: Preparation of methyl esters

ICS 65.120



BSI British Standards

# National foreword

This Draft for Development is the official English language version of CEN ISO/TS 17764-1:2006. It is identical with ISO/TS 17764-1:2002.

**This publication is not to be regarded as a British Standard.**

It is being issued in the Draft for Development series of publications and is of a provisional nature because of the need to carry out collaborative trials to validate this method. It should be applied on this provisional basis, so that information and experience of its practical application may be obtained.

Comments arising from the use of this Draft for Development are requested so that UK experience can be reported to the international organization responsible for the Technical Specification. A review of this publication will be initiated not later than 3 years after its publication by the international organization so that a decision can be taken on its status at the end of its 3-year life. Notification of the start of the review period will be made in an announcement in the appropriate issue of *Update Standards*.

According to the replies received by the end of the review period, the responsible BSI Committee will decide whether to support the conversion into an international standard, to extend the life of the Technical Specification for another 3 years or to withdraw it. Comments should be sent in writing to the Secretary of BSI Technical Committee AW/10, Animal feeding stuffs, at British Standards House, 389 Chiswick High Road, London W4 4AL, giving the document reference and clause number and proposing, where possible, an appropriate revision of the text.

A list of organizations represented on this committee can be obtained on request to its secretary.

This Draft for Development, having been prepared under the direction of the Consumer Products and Services Sector Policy and Strategy Committee, was published under the authority of the Standards Policy and Strategy Committee on 20 December 2002



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TECHNICAL SPECIFICATION

SPÉCIFICATION TECHNIQUE

TECHNISCHE SPEZIFIKATION

# CEN ISO/TS 17764-1

October 2006

ICS 65.120

English Version

# Animal feeding stuffs - Determination of the content of fatty acids - Part 1: Preparation of methyl esters (ISO/TS 17764-1:2002)

Aliments des animaux - Détermination de la teneur en acides gras - Partie 1: Préparation des esters méthyliques (ISO/TS 17764-1:2002)

Futtermittel - Bestimmung des Gehaltes an Fettsäuren - Teil 1: Vorbereitung von Methylestern (ISO/TS 17764-1:2002)

This Technical Specification (CEN/TS) was approved by CEN on 13 February 2006 for provisional application.

The period of validity of this CEN/TS is limited initially to three years. After two years the members of CEN will be requested to submit their comments, particularly on the question whether the CEN/TS can be converted into a European Standard.

CEN members are required to announce the existence of this CEN/TS in the same way as for an EN and to make the CEN/TS available promptly at national level in an appropriate form. It is permissible to keep conflicting national standards in force (in parallel to the CEN/TS) until the final decision about the possible conversion of the CEN/TS into an EN is reached.

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**Management Centre: rue de Stassart, 36 B-1050 Brussels**



Ref. No. CEN ISO/TS 17764-1:2006: E

## Foreword

This document (CEN ISO/TS 17764-1:2006) 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, in collaboration with Technical Committee ISO/TC 34 "Agricultural food products".

According to the CEN/CENELEC Internal Regulations, the national standards organizations of the following countries are bound to announce this CEN Technical Specification: Austria, Belgium, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, Switzerland and United Kingdom.

CEN ISO/TS 17764-1:2006

TECHNICAL SPECIFICATION

**ISO/TS 17764-1**

First edition
2002-11-01

# Animal feeding stuffs — Determination of the content of fatty acids —

## Part 1: Preparation of methyl esters

*Aliments des animaux — Détermination de la teneur en acides gras —*

*Partie 1: Préparation des esters méthyliques*

Reference number
ISO/TS 17764-1:2002(E)

## Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

In other circumstances, particularly when there is an urgent market requirement for such documents, a technical committee may decide to publish other types of normative document:

— an ISO Publicly Available Specification (ISO/PAS) represents an agreement between technical experts in an ISO working group and is accepted for publication if it is approved by more than 50 % of the members of the parent committee casting a vote;

— an ISO Technical Specification (ISO/TS) represents an agreement between the members of a technical committee and is accepted for publication if it is approved by 2/3 of the members of the committee casting a vote.

An ISO/PAS or ISO/TS is reviewed after three years in order to decide whether it will be confirmed for a further three years, revised to become an International Standard, or withdrawn. If the ISO/PAS or ISO/TS is confirmed, it is reviewed again after a further three years, at which time it must either be transformed into an International Standard or be withdrawn.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO/TS 17764-1 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 10, *Animal feeding stuffs*.

ISO/TS 17764 consists of the following parts, under the general title *Animal feeding stuffs — Determination of the content of fatty acids*:

— *Part 1: Preparation of methyl esters*

— *Part 2: Gas chromatographic method*

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# Animal feeding stuffs — Determination of the content of fatty acids —

# Part 1: Preparation of methyl esters

## 1 Scope

ISO/TS 17764 specifies methods for the quantitative determination of individual fatty acids and of the sum of the fatty acids (elutable fatty acids).

This part of ISO/TS 17764 specifies two methods for preparing the methyl esters of fatty acids of animal and vegetable fats, oils and fatty acid mixtures for raw materials for compound animal feeds, and fatty acids originating from fat extracts of animal feeding stuffs, including fats and fatty acid mixtures containing butyric acid.

The general method, the boron trifluoride ($BF_3$) method, is concerned with the preparation of methyl esters of fatty acids with six or more C atoms, originating from fats, oils and free fatty acids.

The KOH/HCl method is concerned with the preparation of methyl esters of fatty acids with four or more C atoms. This method can also be used for the quantitative determination of fatty acids with a chain length shorter than ten C atoms in free fatty acid mixtures.

The methyl esters produced can be used for gas-liquid chromatography (GLC).

NOTE 1 Unsaponifiable materials are not removed and can, when present in considerable amounts, interfere with the chromatographic analysis.

NOTE 2 ISO/TS 17764-2 describes the application of gas chromatography with capillary columns and flame ionization detection for the determination of the content of fatty acids in a fat by making use of the methyl esters of the fatty acids obtained in accordance with the methods specified in this part of ISO/TS 17764.

## 2 Normative references

The following referenced documents are indispensable for the application 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.

ISO 661:1989, *Animal and vegetable fats and oils — Preparation of test sample*

ISO 3696:1987, *Water for analytical laboratory use — Specification and test methods*

ISO 6492:1999, *Animal feeding stuffs — Determination of fat content*

# 3 Fat extraction

## 3.1 General

Extract the fat for determination of the content of the saponifiable fatty acids in an animal feeding stuff or mixed feed in accordance with the category of the feeding stuff, as described in ISO 6492:1999, with the following amendments.

## 3.2 Samples of category A

Perform a fat extraction in accordance with 9.5.1 of ISO 6492:1999.

Evaporate the solvent with a rotating evaporator in a water bath at a temperature of not more than 40 °C. Then dry the residue for 2 h in a vacuum drying oven at 40 °C ± 2 °C.

## 3.3 Samples of category B

Extract the fat in two steps. For the first step, proceed in accordance with 9.5.1 of ISO 6492:1999, treating the test portion as described in 9.3 of ISO 6492:1999 as a sample of category A.

Collect the fat extract in a dry flask. Allow the solvent to evaporate from the residue by exposing the thimble to the air.

Perform a hydrolysis of the residue in accordance with 9.4 of ISO 6492:1999.

After hydrolysis, dry the residue in an extraction thimble for 60 min in a vacuum drying oven at 40 °C ± 2 °C.

Extract the residue according to 9.5.1 of ISO 6492:1999.

Add the fat extract to the first extract.

Evaporate the solvent with a rotating evaporator in a water bath at a temperature of not more than 40 °C and dry the residue for 2 h in a vacuum drying oven at 40 °C ± 2 °C.

# 4 Preparation of test sample of fat or fat extract

If the fat sample or the fat extract is not completely molten, heat the sample to a temperature of not more than 10 °C above the melting temperature. See ISO 661.

# 5 Method for the preparation of methyl esters of fatty acids with six or more C atoms ($BF_3$ method)

## 5.1 Principle

The glycerides are saponified with methanolic sodium hydroxide. The soaps are converted to methyl esters by reaction with a boron trifluoride/methanol complex.

## 5.2 Reagents

Use only reagents and solvents of recognized analytical grade.

**5.2.1 Water**, complying with at least grade 3 in accordance with ISO 3696:1987.

**5.2.2 Heptadecanoic acid (internal standard)**, of purity at least 99 %.

**5.2.3 Sodium hydroxide**, methanolic solution, $c$(NaOH) ≈ 0,5 mol/l.

Dissolve 2 g of sodium hydroxide in 100 ml of methanol containing not more than 0,5 % (mass fraction) of water. If the solution has to be stored for a considerable time, a small amount of white precipitate of sodium carbonate can form; this has no effect on the preparation of the methyl esters.

Instead of sodium hydroxide, a methanolic solution of potassium hydroxide with the same concentration may be used.

**5.2.4 Boron trifluoride** ($BF_3$), methanolic solution, 10 % to 15 % (mass fraction).

**WARNING — Boron trifluoride is poisonous. For this reason, it is not recommended that the analyst prepare the methanolic solution of boron trifluoride from methanol and boron trifluoride.**

Solutions are available commercially.

In gas chromatographic analysis of the methyl esters, certain reagents can give rise to peaks which interfere with the analysis. In particular, $BF_3$ solutions can cause peaks in the area of the methyl esters of fatty acids with 20 or 22 C atoms. It is recommended to check the reagents by preparing the methyl ester of oleic acid followed by a gas chromatographic analysis. Reagents shall not give rise to peaks which interfere with the gas chromatographic analysis of the fatty acid methyl esters.

**5.2.5 *n*-Hexane** or ***n*-heptane**.

**5.2.6 Sodium chloride**, saturated aqueous solution.

**5.2.7 Sodium sulfate**, anhydrous.

## 5.3 Apparatus

Usual laboratory equipment and, in particular, the following.

**5.3.1 Round-bottom flask**, of capacity 50 ml, with ground neck and fitted with a ground glass stopper.

**5.3.2 Boiling aid**, fat-free.

**5.3.3 Reflux condenser**, of effective length 20 cm to 30 cm, with a ground joint to fit the flask.

**5.3.4 Graduated pipette**, of capacity at least 10 ml and fitted with a rubber bulb, or an automatic pipette.

**5.3.5 Vials** with screw caps.

## 5.4 Procedure

### 5.4.1 General

Because of the toxic character of boron trifluoride, the methylation is best performed under a ventilated hood. It is essential to wash all glassware with water immediately after use.

If the fatty acids contain more than two double bonds, it is recommended to remove the air from the flask by bubbling nitrogen with an oxygen content of less than 5 mg/kg through the solution for a few minutes and then maintain a current of nitrogen in the upper part of the condenser during the following saponification.

When preparing methyl esters of fatty acids for gas-liquid chromatography, do not remove the solvent from the solution of methyl esters.

#### 5.4.2 Test portion

Weigh, to the nearest 0,1 mg, 100 mg to 250 mg of the prepared test sample (Clause 4) in a round-bottom flask (5.3.1). Add to the flask, as internal standard, heptadecanoic acid (5.2.2) with a mass of about 20 % of the mass of the test portion and weighed to the nearest 0,1 mg. Weigh in a second round-bottom flask approximately the same amount of the prepared test sample.

Treat both flasks in the same way, as follows.

If the test portion concerns an oil or fat, proceed in accordance with 5.4.3.

If the test portion is exclusively composed of free fatty acids or soaps, proceed in accordance with 5.4.5.

If there is not enough material, a test portion of less than 100 mg may be used. The prescribed reagents and solvents shall be reduced proportionally and the volume of the apparatus adjusted.

#### 5.4.3 Saponification

Add 4 ml of sodium hydroxide, methanolic solution (5.2.3) and a boiling aid (5.3.2). Fit the condenser (5.3.4) to the flask.

Boil under reflux until the droplets of fat disappear and then for 30 min more.

If any unsaponifiable materials remain which could interfere with the analysis, the solution after saponification may be diluted with water and then extracted with diethyl ether, hexane or petroleum ether. The extract is discarded. After acidification of the remaining soap solution, the fatty acids can be separated and methylated according to 5.4.5.

#### 5.4.4 Conversion to methyl esters in the case of fat or oil samples

Add 5 ml of the boron trifluoride/methanol solution (5.2.4) to the boiling solution through the top of the condenser, with the help of a graduated pipette (5.3.4). Continue the boiling for 3 min.

Proceed in accordance with 5.4.6.

#### 5.4.5 Conversion to methyl esters if the sample contains exclusively free fatty acids or soaps

Add with the graduated pipette (5.3.4), 5 ml of boron trifluoride/methanol solution (5.2.4) to the flask. Fit the condenser (5.3.3) to the flask. Boil for 3 min.

#### 5.4.6 Extraction

Remove the flask from the heat source. Add 1 ml to 3 ml of *n*-hexane (5.2.5) through the top of the condenser.

NOTE The volume of the added solvent is not critical.

Allow to cool to room temperature. Remove the condenser and add about 15 ml of saturated sodium chloride (5.2.6).

Stopper the flask and shake it vigorously. Add more of the saturated sodium chloride solution (5.2.6) to bring the liquid level of the mixture into the neck of the flask. Allow the two phases to separate. Transfer most of the upper layer with a pipette into a vial (5.3.5). Extract the saline solution three times more with 1 ml to 3 ml of *n*-hexane (5.2.5) by shaking several times and transfer the upper layer into the vial.

Add a small amount of anhydrous sodium sulfate (5.2.7) to remove traces of water from the solution.

If the mass of the test portion was between 100 mg to 250 mg, the concentration of methyl esters in the solution will be about 3 % (mass fraction). The solution is ready for gas chromatographic analysis.

If cold on-column injection is used in the gas chromatographic analysis, prepare a diluted solution by adding with a pipette 0,25 ml of the solution into a 25 ml flask and fill the flask to the mark with *n*-hexane (5.2.5).

## 6 Method for the preparation of methyl esters of fatty acids with 4 or more C-atoms (KOH-HCl method)

### 6.1 Principle

The glycerides are converted to methyl esters by transesterification with potassium methanolate in absolute methanol. Free fatty acids are esterified by hydrochloric acid methanolic solution.

### 6.2 Reagents

Use only reagents and solvents of recognized analytical grade.

**6.2.1 Water**, complying with at least grade 3 in accordance with ISO 3696:1987.

**6.2.2 Heptadecanoic acid (internal standard)**, of purity *ca*. 99 %.

**6.2.3 *n*-Hexane** or ***n*-heptane**.

**6.2.4 *n*-Pentane**.

**6.2.5 Potassium methanolate**, methanolic solution, $c(CH_3OK) \approx 2$ mol/l.

Dissolve 7,8 g of metallic potassium in 100 ml of absolute methanol. Prepare fresh daily.

Instead of potassium methanolate, a solution of sodium methanolate in methanol with the same concentration may be used.

**6.2.6 Sodium sulfate**, anhydrous

**6.2.7 Methanol**, anhydrous

Add 5 g of sodium sulfate (6.2.6) to a flask containing 250 ml of methanol. Close the flask and shake vigorously. Filter the solution through a paper filter into a conical flask (6.3.1) and close the flask tightly.

**6.2.8 Hydrochloric acid**, methanolic solution, $w(HCl) \approx 20$ % (mass fraction).

Weigh, to the nearest 0,1 g, 80 g of methanol (6.2.7) into a conical flask (6.3.1).

Direct a current of hydrochloric acid gas through the stirred solvent under cooling until the mass of the solution has increased by 20 g. Allow the solution to cool further.

The solution may be kept for 3 months if the flask is tightly closed and stored in the dark.

### 6.3 Apparatus

Usual laboratory equipment and, in particular, the following.

**6.3.1 Conical flasks**, of capacity 250 ml, with a ground neck and fitted with a ground glass stopper.

**6.3.2 Reaction vials**, of capacity *ca*. 10 ml, fitted with a septum and a screw cap.

**6.3.3 Graduated cylinders** with a capacity of 10 ml.

**6.3.4 Electrically heated block**, capable of being maintained at (85 ± 3) °C, and equipped with a magnetic stirrer.

### 6.4 Procedure

Weigh, to the nearest 0,1 mg, 50 mg to 75 mg of the prepared test sample (Clause 4) in each of two reaction vials (6.3.2). Add to one of the flasks, as internal standard, heptadecanoic acid (6.2.2) with a mass of about 20 % of the mass of the test portion and weighed to the nearest 0,1 mg.

Treat both vials in the same way as follows.

Add 4 ml of *n*-hexane (6.2.3). Use 4 ml of *n*-pentane instead of n-hexane if cold on-column injection is used for the gas chromatographic analysis of fatty acids with less than 10 C atoms.

Add *ca*. 75 mg of anhydrous sodium sulfate (6.2.6) and dissolve the test portion by shaking.

Add 0,20 ml of potassium methanolate (6.2.5), close the reaction vial and shake vigorously for 20 s to 50 s. The solution immediately becomes turbid due to the formation of glycerol which quickly settles out. Add 2 ml of hydrochloric acid solution (6.2.8) and a magnetic stirring rod. Close the reaction vial and place it in the heating block (6.3.4), brought to a temperature of 85 °C beforehand. Heat for 20 min under constant stirring. Shake the mixture several times during this period.

Cool the vial with its contents down to room temperature under a flow of cold tap water and shake vigorously.

Decant the upper layer with the methyl esters. Do not remove the solvent from the solution of methyl esters.

If the mass of the test portion was between 50 mg to 75 mg, the concentration of methyl esters in the solution will be about 2 % (mass fraction). The solution is ready for gas chromatographic analysis.

If cold on-column injection is used in the gas chromatographic analysis, prepare a diluted solution by adding with a pipette 0,25 ml of the solution to a 25 ml flask and fill the flask to the mark with *n*-hexane (6.2.4).

## 7 Storage

The acquired solutions with methyl esters are suited for immediate gas chromatographic analysis. If necessary the solution of methyl esters can be stored for several weeks under an inert gas at a temperature of 4 °C to 8 °C.

In case of a longer storage time it is recommendable, in order to prevent oxidation of the methyl esters, to add to the solution an antioxidant in a concentration which has no influence on the gas chromatographic analysis. For instance 0,05 g of BHT (butyl hydroxy toluene) per litre.

## 8 Test report

The test report shall specify:

— all information necessary for the complete identification of the sample;

— the sampling method used, if known;

— the method of esterification followed (i.e. the $BF_3$ method or the KOH/HCl method), with reference to this part of ISO/TS 17764;

— all operating details not specified in this part of ISO/TS 17764, or regarded as optional, together with details of any incidents which may have influenced the test result(s).

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