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

**Part 2:  
Gas chromatographic method**

*Aliments des animaux — Détermination de la teneur en acides gras —  
Partie 2: Méthode par chromatographie en phase gazeuse*



Reference number  
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## Foreword

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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order to decide whether it will be confirmed for a further three years, revised to become an International Standard, or withdrawn.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again after a further three years, at which time it must either be transformed into an International Standard or be withdrawn.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 17764-2 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 2: Gas chromatographic method

### 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 the application of gas chromatography with capillary columns and flame ionization detection for the determination of the quantitative content of fatty acids in a fat by making use of the methyl esters of the fatty acids obtained in accordance with the method specified in ISO/TS 17764-1.

This part of ISO/TS 17764 is applicable to the investigation of animal and vegetable fats, oils and fatty acid mixtures for incorporation in animal feeding stuffs and fat extracts of animal feeding stuffs and raw materials for compound animal feeds, including fats and fatty acid mixtures containing butyric acid.

This method is not applicable to polymerized fatty acids.

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

ISO/TS 17764-1, *Animal feeding stuffs — Determination of the content of fatty acids — Part 1: Preparation of methyl esters*

### 3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

#### 3.1

##### **fatty acid content**

mass fraction of the fatty acids in the test portion of oil, fat, fat extract, free fatty acids or soaps

NOTE The fatty acid content is expressed in grams per kilogram.

#### 3.2

##### **content of elutable material**

mass fraction of the sum of all the fatty acids elutable using a gas chromatographic column as described in this part of ISO/TS 17764

## 4 Principle

The methyl esters prepared from fatty acids in accordance with ISO/TS 17764-1 are separated with gas-liquid chromatography, making use of a capillary column. The peaks in the chromatogram are defined with the help of a reference sample of known composition and are quantified by means of an internal standard.

## 5 Reagents

Use only reagents and solvents of recognized analytical grade.

**5.1 Water**, complying with at least grade 3 in accordance with ISO 3696:1087.

**5.2 *n*-Hexane** or ***n*-heptane**.

**5.3 *n*-Pentane**.

**5.4 Reference sample**: an oil or fat sample with exactly known fatty acid pattern, or a mixture of reference fatty acid methyl ester materials or reference fatty acids materials.

**NOTE** If the  $\text{BF}_3$  method for esterification is used, a mixture of reference fatty acid methyl esters cannot be used for calibration or correction factors for fatty acids with a chain length of less than 10 carbon atoms, because of possible solubility of the methyl esters in the water phase.

## 6 Apparatus

Usual laboratory equipment and, in particular, the following.

**6.1 Gas chromatograph**, comprising a capillary column and an injection system specially designed for use with such columns.

It may be of the split or the split-less type or a cold on-column injector. However, a warm split-less injector is not suitable for the analysis of milk fats due to overlap of the solvent peak with the butyric acid peak.

**6.2 Column**, constructed of inert material (fused silica or glass) with a stationary phase preferable chemically bonded to the wall of the column.

Column dimensions and film thickness are important factors in determining the separation efficiency and capacity of the column. A resolution of at least 1,25 for the fatty acids C16:0 and C16:1, and C18:0 and C18:1 should be accomplished.

**NOTE** In most cases a moderately polar phase will suit. In special cases, for instance for the separation of *cis-trans*-isomers and/or positional isomers, or if one must be sure that no peaks coincide, a more polar phase is warranted. The desirable effectiveness and capacity of the column should also be considered for column dimensions and film thickness. Moderately polar phases are, for instance, various esters of poly(ethylene glycol). More polar phases are often of the cyano-propyl-polysiloxane type.

**6.3 Injection system**, for manual injection, with a capacity of at most 10  $\mu\text{l}$ , and graduated in 0,1  $\mu\text{l}$  divisions, suited for the injector (6.2), or an automatic injection system.

**NOTE** The use of an automatic system is preferable and can improve repeatability and reproducibility.

**6.4 Signal evaluation apparatus**: an electronic system fitted with a recorder to transform the detector signal to a chromatogram (an integrator or a data station).

## 7 Procedure

### 7.1 Preparation of methyl esters

Prepare the methyl esters of the fatty acids of the test portion and the reference sample (5.4) in accordance with ISO/TS 17764-1.

### 7.2 Selection of optimum operating conditions

Optimize the equipment in accordance with the instructions given by the manufacturer.

Optimize the flow of carrier gas in accordance with the recommendations of the column manufacturer for the chosen column and the carrier gas.

Maintain a detector temperature of 20 °C to 50 °C above the highest temperature of the column in a programmed heating, but at least at 150 °C.

The injector temperature depends on the type of the injector; follow the instructions given in the equipment manual.

When using a split injector, set the split ratio between 1:30 and 1:100.

### 7.3 Analysis

**7.3.1** Dissolve the fatty acid methyl esters of the test portion and the test portion with added internal standard in *n*-hexane (5.2) to a content of 1 % (mass fraction) when using a split injector, or 0,05 % (mass fraction) in the case of a split-less injector or a cold on-column injector.

Prepare a solution of the fatty acid methyl esters of the reference sample (7.1) in *n*-hexane with a comparable concentration.

Inject separately 0,1 µl to 1 µl of the test sample, the test sample with internal standard, and when necessary the reference sample.

When using cold on-column injection, the use of *n*-pentane as solvent is necessary for a good separation of the fatty acid methyl esters with a chain length of less than 10 carbon atoms. Dissolve the methyl esters of the fatty acids in the test portions and the reference sample in the same solvent.

**7.3.2** Select a temperature programme depending on the fatty acid composition, allowing an effective resolution in the shortest possible time. Take into account the criteria mentioned in 6.3.

Programme the oven temperature starting from 60 °C if the sample contains fatty acids with a chain length shorter than 12 carbon atoms.

If necessary, progress isothermally after the highest temperature in the programme has been reached until all components have been eluted.

When using a cold on-column injector, start with an oven temperature of not more than 10 °C higher than the boiling point of the solvent at the prevailing pressure (50 °C for *n*-pentane).

Start the temperature programme immediately after the injection. Follow the manufacturer's instructions.

## 8 Peak identification

Identify the methyl ester peaks of the test portion according to the retention times in comparison with the retention times of the peaks of known fatty acid methyl esters in the reference sample. Peaks in the chromatogram of the test portion with the same retention time as peaks in the reference sample are considered to represent the same fatty acids.

## 9 Calculation

### 9.1 Correction for heptadecanoic acid in the test portion

Correct the peak area of heptadecanoic acid in the test portion with the added internal standard for heptadecanoic acid originating from the test sample, using the equation:

$$A_{\text{rsr}} = A_{\text{sr}17:0} - \left( \frac{A_{\text{s}17:0} (A_{\text{sr}16:0} + A_{\text{sr}18:0} + A_{\text{sr}18:1})}{(A_{\text{s}16:0} + A_{\text{s}18:0} + A_{\text{s}18:1})} \right)$$

where

- $A_{\text{rsr}}$  is the corrected area under the peak of the internal standard in the test portion with added internal standard, in area units;
- $A_{\text{sr}16:0}$  is the area under the peak of hexadecanoic acid (palmitic acid) in the test portion with added internal standard, in area units;
- $A_{\text{sr}17:0}$  is the area under the peak of heptadecanoic acid (margarinic acid) in the test portion with added internal standard, in area units;
- $A_{\text{sr}18:0}$  is the area under the peak of octadecanoic acid (stearic acid) in the test portion with added internal standard, in area units;
- $A_{\text{sr}18:1}$  is the area under the peak of octadecenoic acid (oleic acid) in the test portion with added internal standard, in area units;
- $A_{\text{s}16:0}$  is the area under the peak of hexadecanoic acid (palmitic acid) in the test portion of the analysed sample without added internal standard, in area units;
- $A_{\text{s}17:0}$  is the area under the peak of heptadecanoic acid (margarinic acid) in the test portion of the analysed sample without added internal standard, in area units;
- $A_{\text{s}18:0}$  is the area under the peak of octadecanoic acid (stearic acid) in the test portion of the analysed sample without added internal standard, in area units;
- $A_{\text{s}18:1}$  is the area under the peak of octadecenoic acid (oleic acid) in the test portion of the analysed sample without added internal standard, in area units.

Correction for heptadecanoic acid in the test sample is not necessary if the relative quantity does not exceed 0,5 % of total fatty acids.

### 9.2 Determination of relative calibration factors

Determine the relative calibration factors for fatty acids with a chain length shorter than 10 carbon atoms.

If another than cold on-column injection is used, it is necessary to account for selective evaporation of fatty acid methyl esters. In this case, determine the relative calibration factors for the whole range of fatty acid methyl esters.

Calibration factors are used to convert peak areas into mass fractions. Determine the calibration factors with the help of a chromatogram derived from the analysis of the reference mixture (5.4) carried out under operating conditions identical to those used for the test sample.

Calculate the calibration factor for the fatty acid  $i$  by the equation:

$$k_i = \frac{m_i}{A_i}$$



where

$k_i$  is the calibration factor for fatty acid  $i$ , in mass units per area unit;

$m_i$  is the mass of fatty acid  $i$  in the reference sample, in mass units;

$A_i$  is the peak area of fatty acid  $i$  in the reference sample, in area units.

If it is not possible to determine a calibration factor because a reference fatty acid is not available, use the calibration factor of the nearest preceding fatty acid for which a reference fatty acid is available.

Calibration factors are expressed relative to the calibration factor of the internal standard C17:0. The resulting relative calibration factor is:

$$k'_i = \frac{k_i}{k_r}$$

where

$k'_i$  is the relative calibration factor of fatty acid  $i$ ;

$k_i$  is the calibration factor of fatty acid  $i$ , in mass units per area unit;

$k_r$  is the calibration factor of the internal standard fatty acid, in mass units per area unit.

### 9.3 Range of relative calibration factors

The relative calibration factors can differ slightly from the reciprocal values of the relative response factors. The response is considered to be the magnitude of the signal from the flame ionization detector for a certain fatty acid.

Calculate the theoretical values of the relative response factors of methyl esters of straight-chain saturated fatty acids by the equation:

$$R_i = \frac{M_r(n_i - 1)}{M_i(n_r - 1)}$$

where

$R_i$  is the theoretical relative response factor of fatty acid  $i$ ;

$M_r$  is the molar mass of the internal standard fatty acid (C17:0), in grams per mole;

$M_i$  is the molar mass of fatty acid  $i$ , in grams per mole;

$n_r$  is the number of carbon atoms of the internal standard fatty acid;

$n_i$  is the number of carbon atoms of fatty acid  $i$ .

The relative calibration factor ( $k'_i$ ) should not differ more than 5 % from the value of  $R_i^{-1}$ . In the case of a greater divergence, check whether systematic divergences have occurred. These are allowed if one or more reference samples have been used correctly.

NOTE The most common systematic errors are selective evaporation of components from the syringe needle on injection in a warm injector, or selective splitting in the case of a splitting injector. In these cases, short-chain fatty acids are relatively overestimated. This results in calibration factors for short-chain fatty acids that are lower than the theoretical values. Another cause of systematic divergences can be the incomplete extraction of methyl esters of short-chain fatty acids in the alkane phase.

#### 9.4 Calculation of fatty acid contents

Calculate the content of individual fatty acids in the fat by the equation:

$$w_i = \frac{A_{iSR} \times m_r}{A_{rSR} \times m_s} \times k'_i \times 1000$$

where

- $w_i$  is the mass fraction of fatty acid  $i$  in the fat sample, in grams per kilogram;
- $A_{iSR}$  is the area under the peak corresponding to fatty acid  $i$  in the fat sample with added internal standard, in area units;
- $A_{rSR}$  is the corrected area under the peak of the internal standard in the test portion with added internal standard, in area units;
- $m_r$  is the mass of the internal standard added to the test portion of the fat sample, in grams;
- $m_s$  is the mass of the test portion of the fat sample, in grams;
- $k'_i$  is the relative calibration factor of fatty acid  $i$ .

Express the results to the nearest 1 g/kg.

#### 9.5 Calculation of elutable material

Calculate the content of elutable material by summation of the values  $w_i$  of all the individual fatty acids.

#### 9.6 Calculation of fatty acids in fat-containing material

Calculate the content of individual fatty acids by multiplying the content of the fatty acid in the fat by the fat content in the material.

### 10 Precision

#### 10.1 General

The precision of the method was established in 1999 by interlaboratory tests carried out in accordance with ISO 5725 [1]. Details of the tests are given in Annex A. The values derived from these tests may not be applicable to concentration ranges and matrices other than those given.

#### 10.2 Repeatability

The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will in not more than 5 % of cases exceed the repeatability limit  $r$  given in or derived from Table 1.

Table 1 — Repeatability limit ( $r$ ) and reproducibility limit ( $R$ )

Sample/fatty acid	$r$ g/kg	$R$ g/kg
<b>Category B (hydrolysis necessary)</b>		
C16:0 (Palmitic acid; hexadecanoic acid)	9	30
C18:1 (Oleic acid; <i>cis</i> -9-octadecenoic acid) (content < 200 g/kg)	3	10
C18:1 (Oleic acid; <i>cis</i> -9-octadecenoic acid) (content > 200 g/kg)	22	38
<b>Category A (hydrolysis not necessary)</b>		
C16:0 (Palmitic acid; hexadecanoic acid)	8	15
C18:1 (Oleic acid; <i>cis</i> -9-octadecenoic acid) (content < 200 g/kg)	4	15
C18:1 (Oleic acid; <i>cis</i> -9-octadecenoic acid) (content > 200 g/kg)	9	40

### 10.3 Reproducibility

The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories by different operators using different equipment, will in not more than 5 % of cases exceed the reproducibility limit  $R$  given in or derived from Table 1.

## 11 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 used, 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);
- the test result obtained, or the two test results obtained if the repeatability has been checked.

## Annex A (informative)

### Results of an interlaboratory test

The precision of the method was established by an interlaboratory test organized by NEN in 2001 and carried out in accordance with ISO 5725<sup>1)</sup>. In the test, 11 laboratories participated. However four laboratories had no experience with the method and were not able to deliver data which was asked for. Samples were investigated of crude fish oil, used frying oil, coconut oil, fat extracted from coconut expeller and fat extracted from meat- and bone-meal.

Tables A.1 to A.3 give a summary of the statistical results of the tests.

NOTE More detailed information is given in document ISO/TC 34/SC 10 N 880.

**Table A.1 — Precision data for sum of fatty acids <sup>a</sup>**

Parameter	Samples <sup>b</sup>				
	1	2	3	4	5
Number of laboratories retained after eliminating outliers	6	6	6	6	6
Mean fatty acid content, g/kg	787	851	868	830	786
Repeatability standard deviation ( $s_r$ ), g/kg	6,0	10,1	15,8	13,2	58,1
Repeatability coefficient of variation, %	0,8	1,2	1,8	1,6	2,6
Repeatability limit ( $r$ ) [ $r = 2,8 s_r$ ], g/kg	16,7	28,3	44,2	36,9	58,1
Reproducibility standard deviation ( $s_R$ ), g/kg	52,7	42,0	34,4	26,2	34,4
Reproducibility coefficient of variation, %	6,7	4,9	4,0	3,2	4,4
Reproducibility limit ( $R$ ) [ $R = 2,8 s_R$ ], g/kg	147,0	118,0	96,2	73,3	96,2
<sup>a</sup> Sum of all peaks in the chromatogram identified as fatty acids. <sup>b</sup> Sample 1: crude fish oil Sample 2: used frying oil Sample 3: coconut oil Sample 4: fat extracted from coconut expeller Sample 5: fat extracted from meat- and bonemeal					

1) ISO5725:1986 (now withdrawn) was used to obtain the precision data.

Table A.2 — Precision data for palmitic acid (hexadecanoic acid)

Parameter	Sample <sup>a</sup>						
	1	2	3	4	5	6	7
Number of laboratories retained after eliminating outliers	7	7	7	7	7		
Mean fatty acid content, g/kg	149	180	82	64	201		
Repeatability standard deviation ( $s_r$ ), g/kg	1,6	2,8	1,3	3,1	3,2		
Repeatability coefficient of variation, %	1,1	1,6	1,6	3,7	1,6		
Repeatability limit ( $r$ ) [ $r = 2,8 s_r$ ], g/kg	4,5	7,9	3,5	8,6	9,0		
Reproducibility standard deviation ( $s_R$ ), g/kg	3,1	5,9	2,9	7,0	10,7		
Reproducibility coefficient of variation, %	2,1	3,3	3,6	8,4	5,3		
Reproducibility limit ( $R$ ) [ $R = 2,8 s_R$ ], g/kg	8,6	16,6	8,2	19,7	29,9		
<sup>a</sup> Sample 1: crude fish oil Sample 2: used frying oil Sample 3: coconut oil Sample 4: fat extracted from coconut expeller Sample 5: fat extracted from meat- and bonemeal							

Table A.3 — Precision data for oleic acid (*cis*-9-octadecenoic acid)

Parameter	Sample <sup>a</sup>						
	1	2	3	4	5	6	7
Number of laboratories retained after eliminating outliers	6	5	6	5	6		
Mean fatty acid content, g/kg	146	329	59	56	282		
Repeatability standard deviation ( $s_r$ ), g/kg	1,1	3,1	1,2	1,1	7,9		
Repeatability coefficient of variation, %	0,8	0,9	2,0	1,9	2,8		
Repeatability limit ( $r$ ) [ $r = 2,8 s_r$ ], g/kg	3,2	8,7	3,3	3,0	22,2		
Reproducibility standard deviation ( $s_R$ ), g/kg	6,2	14,1	3,2	2,2	13,7		
Reproducibility coefficient of variation, %	4,3	4,3	5,5	4,0	4,9		
Reproducibility limit ( $R$ ) [ $R = 2,8 s_R$ ], g/kg	17,4	39,4	9,1	6,3	38,4		
<sup>a</sup> Sample 1: crude fish oil Sample 2: used frying oil Sample 3: coconut oil Sample 4: fat extracted from coconut expeller Sample 5: fat extracted from meat- and bonemeal							

## Bibliography

- [1] ISO 5725:1986, *Precision of test methods — Determination of repeatability and reproducibility for a standard test method by inter-laboratory tests*
- [2] ISO 5725-1:1994, *Accuracy (trueness and precision) of measurement methods and results — Part 1: General principles and definitions*
- [3] ISO 5725-2:1994, *Accuracy (trueness and precision) of measurement methods and results — Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method*
- [4] ISO 6492, *Animal feeding stuffs — Determination of fat content*
- [5] ISO 6497, *Animal feeding stuffs — Sampling*
- [6] ISO 6498, *Animal feeding stuffs — Preparation of test samples*

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