

SRI LANKA STANDARD 313 PART 4/ SECTION 2: 2017
(ISO 12966-1: 2014)
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METHODS FOR
ANALYSIS OF ANIMAL AND VEGETABLE
FATS AND OILS
PART 4 - DETERMINATION OF PRINCIPLE
CONSTITUENTS AND NATURAL CONSTITUENTS
Section 2: Guidelines on modern gas chromatography of fatty acid
methyl esters
(Third Revision)

SRI LANKA STANDARDS INSTITUTION

Sri Lanka Standard
METHODS FOR ANALYSIS OF ANIMAL AND VEGETABLE FATS AND OILS
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CONSTITUENTS

Section 2: Guidelines on modern gas chromatography of fatty acid methyl esters
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SLS 313 Part 4/ Section 2: 2017
(ISO 12966-1: 2014)

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Sri Lanka Standard
METHODS FOR ANALYSIS OF ANIMAL AND VEGETABLE FATS AND OILS
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CONSTITUENTS
Section 2: Guidelines on modern gas chromatography of fatty acid methyl esters
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NATIONAL FOREWORD

This Sri Lanka Standard was approved by the Sectoral Committee on Food Products and was authorized for adoption and publication as a Sri Lanka Standard by the Council of the Sri Lanka Standards Institution on 2017-12-04.

This Standard was first published in 1976 and subsequently revised in 1993 and in 2010. The Standard prescribes the general methods for analysis of animal and vegetable fats and oils. Considering the experience gained during the years on the subject of testing animal and vegetable fats and oils, it was decided to revise the Standard with a view of providing guidelines on modern gas chromatography of fatty acid methyl esters and also of cancelling and replacing **SLS 313: Part 4 – Section 2: 2010**.

In order to accommodate large number of test methods within the scope of one Standard, this Standard is published in four parts covering different characteristics as indicated below.

- Part 1 Determination of physical characteristic
- Part 2 Determination of chemical characteristics
- Part 3 Determination of foreign substances and parameters affecting quality and stability
- Part 4 Determination of principal constituents and natural constituents

Part 4 of the Standard consists of several sections.

Section 2 of this Standard is identical with **ISO 12966-1: 2014** Animal and vegetable fats and oils – Gas chromatography of fatty acid methyl esters – Guidelines on modern gas chromatography of fatty acid methyl esters published by the International Organization for Standardization (ISO).

Terminology and Conventions:

The text of the International Standard has been accepted as suitable for publication, without deviation, as a Sri Lanka Standard. However, certain terminology and conventions are not identical with those used in Sri Lanka Standards. Attention is therefore drawn to the following:

- a) Wherever the words “International Standard” appear referring to this Standard should be interpreted as “Sri Lanka Standard”.
- b) The comma has been used throughout as a decimal marker. In Sri Lanka Standards it is the current practice to use the full point on the base line as the decimal marker.
- c) Wherever page numbers are quoted, they are ISO page numbers.

The test temperature adopted in Sri Lanka is 27 ± 2 °C and relative humidity 65 ± 5 per cent is recommended as per **ISO 554: 1976** Standard atmospheres for conditioning and/ or testing - Specifications.

Cross References

International Standard

Corresponding Sri Lanka Standard

ISO 12966-2, Animal and vegetable fats and oils – Gas chromatography of fatty acid methyl esters – Part 2: Preparation of methyl of fatty acids.

SLS 313: part 4/ Section1, Methods for analysis of animal and vegetable fats and oils: Determination of principle constituents and natural constituents/ preparation of methyl esters of fatty acids

ISO 12966-3, Animal and vegetable fats and oils – Gas chromatography of fatty acid methyl esters – Part 3: Preparation of methyl esters using trimethylsulfonium hydroxide (TMSH).

SLS 313: Part1/ Section 9, Methods for analysis of animal and vegetable fats and oils: Determination of principle constituents and natural constituents/ Gas chromatography of fatty acid methyl esters - preparation of methyl esters using trimethylsulfonium hydroxide (TMSH)

ISO 12966-4, Animal and vegetable fats and oils – Determination of methyl esters of fatty acids – Part 4: Capillary gas chromatographic method.

SLS 313: Part 4/ Section 10, Methods for analysis of animal and vegetable fats and oils: Determination of principle constituents and natural constituents/ Gas chromatography of fatty acid methyl esters - Determination by capillary gas chromatography

**Animal and vegetable fats and oils —
Gas chromatography of fatty acid
methyl esters —**

Part 1:
**Guidelines on modern gas
chromatography of fatty acid methyl
esters**

*Corps gras d'origines animale et végétale — Chromatographie en
phase gazeuse des esters méthyliques d'acides gras —*

*Partie 1: Lignes directrices relatives à la chromatographie en phase
gazeuse moderne des esters méthyliques d'acides gras*





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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.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the WTO principles in the Technical Barriers to Trade (TBT) see the following URL: Foreword - Supplementary information

The committee responsible for this document is ISO/TC 34, *Food products*, Subcommittee SC 11, *Animal and vegetable fats and oils*.

This first edition of ISO 12966-1, together with ISO 12966-4, cancels and replaces ISO 5508:1990 and ISO 15304:2002 which have been technically revised.

ISO 12966 consists of the following parts, under the general title *Animal and vegetable fats and oils — Gas chromatography of fatty acid methyl esters*:

- *Part 1: Guidelines on modern gas chromatography of fatty acid methyl esters*
- *Part 2: Preparation of methyl esters of fatty acids*
- *Part 3: Preparation of methyl esters using trimethylsulfonium hydroxide (TMSH)*
- *Part 4: Capillary gas chromatographic method*

Introduction

This part of ISO 12966 is one of a suite of four International Standards for the preparation and determination of fatty acid methyl esters by gas chromatography in animal and vegetable fats and oils. ISO 12966 (all parts) is applicable to crude, refined, partially hydrogenated or fully hydrogenated fats, oils and fatty acids derived from animal and vegetable sources.

ISO 12966 (all parts) is not suitable for the analysis of dairy, ruminant fats and oils (including milk and milk products or fat coming from milk and milk products), or products supplemented with conjugated linoleic acid (CLA). Furthermore it is not intended to be applied to polymerized and oxidized fats and oils.

This part of ISO 12966 is a guideline to the modern gas chromatography of fatty acid methyl esters, while ISO 12966-2 and ISO 12966-3 cover the preparation of fatty acid methyl esters by different methods. In ISO 12966-4, the conditions for the analysis of fatty acid methyl esters by capillary gas chromatography are given.

This suite of International Standards replaces the following International Standards:

- ISO 5508:1990 is replaced by ISO 12966-1 and ISO 12966-4
- ISO 15304:2002 is replaced by ISO 12966-1 and ISO 12966-4
- ISO 5509:2000 is replaced by ISO 12966-2 and ISO 12966-3

Animal and vegetable fats and oils — Gas chromatography of fatty acid methyl esters —

Part 1: Guidelines on modern gas chromatography of fatty acid methyl esters

1 Scope

This part of ISO 12966 gives an overview of the gas chromatographic determination of fatty acids, free and bound, in animal and vegetable fats and oils following their conversion to fatty acid methyl esters (FAMES).

The qualitative and quantitative determination of the composition of fatty acids by gas liquid chromatography (GLC) is a widely used application in lipid analysis. It is used for the characterization of fats and oils, or fatty foodstuffs after the extraction of the oil from the matrix. The bound fatty acids of the triacylglycerols (TAGs) and, depending on the esterification method, the free fatty acids (FFA) and other lipids, are converted to fatty acid methyl esters (FAMES), which are determined by capillary gas chromatography. Depending on the number of different fatty acids (theoretically more than 50 different fatty acids can be present) capillary columns with a length of 10 m to 100 m are used for a separation.

The GLC of FAMES is applicable to all natural and synthetic mixtures of tri-, di- and monoacylglycerols, to fatty acid esters, free fatty acids, soaps and other fatty compounds. With this suite of standards, FAMES from C4 to C26 can be determined, including saturated fatty acid methyl esters, *cis*- and *trans*-monounsaturated fatty acid methyl esters, and *cis*- and *trans*-polyunsaturated fatty acid methyl esters.

For the determination of short chain fatty acids, isopropyl and butyl esters are often used so as to avoid interferences with the solvent peak and in order to reduce differences in detector responses.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 12966-2, *Animal and vegetable fats and oils — Gas chromatography of fatty acid methyl esters — Part 2: Preparation of methyl esters of fatty acids*

ISO 12966-3, *Animal and vegetable fats and oils — Gas chromatography of fatty acid methyl esters — Part 3: Preparation of methyl esters using trimethylsulfonium hydroxide (TMSH)*

ISO 12966-4:—¹⁾, *Animal and vegetable fats and oils — Determination of methyl esters of fatty acids — Part 4: Capillary gas chromatographic method*

3 Principle

Gas liquid chromatography (GLC) is used for the qualitative and quantitative analysis of FAMES. The FAMES are prepared in accordance with ISO 12966-2 or ISO 12966-3 and the dissolved FAMES are then injected into and vaporized within the injector. The separation of FAMES is achieved on analytical

1) Under preparation. To be published.

columns of different polarity and lengths. For the detection of the FAMES, a flame ionization detector (FID) is used.

In the gas chromatography of FAMES with FID, hydrogen should be used as the carrier gas (mobile phase). For MSD (mass selective detector) applications helium shall be used. The separation can be done in a shorter time with sharper peaks by using hydrogen. The stationary phase is a microscopic layer of a thin liquid film on an inert solid surface, made of steel, glass or fused silica.

The volatilized compounds being analysed interact, during their passage through the capillary tubing, with the stationary phase coating the inner surface of the column. Due to this different interaction of different compounds, they exit the column, or elute, at a different time, which is called the retention time of the compound at a given set of analysis parameters. The comparison of retention times is used for the identification of the different compounds.

CAUTION — When a mass selective detector is used, special care shall be taken in order to achieve quantitative results due to differences in fragmentation patterns of individual FAMES. Also, for mass spectrometry, derivatives other than the FAMES discussed in this part of ISO 12966 are used, as for example, picolinyl esters or dimethyl oxazolidines and others.

4 Preparation of FAME

The preparation of the fatty acid methyl esters shall be carried out in accordance with ISO 12966-2 and ISO 12966-3. An overview is given in [Table 1](#).

Table 1 — Overview on the different esterification methods

Method	Principle	GC-Injector	Note	–
ISO 12966-2, Subclause 4.2	Rapid transmethylation method under alkali-catalysed conditions with KOH FFA are not converted to FAME Danger of soap formation	On-column Split/ Splitless	Applicable to fats and oils containing fatty acids down to butanoic (C4:0) Transesterification at room temperature, no solvent evaporation necessary	Also for medium chain triglycerides (MCTs) and short chain fatty acids, formation of artefacts insignificant. Internal standard for butanoic/hexanoic acid determination Short chain FAMES are easily lost during saline/solvent partitioning.
ISO 12966-2, Subclause 4.3	General transmethylation/methylation under sequential alkaline and acid condition (NaOCH ₃) FFA are converted to FAME	On-column Split/ Splitless	Applicable to fats and oils Not recommended for lauric oils	Short chain FAMES are easily lost during reflux and saline / solvent partitioning
ISO 12966-2, Subclause 4.4	Boron trifluoride (BF ₃) transmethylation/methylation Reagent is very toxic. Use only in closed vials and fume cupboards Formation of artefacts possible	On-column Split/ Splitless	14 % methanolic solution of BF ₃ , shall be bought and not prepared due to toxicity. The solution has a limited shelf life (ageing)	Applicable to fish oils. Not applicable to compounds with secondary oxygen groups.

Table 1 (continued)

Method	Principle	GC-Injector	Note	-
ISO 12966-2, Subclause 4.5	Methanolic sulphuric acid or hydrochloric acid Formation of artefacts possible Evaporation necessary, not for epoxy fatty acids	On-column Split / Splitless	Reagent must be prepared; esterification under boiling conditions in a sealed ampoule for 3h	Also for samples with higher content of free fatty acids
ISO 12966-3	Trimethylsulfonium hydroxide (TMSH) Disturbing peaks of by-products of the reaction occur in chromatogram close to the signals of the short chain FAME	Only split injection possible	TMSH-solution is available as ready for use solution	Very quick method for C4 to C26, Esterification of free fatty acids is about 80 %, formation of small amounts of <i>trans</i> -FA.

5 Columns

At the time of publication of this part of ISO 12966, for the separation of FAMES, wall coated open tubular (WCOT) capillaries are used as they offer a number of advantages over a packed column. This includes vastly improved separations with higher resolution, reduced time of analysis, smaller sample size and higher sensitivities. All this is enabled by the possibility of using very long columns with a large number of theoretical plates.

Sample capacity increases with the column diameter, smaller diameters giving greater efficiency and better resolutions. Therefore for complex samples columns with small diameters and small sample capacity are used. Common available internal diameters (ID) are 0,1 mm (fast GC columns) to 0,53 mm (wide bore column), film thickness is between 0,1 µm and 0,3 µm. The column diameter shall suit the type of sample inlet system: 0,20 mm, 0,25 mm or 0,32 mm ID column are for split and splitless injection systems, 0,32 mm ID for splitless and on-column injections, and 0,53 mm ID for direct injection systems. Retention and sample capacity increase with increasing film thickness, at the same time the column efficiency decreases. Film thickness is inversely related to plate number, but directly proportional to the time of analysis. This also means that a greater film thickness gives greater retention, which requires a higher oven temperature in isothermal conditions.

The length of the columns is 10 m to 100 m depending on the required resolution and separation problem. With short columns limited, but fast, information can be obtained, in process control, etc. Different types of capillary columns with non-polar, polar, and highly polar stationary phases are used for the separation of FAMES. The elution order for columns with different polarity are shown in ISO 12966-4 (see Annex B and Annex C).

Fused silica columns coated with highly polar stationary phases of cyanoalkyl polysiloxane are used for the analysis of samples with complex mixtures of geometrical and positional isomers of polyunsaturated fatty acids (PUFA). The main advantage of these high-polar phases compared to non-polar phases is their high-resolution capability of unsaturated FAME, especially for the separation of *cis* and *trans* FA isomers. However, the polarity of this column shows less thermal stability compared to other stationary phases.

Column length is a significant factor for the separation of FAMES, especially if as mentioned in this clause, the separation of isomers is required. A longer column will provide a better resolution compared with a shorter column, nevertheless there are some practical limits for increasing the column length. Doubling column length (e.g. 30 m to 60 m) will increase the resolution by a factor of $\sqrt{2} = 1,41$, which is 40 % only. At the same time, the time of analysis will increase as well as the head pressure. Shorter columns are a compromise between speed and resolution. However, the separation of geometrical and positional isomers of FAMES requires in some cases a 100 m column. The use of 100 m, 0,20 mm or 0,25 mm ID, 0,20 µm film thickness columns with a highly polar biscyanopropyl (SP-2560) or a highly substituted cyanopropyl stationary phase (CP-Sil 88) is recommended, as the separation capacity of these columns

is sufficient to separate most C18:1 *trans*- and *cis*-isomers. Some 50 m or 60 m long columns may also achieve this separation mostly for vegetable oils. Other types of columns with different cyanopropyl polysilphenylene-siloxanes (BPX70, DB-23, HP-23, Rtx-2330, SP-2330, SP-2380, SLB-IL111, etc.) may also be used, but a change in the elution order is possible. The AOCS method Ce 1h-05 (2009) recommends a 100 m SP-2560 or CP-Sil 88 capillary columns for the determination of *cis*-, *trans*-, and unsaturated FAMES in vegetable or non-ruminant animal oils. For the determination of FAMES in marine and other oils containing long-chain PUFAs, the AOCS method Ce 1i-07 (2009) recommends a highly polar Supelcowax 10 capillary column with a polyethylene glycol phase; and AOCS method Ce 1b-89 (2009) uses a Omegawax for the determination of long-chain omega-3 FAMES.²⁾

The stationary phase of all these columns is a thin film on the inner surface of capillary tube, which is made of a special quartz glass, coated with polyimide for stabilization (fused silica). Also, capillary columns made of steel, which are more robust, or glass are acceptable.

NOTE 1 The use of packed columns is no longer “state of the art”, but can be used for special applications.

The type of stationary phase is important for the selectivity of the column, resolution and separation. The more polar it is, the more unsaturated fatty acids are retained.

Trans fatty acids have a behaviour between those of saturated and *cis* unsaturated fatty acids. On non-polar columns, unsaturated fatty acids elute before the corresponding saturated fatty acid.

NOTE 2 The elution order also depends on the temperature of the column. The elution pattern for some FAME is changeable using different oven temperature programs.

If the analysed fats and oils do contain polymerized and/or oxidized fatty acids, these are not all converted to FAMES. Polymerized FAME are not volatile at recommended analysis conditions and will decompose in the injector. This can lead to unexpected losses of FAME during the following analyses.

6 GLC of FAMES

For the qualitative and quantitative determination of FAMES ISO 12966-4 shall be applied. Depending on the required resolution and separation, isothermal or temperature programmed techniques for columns with different polarity and length are used.

7 Evaluation of the chromatograms

7.1 Peak area and area per cent

Common, non-lauric, fats and oils are composed mainly of fatty acids with a chain length from 16 to 22 carbon atoms. In this case, the use of response or correction factors may not be necessary as the theoretical FID signal from these FAMES is close to the peak area of the FAME. However, the instrumental method should always be checked against certified and appropriate reference standards to determine the actual/instrumental response factors. If these response factors differ significantly from 1 or higher-precision results are required, the instrumental response factors should be applied.

For coconut, palm kernel, other lauric oils and fish oils instrumental response factors shall always be applied; otherwise, serious errors will result.

Theoretical FID correction factors are given in Annex A of ISO 12966-4:—.

7.2 Evaluation by means of an internal standard or correction factors

In certain analyses, for instance if not all of the FAMES are quantified, the use of an internal standard is recommended. But also for fats and oils with short chain fatty acids, and unusual fatty acids, all

2) Supelcowax 10 and Omegawax are trade names of products from different suppliers. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

components should be calibrated by means of an internal standard and correction factors, with reference e.g. to palmitic, stearic or heneicosanoic fatty acid methyl esters or triacylglycerols composed of these standard fatty acids.

In some cases, the use of a pure triacylglycerol as an internal standard is recommended. This internal standard is added prior to the preparation of the FAMES.

8 Test report

The test report for FAME analysis shall specify the following:

- a) the test method used, together with reference to this part of ISO 12966, i.e. ISO 12966-1;
- b) all information necessary for the complete identification of the sample;
- c) the sampling method used, if known;
- d) all operating details not specified in this part of ISO 12966, or regarded as optional;
- e) details of any incidents which might have influenced the test result(s);
- f) the test result(s) obtained; or if the repeatability has been checked, the final quoted result obtained.

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