DRAFT INTERNATIONAL STANDARD
ISO/DIS 12966-1

ISO/TC **34**/SC **11** Secretariat: **BSI**

Voting begins on: **2013-09-26** Voting terminates on: **2014-02-16**

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

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

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

Partie 1: Directive de chromatographie en phase gazeuse moderne des esters méthyliques d'acides gras

[Revision of second edition (ISO 5508:1990) and first edition ISO 15304:2002]

ICS: 67.200.10

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ISO/CEN PARALLEL PROCESSING

This draft has been developed within the International Organization for Standardization (ISO), and processed under the **ISO lead** mode of collaboration as defined in the Vienna Agreement.

This draft is hereby submitted to the ISO member bodies and to the CEN member bodies for a parallel five month enquiry.

Should this draft be accepted, a final draft, established on the basis of comments received, will be submitted to a parallel two-month approval vote in ISO and formal vote in CEN.

To expedite distribution, this document is circulated as received from the committee secretariat. ISO Central Secretariat work of editing and text composition will be undertaken at publication stage.

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Reference number
ISO/DIS 12966-1:2013(E)

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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.

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 12966-1 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 11, *Animal and vegetable fats and oils*.

This second/third/... edition cancels and replaces the first/second/... edition (), [clause(s) / subclause(s) / table(s) / figure(s) / annex(es)] of which [has / 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 methy esters*

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Introduction

This International Standard is part 1 of a suite of four standards for the preparation and determination of fatty acid methyl esters by gas chromatography in animal and vegetable fats and oils, fat spreads and in fats and oils isolated from foodstuffs. The standards are not applicable to polymerized and oxidized fats or fatty acids.

Part 1 is a guideline to the modern gas chromatography of fatty acid methyl esters, while parts 2 and 3 cover the preparation of fatty methyl esters by different methods. In part 4 of this International Standard, the conditions for the analysis of fatty acid methyl esters by GLC are given.

This suite of standards replaces the following standards:

— ISO 5508:1990 is replaced by ISO 12996-1 and ISO 12996-4

— ISO 15304:2002 is replaced by ISO 12996-4

— ISO 5509:2000 is replaced by ISO 12996-2 and ISO 12996-3

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Animal and vegetable fats and oils — Gas chromatography of fatty acid methyl esters — Part 1: Guidelines on modern gas chromatography of fatty acid methy esters

1 Scope

This International Standard 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 characterisation 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 meters to 100 meters 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 ester 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, *Animal and vegetable fats and oils — Gas chromatography of fatty acid methyl esters — Part 4: Determination of cis-, trans-, saturated, mono- and polyunsaturated fatty acids in vegetable or non-ruminant oils and fats*

3 Principle

Gas liquid chromatography (GLC) is used for the qualitative and quantitative analysis of FAMEs. The FAMEs are prepared according to ISO 12996 Part 2 or 3 and the dissolved FAMEs are then injected into and vaporized within the injector. The separation of FAMEs is achieved on analytical columns of different polarity and lengths. For the detection of the FAMEs, a Flame Ionisation Detector (FID) is used.

In the gas chromatography of FAMEs with FID hydrogen shall be used as the carrier gas (mobile phase), for MSD applications helium shall be used. The separation can be done in a shorter time with sharper peaks by

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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 volatilised 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.

NOTE When a mass selective detector is used, special care must 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 here 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 according to part 2 and 3 of ISO 12966. An overview is given in table 1

Table 1 — Overview on the different esterification methods

Method	Principle	GC-Injector	Note	
ISO 12966-2 Paragraph 4.2	Rapid transmethylation method under alkali-catalyzed 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 Paragraph 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 Paragraph 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 or milk fats, milk fat containing fats and waxes
ISO 12966-2 Paragraph 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

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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 trans-FA.

5 Columns

Nowadays, for the separation of FAMEs, wall coated open tubular (WCOT) capillaries are used as they offers 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 give greater efficiency and better resolutions. Therefore for complex samples columns with small diameters and small sample capacity are used. Common available internal diameters 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 i.d. column are for split and splitless injection systems, 0,32 mm i.d. for splitless and on-column injections, and 0,53 mm i.d. 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.

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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, e.g. 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, esp. if as mentioned above 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 SP2560 or CPSil88 as the stationary phase 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 (e.g. 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 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.

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 stabilisation (fused silica). Also, capillary columns made of steel, which are more robust, or glass are acceptable.

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