
**Animal and vegetable fats and oils —
Determination of fatty-acid-bound
chloropropanediols (MCPDs) and
glycidol by GC/MS —**

Part 4:

**Method using fast alkaline
transesterification and measurement
for 2-MCPD, 3-MCPD and glycidol by
GC-MS/MS**

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*Corps gras d'origines animale et végétale — Détermination des
esters de chloropropanediols (MCPD) et d'acides gras et des esters de
glycidol et d'acides gras par CPG/SM —*

*Partie 4: Méthode par transestérification alcaline rapide et mesure
pour le 2-MCPD, le 3-MCPD et le glycidol par CPG-SM/SM*

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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 of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 11, *Animal and vegetable fats and oils*, in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 307, *Oilseeds, vegetable and animal fats and oils and their by-products – Methods of sampling and analysis*, in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

A list of all parts in the ISO 18363 series can be found on the ISO website.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

The ISO 18363 series is a family of International Standards which can be used for the determination of ester-bound MCPD and glycidol. This introduction describes the methods specified in the different parts so that the analyst can decide which methods are suitable for application. The detailed application of each method is contained within the scope of the individual method.

ISO 18363-1 is a differential method equivalent to the DGF standard C-VI 18 (10) and identical to AOCs Official Method Cd 29c-13. In brief, it is based on a fast alkaline catalysed release of 3-MCPD and glycidol from the ester derivatives. Glycidol is subsequently converted into induced 3-MCPD. It consists of two parts. The first part (A) allows the determination of the sum of ester-bound 3-MCPD and ester-bound glycidol, whereas the second part (B) determines ester-bound 3-MCPD only. Both assays are based on the release of the target analytes 3-MCPD and glycidol from the ester-bound form by an alkaline-catalysed alcoholysis carried out at room temperature. In part A, an acidified sodium chloride solution is used to stop the reaction and subsequently convert the glycidol into induced 3-MCPD. Thus, 3-MCPD and glycidol become indistinguishable in part A. In part B, the reaction stop is achieved by the addition of an acidified chloride-free salt solution which also prevents the conversion of glycidol into induced MCPD. Consequently, part B allows the determination of the genuine 3-MCPD content. Finally, the glycidol content of the sample is proportional to the difference of both assays (A – B) and can be calculated when the transformation ratio from glycidol to 3-MCPD has been determined. ISO 18363-1 is applicable to the fast determination of ester-bound 3-MCPD and glycidol in refined and non-refined vegetable oils and fats. ISO 18363-1 can also apply to animal fats and used frying oils and fats, but a validation study must be undertaken before the analysis of these matrices. Any free analytes within the sample would be included in the results, but the document does not allow the distinction between free and bound analytes. However, as of publication, research has not shown any evidence of a free analyte content as high as the esterified analyte content in refined vegetable oils and fats. In principle, ISO 18363-1 can also be modified in such a way that the determination of 2-MCPD is feasible, but again a validation study must be undertaken before the analysis of this analyte.

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ISO 18363-2 represents the AOCs Official Method Cd 29b-13. In brief, it is based on a slow alkaline release of MCPD and glycidol from the ester derivatives. Glycidol is subsequently converted into 3-MBPD. ISO 18363-2 consists of two sample preparations that differ in the use of internal standards. Both preparations are used for the determination of ester-bound 2-MCPD and 3-MCPD. In part A, a preliminary result for ester-bound glycidol is determined. Because the 3-MCPD present in the sample is converted to some minor extent into induced glycidol by the sample preparation, part B serves to quantify this amount of induced glycidol that is subsequently subtracted from the preliminary glycidol result of part A. By the use of isotopically labelled free MCPD isomers in assay A and isotopically labelled ester-bound 2-MCPD and 3-MCPD in part B, the efficiency of ester cleavage can be monitored. Both assays, A and B, are based on the release of the target analytes 2-MCPD, 3-MCPD, and glycidol from the ester-bound form by a slow alkaline catalysed alcoholysis in the cold. In both sample preparations, the reaction is stopped by the addition of an acidified concentrated sodium bromide solution so as to convert the unstable and volatile glycidol into 3-MBPD, which shows comparable properties to 3-MCPD with regard to its stability and chromatographic performance. Moreover, the major excess of bromide ions prevents the undesired formation of 3-MCPD from glycidol in the case of samples which contain naturally occurring amounts of chloride. ISO 18363-2 is applicable to the determination of ester-bound 3-MCPD, 2-MCPD and glycidol in refined and unrefined vegetable oils and fats. It also applies to animal fats and used frying oils and fats, but a validation study must be undertaken before the analysis of these matrices. Any free analytes within the sample are included in the results, but the document does not allow a distinction between free and bound analytes. However, as of publication of this document, research has not shown any evidence of a free analyte content as high as the esterified analyte content in vegetable oils and fats.

ISO 18363-3 represents AOCs Official Method Cd 29a-13. In brief, it is based on the conversion of glycidyl esters into 3-MBPD esters and a slow acidic catalysed release of MCPD and MBPD from the ester derivatives. ISO 18363-3 is based on a single sample preparation in which glycidyl esters are converted into MBPD monoesters and, subsequently, the free analytes 2-MCPD, 3-MCPD and 3-MBPD are released by a slow acid-catalysed alcoholysis. The 3-MBPD represents the genuine content of bound glycidol. ISO 18363-3 is applicable to the determination of ester-bound 2-MCPD, 3-MCPD and glycidol in refined

and non-refined vegetable oils and fats. It also applies to animal fats and used frying oils and fats, but a validation study must be undertaken before the analysis of these matrices. The method is suited for the analysis of bound (esterified) analytes, but if required ISO 18363-3 can be also performed without the initial conversion of glycidyl esters. In such a setup, both free and bound 2-MCPD and 3-MCPD forms are included in the results and the amount of free analytes can be calculated as the difference between two determinations performed in both setups. However, as of publication, research has not shown any evidence of a free analyte content as high as the esterified analyte content in vegetable oils and fats.

This document specifies a rapid procedure based on fast alkaline cleavage of the MCPD and glycidyl esters. The released glycidol is subsequently converted into 3-MBPD. The pH of the fast alkaline cleavage generally causes the released MCPD to partially convert to glycidol during the cleavage of the esters, leading to overestimation of the glycidyl ester content of the sample. By adding two distinct isotopically labelled ester-bound 3-MCPD and glycidol internal standards, it is possible to quantify the amount of labelled glycidol resulting from the degradation of the released internal standard. This information can be used to correct for overestimation of the glycidyl ester induced glycidol by 3-MCPD induced glycidol. The same two internal standards are used for quantification of the bound MCPD and glycidol, requiring a single sample preparation to quantify bound 2-MCPD-, 3-MCPD- and glycidol esters. In analogue with ISO 18363-1, ISO 18363-2 and ISO 18363-3, the released MCPDs and 3-MBPD are derivatized with phenylboronic acid before GC-MS/MS analysis. In contrast to the other parts of the ISO 18363 series, this document requires GC-MS/MS instrumentation to unambiguously detect each of the (isotopically labelled) MBPDs required for correct quantification of the glycidyl ester induced glycidol. This document is applicable to the determination of ester-bound 3-MCPD, 2-MCPD and glycidol in refined and unrefined vegetable oils and fats. It also applies to animal fats and used frying oils and fats, but a validation study must be undertaken before analysis of these matrices. Any free analytes within the sample are included in the results, but the document will not allow the distinction between free and bound analytes. However, as of publication of this document, research has not shown any evidence of a free analyte content as high as the esterified analyte content in vegetable oils and fats.

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Animal and vegetable fats and oils — Determination of fatty-acid-bound chloropropanediols (MCPDs) and glycidol by GC/MS —

Part 4:

Method using fast alkaline transesterification and measurement for 2-MCPD, 3-MCPD and glycidol by GC-MS/MS

1 Scope

This document specifies a rapid procedure for the simultaneous determination of 2-MCPD esters (bound 2-MCPD), 3-MCPD esters (bound 3-MCPD) and glycidyl esters (bound glycidol) in a single assay, based on alkaline catalysed ester cleavage and derivatization of cleaved (free) analytes with phenylboronic acid (PBA) prior to GC-MS/MS analysis. Glycidyl ester overestimation is corrected by addition of an isotopic labelled ester bound 3-MCPD which allows the quantification of 3-MCPD induced glycidol during the procedure.

This method is applicable to solid and liquid fats and oils. This document also applies to animal fats and used frying oils and fats, but these matrices were not included in the validation. For all three analytes the limit of quantification (LOQ) is 0,1 mg/kg and the limit of detection (LOD) is 0,03 mg/kg.

Milk and milk products (on fat coming from milk and milk products), infant formulas, emulsifiers, free fatty acids and other fats- and oils-derived matrices are excluded from the scope of this document.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements 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, *Water for analytical laboratory use — Specification and test methods*

3 Terms and definitions

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

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

- ISO Online browsing platform: available at <https://www.iso.org/obp>
- IEC Electropedia: available at <http://www.electropedia.org/>

3.1

bound 2-MCPD

amount of 2-MCPD cleaved from its esterified (bound) forms by alkaline-catalysed transesterification according to the reference method

Note 1 to entry: The content of 2-MCPD is calculated and reported as a mass fraction, in milligrams per kilogram (mg/kg).

3.2

bound 3-MCPD

amount of 3-MCPD cleaved from its esterified (bound) forms by alkaline-catalysed transesterification according to the reference method

Note 1 to entry: The content of 3-MCPD is calculated and reported as a mass fraction, in milligrams per kilogram (mg/kg).

3.3

bound glycidol

amount of glycidol cleaved from its esterified (bound) forms by alkaline-catalysed transesterification according to the reference method

Note 1 to entry: The content of glycidol is calculated and reported as a mass fraction, in milligrams per kilogram (mg/kg).

4 Principle

The oil or fat sample is dissolved in toluene and tert-butyl-methyl-ether, and the internal standards (3-MCPD- $^{13}\text{C}_3$ diester and pentadeuterated glycidyl ester) are added. The sample is then cooled down to 10 °C before the alkaline transesterification is initiated by the addition of a sodium methoxide solution in methanol. After 12 min incubation at 10 °C, the sample mixture is acidified with an acidic solution of sodium bromide to convert the released glycidol to 3-MBPD. The fatty acid methyl esters generated during the transesterification are removed by duplicate extraction of the organic layer. Finally, the purified sample – containing cleaved (free) analytes – is derivatized with phenylboronic acid prior to GC-MS/MS analysis (see ISO 3696).

The quantification of ester bound 2-MCPD and 3-MCPD is based on the 2-MCPD/3-MCPD- $^{13}\text{C}_3$ and 3-MCPD/3-MCPD- $^{13}\text{C}_3$ signal ratio, respectively. The quantification of ester-bound glycidol is based on the 3-MBPD/3-MBPD-d5 signal ratio. The amount of 3-MBPD- $^{13}\text{C}_3$ formed after the transesterification reaction signifies the amount of released 3-MCPD- $^{13}\text{C}_3$ that has degraded to glycidol due to the conditions of the alkaline transesterification. Because no difference in degradation speed between 3-MCPD and 3-MCPD- $^{13}\text{C}_3$ has been observed, it is then used to correct for overestimation of the glycidyl ester induced glycidol caused by this degradation of 3-MCPD. Under the conditions used the 2-MCPD is considered stable and thus will not significantly contribute to possible glycidol overestimation (see ISO 5555).

This method allows the simultaneous quantification of all three analytes in a single assay.

5 Reagents

5.1 General

WARNING — Attention is drawn to the regulations which specify the handling of hazardous substances. Technical, organizational and personal safety measures shall be followed.

Unless otherwise stated analytically, pure reagents shall be used. Water shall conform to grade 3 of ISO 3696.

5.2 Standard and reference compounds

5.2.1 1,2-Dipalmitoyl-3-chloropropanediol (PP-3-MCPD), purity $\geq 95\%$.

NOTE 1,2-Dipalmitoyl-3-chloropropanediol can be substituted with 1,2-dioleoyl-3-chloropropanediol or other fatty acid diesters of 3-MCPD with similar chain length (C16 to C18 are preferred as they are the most abundant in the majority of oils or fats).

5.2.2 1,3-Distearoyl-2-chloropropanediol (SS-2-MCPD), purity $\geq 95\%$.

NOTE In analogy with the recommendations given for PP-3-MCPD, 1,3-distearoyl-2-chloropropanediol can be substituted by other fatty acid diesters of 2-MCPD with similar chain length (C16 to C18 are preferred as they are the most abundant in the majority of oils or fats).

5.2.3 Carbon-13 labelled 1,2-dipalmitoyl-3-chloropropanediol (PP-3-MCPD- $^{13}\text{C}_3$), purity $\geq 95\%$.

NOTE The same consideration applied to 1,2-dipalmitoyl-3-chloropropanediol is valid also for its carbon-13 labelled analogue, see Note in [5.2.1](#).

5.2.4 Glycidyl stearate (Gly-S), purity $\geq 98\%$.

NOTE Glycidyl stearate can be substituted by glycidyl oleate or other fatty acid esters of glycidol with similar chain length (C16 to C18 are preferred as they are the most abundant in the majority of oils or fats).

5.2.5 Pentadeuterated glycidyl stearate (Gly-S-d5), purity $\geq 98\%$.

NOTE The same consideration applied to glycidyl palmitate is valid also for its pentadeuterated analogue, see Note in [5.2.4](#).

5.3 Standard solutions

5.3.1 General

All standard solutions are prepared with toluene ([5.4.4](#)). All standards are prepared using ester-bound reference compounds ([5.2](#)). Concentrations are given in the free component equivalent concentration and shall be corrected for reference compounds ([5.2](#)) purity. For an exemplary calculation of ester-bound to free equivalent concentration conversion, see [10.2.a\)](#)

5.3.2 Stock solutions

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NOTE Stock solutions are stable for at least 12 months when stored at $-18\text{ }^\circ\text{C}$. Using an ultrasonic bath can help to ensure all standards are completely dissolved.

5.3.2.1 Calibration stock (3-MCPD: 52,7 $\mu\text{g/ml}$, glycidol: 52,2 $\mu\text{g/ml}$, 2-MCPD: 48,1 $\mu\text{g/ml}$). Weigh 14,0 mg of PP-3-MCPD ([5.2.1](#)), 12,0 mg of Gly-S ([5.2.4](#)) and 14,0 mg of SS-2-MCPD ([5.2.2](#)) in a 50 ml volumetric flask. Fill up to the mark, making sure that the standards are completely dissolved in the solvent.

5.3.2.2 Spike stock (3-MCPD: 52,7 $\mu\text{g/ml}$, glycidol: 52,2 $\mu\text{g/ml}$, 2-MCPD: 34,4 $\mu\text{g/ml}$). Weigh 14,0 mg of PP-3-MCPD ([5.2.1](#)), 12,0 mg of Gly-S ([5.2.4](#)) and 10,0 mg of SS-2-MCPD ([5.2.2](#)) in a 50 ml volumetric flask. Fill up to the mark, making sure that the standards are completely dissolved in the solvent.

5.3.2.3 PP-3-MCPD- $^{13}\text{C}_3$ stock (3-MCPD- $^{13}\text{C}_3$: 38,5 $\mu\text{g/ml}$). Weigh 20 mg of PP-3-MCPD- $^{13}\text{C}_3$ ([5.2.3](#)) in a 100 ml volumetric flask. Fill up to the mark, making sure that the standard is completely dissolved in the solvent.

5.3.2.4 Gly-S-d5 stock (Glycidol-d5: 45,8 $\mu\text{g/ml}$). Weigh 10 mg of Gly-S-d5 ([5.2.5](#)) in a 50 ml volumetric flask. Fill up to the mark, making sure that the standard is completely dissolved in the solvent.

5.3.3 Working solutions

It is advisable to freshly prepare the calibration working solutions on the day they are to be used.

The concentrations of all stock and standard solutions shall be corrected for the purity of the used standards.

NOTE The spike solution (5.3.3.4) and internal standard solution (5.3.3.5) can be stored in the refrigerator for at least three months.

5.3.3.1 Calibration working solution I (3-MCPD: 7,9 µg/ml, glycidol: 7,8 µg/ml, 2-MCPD: 7,2 µg/ml). Pipette 300 µl of the stock solution (5.3.2.1) into a 2,5 ml GC vial containing 1 700 µl of toluene (5.4.4) and homogenize using a vortex mixer.

5.3.3.2 Calibration working solution II (3-MCPD: 3,2 µg/ml, glycidol: 3,1 µg/ml, 2-MCPD: 2,9 µg/ml). Pipette 120 µl of the stock solution (5.3.2.1) into a 2,5 ml GC vial containing 1 880 µl of toluene (5.4.4) and homogenize using a vortex mixer.

5.3.3.3 Calibration working solution III (3-MCPD: 0,16 µg/ml, glycidol: 0,16 µg/ml, 2-MCPD: 0,14 µg/ml). Pipette 40 µl of the stock solution (5.3.2.1) into a 2,5 ml GC vial containing 1 960 µl of toluene (5.4.4) and homogenize using a vortex mixer.

5.3.3.4 Spike solution (3-MCPD: 1,05 µg/ml, glycidol: 1,04 µg/ml, 2-MCPD: 0,69 µg/ml). Pipette 5,0 ml of the spike stock solution (5.3.2.2) into a 250 ml volumetric flask and fill up to the mark with the solvent.

5.3.3.5 Internal standard solution (3-MCPD-¹³C₃: 1,54 µg/ml, glycidol-d5: 0,92 µg/ml). Pipette 5,0 ml of the Gly-S-d5 (5.3.2.4) and 10,0 ml of PP-3-MCPD-¹³C₃ (5.3.2.3) into a 250 ml volumetric flask and fill up to the mark with the solvent.

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5.4 Other reagents

5.4.1 **Methanol**, analytical grade.

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5.4.2 **Iso-octane**, analytical grade.

5.4.3 **Acetone**, analytical grade.

5.4.4 **Toluene**, analytical grade.

5.4.5 **Tert-butyl-methyl-ether**, analytical grade.

5.4.6 **Water**, ultra-pure.

5.4.7 **Sulfuric acid** (purity ≥ 95 %).

5.4.8 **Phenylboronic acid** (purity ≥ 97 %).

5.4.9 **Sodium bromide** (purity ≥ 99,5 %).

5.4.10 **Sodium methoxide solution in methanol** (mass fraction of 25 %).

5.4.11 **Non-thermally treated, cold pressed vegetable oil** (blank oil, see 9.4).

5.5 Reagent solutions

5.5.1 Aqueous sulfuric acid solution (25 %). Transfer 25 ml of sulfuric acid (5.4.7) to a 100 ml volumetric flask containing 50 ml of H₂O (5.4.6). Fill up to the mark with H₂O (5.4.6) and homogenize.

5.5.2 Acid aqueous solution of sodium bromide (sodium bromide 600 mg/ml, sulfuric acid volume fraction of 0,9 %). Dissolve 600 g of sodium bromide (5.4.9) into 700 ml of ultrapure water (5.4.6). Transfer the sodium bromide solution to a 1 000 ml volumetric flask containing 36 ml of sulfuric acid solution (5.5.1). Fill up to the mark with H₂O (5.4.6) and homogenize.

5.5.3 Sodium methoxide solution (0,35M). Transfer 20 ml of sodium methoxide (mass fraction of 25 %) (5.4.10) to a 250 ml volumetric flask, fill up to the mark with methanol (5.4.1) and homogenize.

NOTE The sodium methoxide solution (0,35M) can be stored in a refrigerator for at least three months.

5.5.4 Phenylboronic acid solution (saturated). Weigh 12,0 g of phenylboronic acid (5.4.8) and add 100 ml volume fraction of 5 % water (5.4.6) in acetone (5.4.3) mixture. Shake vigorously.

NOTE The phenylboronic acid does not dissolve completely in the solvent mixture. Only the supernatant is used for the derivatization step (see 8.1.11). The solution can be stored at room temperature for at least three months.

6 Apparatus

6.1 Vortex mixer.

6.2 Cooled sample tray, set to 10 °C ± 0,5 °C.

6.3 Heated sample tray, with agitator capabilities set to 80 °C ± 4,0 °C.

6.4 Ultrasonic bath.

6.5 GC-MS/MS system, with split/splitless injector and backflush option.

6.6 Fused-silica-GC-column, stationary phase 5 % diphenyl to 95 % dimethylpolysiloxane or similar polarity, length 20 m, ID 0,18 mm, film thickness 0,18 µm. Pre-column: stationary phase 5 % diphenyl to 95 % dimethylpolysiloxane or similar polarity, length 2 m, ID 0,53 mm, film thickness 0,10 µm.

The pre-column is periodically exchanged to retain good peak shape and sensitivity.

6.7 Electronic pipette, capable of pipetting volumes of 1,0 µl to 1 000 µl.

Using an electronic pipette is recommended for the sequential addition of accurate amounts of internal standard solutions or the dilution of standards for calibration.

7 Sample and storage

7.1 Sampling

Sampling is not part of this method. A recommended sampling method is given in ISO 5555.

7.2 Preparation of the test sample

Liquid samples shall be used without additional treatment. Solid or turbid fats shall be carefully melted at approximately 60 °C in a drying oven or water bath. For high-melting fats, the temperature shall be carefully increased in 10 °C steps until the melting process starts. Samples containing high amounts of water shall be dried (e.g. by anhydrous Na₂SO₄) before sampling.

Oils and fats with higher melting points > 60 °C often show solidification when incubated at 10 °C in the presence of the reaction medium (see 8.1.4). This influences the completeness of the ester cleavage as

the reaction speed decreases substantially. It is important that high melting fats form a milky solution to keep the reaction going. Samples that completely solidify typically show low signal intensities and the results are not reproducible. The internal standard signals are effective markers to spot samples that did not readily undergo the cleavage reaction. If the internal standard signals of the sample are < 50 % of the mean internal standard signals of the recovery samples, the sample preparation shall be restarted with less fat or oil.

7.3 Storage conditions

The glycidyl ester concentrations are subject to storage conditions whereas the MCPD esters are not. Room temperature (22 °C) has proven to provide the best stability for both glycidyl and MCPD esters and thus samples should be stored under such conditions. Samples cannot be stored under refrigerated conditions (4 °C) as the degradation of glycidyl esters is likely to occur over time.

8 Procedure

8.1 Test sample preparation

NOTE The procedure has been given for a single sample. A timetable for the parallel sample preparation of a batch of 20 samples for steps 8.1.5 and 8.1.6 is given in Table A.2.

8.1.1 Weigh 100 mg to 120 mg of oil or fat sample (to a precision of 0,01 mg) in a 2,5 ml GC vial.

8.1.2 Add 100 µl of toluene (5.4.4) and 200 µl of tBME (5.4.5) followed by 100 µl of internal standard working solution I (5.3.3.5) to all samples.

Steps 8.1.3 to 8.1.9 shall be executed uninterrupted to ensure correct quantification.

8.1.3 Agitate all samples using a heated agitator (80 °C, 250 r/min) for 120 s or until all fat has been melted and dissolved.

8.1.4 Homogenize for 10 s using a vortex mixer and place the vials in a cooled sample tray (10 °C). Leave them to cool for exactly 240 s.

8.1.5 Start the transesterification by adding 200 µl of sodium methoxide solution (5.5.3). Homogenize for 10 s after addition of the sodium methoxide solution (5.5.3) using a vortex mixer and place the sample back in the cooled sample tray.

8.1.6 After exactly 12 min, stop the reaction by adding 700 µl of aqueous acidified sodium bromide solution (5.5.2). Homogenize for 10 s using a vortex mixer and let the temperature stabilize at room temperature for at least 5 min for the complete conversion of all glycidol to 3-MBPD.

8.1.7 Add to all samples 300 µl of iso-octane (5.4.2) and homogenize using a vortex mixer for 10 s.

8.1.8 Agitate all samples using a heated agitator (80 °C, 400 r/min) for 270 s ± 10 s or until the (partially) solidified or jellified upper layer has been completely dissolved and homogenized (see 7.2).

8.1.9 Let the samples cool down for 3 min before extracting the organic layer, while being careful not to extract any of the water layer and discard the extract.

8.1.10 Add 600 µl of fresh iso-octane (5.4.2) and homogenize using a vortex mixer for 10 s followed by extraction of the organic layer, while being careful not to extract any of the water layer. Discard the extract.