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

Part 3: Method using acid transesterification and measurement for 2-MCPD, 3-MCPD and glycidol

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 3: Méthode par transestérification acide et mesure du 2-MCPD, du 3-MCPD et du glycidol

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Foreword

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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 document 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).

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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).

This second edition cancels and replaces the first edition (ISO 18363-3:2017), of which it constitutes a minor revision.

The main changes are as follows:

— the text of the Introduction has been revised to be consistent with ISO 18363-4:2021.

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[1] 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 each individual method.

ISO 18363-1 is a differential method equivalent to DGF standard C-VI 18 (10)[2] and identical to AOCS Official Method Cd 29c-13[3]. 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 of this document, 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.

ISO 18363-2 represents AOCS Official Method Cd 29b-13[4]. 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 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 refined vegetable oils and fats.

This document (i.e. ISO 18363-3) represents AOCS Official Method Cd 29a-13[5]. In brief, it is based on the conversion of glycidyl esters into 3-MBPD esters and a slow acid catalysed release of MCPD and MBPD from the ester derivatives. This document 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. This document 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 this document can also be 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 of this document, research has not shown any evidence of a free analyte content as high as the esterified analyte content in refined vegetable oils and fats.

ISO 18363-4 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 this document, 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, ISO 18363-4 requires GC-MS/MS instrumentation to unambiguously detect each of the (isotopically labelled) MBPDs required for correct quantification of the glycidyl ester induced glycidol. ISO 18363-4 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 ISO 18363-4 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 refined vegetable oils and fats.

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

Part 3: Method using acid transesterification and measurement for 2-MCPD, 3-MCPD and glycidol

1 Scope

This document specifies a 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 acid catalysed ester cleavage and derivatization of cleaved (free) analytes with phenylboronic acid (PBA) prior to GC/MS analysis.

This document is applicable to solid and liquid fats and oils. 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.

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 terminology databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

3.1
bound 2-MCPD
amount of 2-MCPD cleaved from its esterified (bound) forms by acid 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 acid 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 acid 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/fat sample is dissolved in tetrahydrofuran, and the internal standards (pentadeuterated 3-MCPD diester and pentadeuterated glycidyl ester) are added. During the first step of sample preparation, glycidyl esters are converted into 3-MBPD monoesters by the addition of an acidified solution of sodium bromide. Upon completion of the reaction, the organic phase, containing 2- and 3-MCPD esters and 3-MBPD esters, is separated and evaporated to dryness. In the second step the residue is dissolved in tetrahydrofuran and the acid transesterification is initiated by the addition of an acid alcoholic solution. After 16 h incubation at 40 °C, the sample mixture is neutralized and the fatty acid methyl esters generated during the transesterification are removed. Finally, the purified sample [containing cleaved (free) analytes] is derivatized with phenylboronic acid prior to GC/MS analysis.

The quantification of 2- and 3-MCPD esters (expressed as bound 2- and 3-MCPD) is based on the 2-MCPD/3-MCPD-d5 and 3-MCPD/3-MCPD-d5 signal ratio, respectively. The quantification of glycidyl esters (expressed as bound glycidol) is based on the 3-MBPD/3-MBPD-d5 signal ratio.

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

5 Reagents

WARNING — This document requires 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.

5.1 Standard and reference compounds

5.1.1 1,2-Dipalmitoyl-3-chloropropanediol (PP-3-MCPD), purity ≥ 95 % (e.g. from a supplier or synthesized from 3-MCPD and palmitoyl chloride as described by Reference [6]).

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

5.1.2 1,3-Dipalmitoyl-2-chloropropanediol (PP-2-MCPD), purity ≥ 95 % (e.g. synthesized from 2-MCPD and palmitoyl chloride as described by Reference [6]).

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

5.1.3 Pentadeuterated 1,2-dipalmitoyl-3-chloropropanediol (PP-3-MCPD-d5), purity ≥ 95 %.

NOTE The same consideration applied to 1,2-dipalmitoyl-3-chloropropanediol is valid also for its pentadeuterated analogue, see the note in 5.1.1.

5.1.4 Glycidyl palmitate (Gly-P), purity ≥ 98 %.

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

5.1.5 Pentadeuterated glycidyl palmitate (Gly-P-d5), purity ≥ 98 %.

NOTE The same consideration applied to glycidyl palmitate is valid also for its pentadeuterated analogue, see the note in 5.1.4.

5.2 Standard solutions

5.2.1 General

All standard solutions can be prepared with either toluene (5.3.5) or tetrahydrofuran (5.3.1). Toluene is preferred for standard solutions containing glycidyl esters.

5.2.2 Stock solutions (1 mg/ml)

a) Weigh 10 mg of PP-3-MCPD (5.1.1) in a 10 ml volumetric flask. Fill up to the mark, making sure that the standard is completely dissolved in the solvent.

b) Weigh 10 mg of PP-2-MCPD (5.1.2) in a 10 ml volumetric flask. Fill up to the mark, making sure that the standard is completely dissolved in the solvent.

c) Weigh 10 mg of PP-3-MCPD-d5 (5.1.3) in a 10 ml volumetric flask. Fill up to the mark, making sure that the standard is completely dissolved in the solvent.

d) Weigh 10 mg of Gly-P (5.1.4) in a 10 ml volumetric flask. Fill up to the mark, making sure that the standard is completely dissolved in the solvent.

e) Weigh 10 mg of Gly-P-d5 (5.1.5) in a 10 ml volumetric flask. Fill up to the mark, making sure that the standard is completely dissolved in the solvent.

NOTE Stock solutions are stable for at least three months when stored at −18 °C.

5.2.3 Working solutions

a) Calibration I (PP-3-MCPD, 55 µg/ml). Pipette 550 µl of the stock solution [5.2.2 a)] into a 10 ml volumetric flask and fill up to the mark with the solvent.

b) Calibration II (PP-3-MCPD, 5,5 µg/ml). Pipette 1 ml of the Calibration I solution [5.2.3 a)] into a 10 ml volumetric flask and fill up to the mark with the solvent.

c) Calibration III (PP-2-MCPD, 55 µg/ml). Pipette 550 µl of the stock solution [5.2.2 b)] into a 10 ml volumetric flask and fill up to the mark with the solvent.

d) Calibration IV (PP-2-MCPD, 5,5 µg/ml). Pipette 1 ml of the Calibration III solution [5.2.3 c)] into a 10 ml volumetric flask and fill up to the mark with the solvent.

e) Calibration V (Gly-P, 100 µg/ml). Pipette 1 ml of the stock solution [5.2.2 d)] into a 10 ml volumetric flask and fill up to the mark with the solvent.

f) Calibration VI (Gly-P, 10 µg/ml). Pipette 1 ml of the Calibration V solution [5.2.3 e)] into a 10 ml volumetric flask and fill up to the mark with the solvent.

g) Internal standard I (PP-3-MCPD-d5, 40 µg/ml). Pipette 400 µl of the stock solution [5.2.2 c)] into a 10 ml volumetric flask and fill up to the mark with the solvent.

h) Internal standard II (Gly-P-d5, 50 µg/ml). Pipette 500 µl of the stock solution [5.2.2 e)] into a 10 ml volumetric flask and fill up to the mark with the solvent.

As an alternative to the preparation of separate standard solutions for each analyte, the three [5.2.3 a), c), and e)] can be combined in one single standard solution at high concentration of all three analytes (“mixed Calibration I”). To prepare the mixed solution, pipette 550 µl of PP-3-MCPD stock solution [5.2.2 a)], 550 µl of PP-2-MCPD stock solution [5.2.2 b)], and 1 ml of Gly-P stock solution [5.2.2 d)] into a 10 ml volumetric flask

and fill up to the mark with the solvent. Also, the solutions 5.2.3 b), d), and f) can be combined in one single standard solution at low concentration of all three analytes ("mixed Calibration II"). To prepare the mixed solution, pipette 1 ml of mixed Calibration I into a 10 ml volumetric flask and fill up to the mark with the solvent.

The internal standard solutions [5.2.3 g and h)] can also be combined in a single solution ("mixed internal standard"). To prepare the mixed solution, pipette 400 µl of PP-3-MCPD-d5 [5.2.2 c)] and 500 µl of Gly-P-d5 [5.2.2 e)] into a 10 ml volumetric flask and fill up to the mark with solvent.

5.3 Other reagents

5.3.1 Tetrahydrofuran, anhydrous.

5.3.2 Methanol, analytical grade.

5.3.3 n-Heptane, analytical grade.

5.3.4 Acetone, analytical grade.

5.3.5 Toluene, analytical grade.

5.3.6 Water, ultra-pure (e.g. obtained by using a purification system).

5.3.7 Sulfuric acid, purity ≥ 95 %.

5.3.8 Sodium hydrogen carbonate, purity ≥ 99 %.

5.3.9 Sodium sulfate, purity ≥ 99 %.

5.3.10 Phenylboronic acid, purity ≥ 97 %.

5.3.11 Sodium bromide, purity ≥ 99,5 %.

5.4 Reagent solutions

5.4.1 Acid aqueous solution of sodium bromide [sodium bromide 3 mg/ml, sulfuric acid 5 % (volume fraction)]. Prepare a concentrated aqueous solution of sodium bromide by dissolving 1 g of sodium bromide (5.3.11) in 10 ml of ultra-pure water (5.3.6). Transfer 180 µl of the concentrated solution into an empty conical flask. Add 0,3 ml of sulfuric acid (5.3.7) and 5,5 ml of ultra-pure water (5.3.6). Shake vigorously.

It is advisable to freshly prepare the solution on daily basis.

5.4.2 Sodium hydrogen carbonate solution (0,6 % mass concentration). Weigh 0,6 g of sodium hydrogen carbonate (5.3.8) in a 100 ml volumetric flask and fill up to the mark with ultra-pure water (5.3.6). Use an ultrasonic bath (6.3) to ensure the complete dissolution of the reagent.

NOTE As an alternative, the solution can be prepared by dilution of the sodium hydrogen carbonate saturated solution (5.4.4).

5.4.3 Sulfuric acid/methanol solution (1,8 % volume fraction). Pipette 1,8 ml of sulfuric acid (5.3.7) in a 100 ml volumetric flask and fill up to the mark with methanol (5.3.2).

It is advisable to freshly prepare the solution on daily basis.

5.4.4 Sodium hydrogen carbonate solution (saturated). Weigh 9,6 g of sodium hydrogen carbonate (5.3.8) in a 100 ml volumetric flask and fill up to the mark with ultra-pure water (5.3.6). Use an ultrasonic bath (6.3) to ensure the dissolution of the reagent.

NOTE The saturated solution of sodium hydrogen carbonate can be substituted with a solution of the exact concentration (9 % mass concentration) to improve the robustness of the method.

5.4.5 Sodium sulfate solution (20 % mass concentration). Weigh 20 g of sodium sulfate (5.3.9) in a 100 ml volumetric flask and fill up to the mark with ultra-pure water (5.3.6). Use an ultrasonic bath (6.3) to ensure the complete dissolution of the reagent.

5.4.6 Phenylboronic acid solution (saturated). Weigh 3 g of phenylboronic acid (5.3.10) and add 12 ml of an acetone (5.3.4)/ultra-pure water (5.3.6) mixture (19/1 volume fraction). Shake vigorously.

NOTE The phenylboronic acid does not dissolve completely in the solvent mixture. Only the supernatant is used for the derivatization step (see 8.1.6).

6 Apparatus

6.1 Vortex mixer.

6.2 Oven, capable of 45 °C ± 5 °C.

6.3 Ultrasonic bath.

6.4 Evaporation unit (nitrogen).

6.5 Centrifuge.

6.6 GC/MS equipment consisting of

a) capillary gas chromatograph coupled with a quadrupole mass selective detector and data processing system, and

b) bonded, poly(dimethylsiloxane) capillary column (e.g. 30 m length x 0,25 mm i.d. x 1,0 µm film thickness).

7 Sample

7.1 Sampling

Sampling is not part of this method. A recommended sampling method is given in ISO 5555[7].

7.2 Preparation of the test sample

Liquid samples shall be used without additional treatment. Solid or turbid fats shall carefully be melted at approximately 80 °C in a drying oven or water bath. For high-melting fats, the temperature shall carefully be increased in 10 °C steps until the melting process starts.

8 Procedure

8.1 Test sample preparation

8.1.1 Weigh 100 mg to 110 mg of oil/fat (to a precision of 0,01 mg) in a screw cap glass tube. Add 50 µl of internal standard working solution I [5.2.3 g)] and II [5.2.3 h)] and 2 ml of tetrahydrofuran (5.3.1). Mix for 15 s (or until complete dissolution) by means of a vortex mixer (6.1).

The presence of water interferes with the conversion of glycidyl esters into MBPD monoesters (see 8.1.2); therefore, it is recommended to use anhydrous tetrahydrofuran (5.3.1).[8]

If the mixed internal standard solution is used (see the note in 5.2.3), 50 µl of this solution shall be added to the sample.

When a sample is suspected to contain high levels of chloride salts, an additional step of liquid/liquid extraction with water is required as described by Reference [9]. Particular attention shall be paid to discarding the water layer completely after the extraction.

8.1.2 Add 30 µl of acid aqueous solution of sodium bromide (5.4.1) to the sample, shake vigorously using a vortex mixer (6.1) and incubate the mixture at 50 °C for 15 min. Stop the reaction by the addition of 3 ml of 0,6 % aqueous solution of sodium hydrogen carbonate (5.4.2). In order to separate the oil/fat from the water phase, add 2 ml of n-heptane (5.3.3) and shake vigorously using a vortex mixer (6.1) for 15 s. After separation of the two phases, transfer the upper layer to an empty glass tube and evaporate to dryness under a nitrogen stream (for a maximum of 15 min to 20 min at 35 °C to 40 °C). Dissolve the residue in 1 ml of tetrahydrofuran (5.3.1).

Both time and temperature of reaction shall be carefully controlled to avoid either sub-optimal conversion of the glycidyl esters or ex-novo formation of 3-MBPD esters.[8]

The clear separation of the organic phase from the water phase can be difficult. Therefore, it is often necessary to centrifuge the sample (for approximately 1 min to 2 min at 250*g*).

The evaporation of the organic phase under nitrogen stream shall be carefully monitored and stopped immediately after the evaporation of the solvent. A small amount of residual solvent (<50 µl) dissolved in the sample does not affect the quality of the results and should be preferred over a prolonged exposure of the sample to a nitrogen stream.

8.1.3 Add 1,8 ml of sulfuric acid/methanol solution (5.4.3) to the sample and shake by means of a vortex mixer (6.1) for 10 s. Close the cap of the glass tube tightly and incubate the mixture at 40 °C for 16 h.

8.1.4 After the incubation period, stop the reaction by adding 0,5 ml of sodium hydrogen carbonate saturated solution (5.4.4) to the sample. Shake by means of a vortex mixer (6.1) for 10 s. Evaporate the organic solvents of the mixture under a nitrogen stream.

NOTE The removal of organic solvents enhances the sensitivity of the method. The evaporation can be stopped when, upon visual check, the volume in the test tube is about 1 ml.

8.1.5 Add 2 ml of sodium sulfate solution (5.4.5) and 2 ml of n-heptane (5.3.3). Shake by means of a vortex mixer (6.1) for 10 s. The two phases will spontaneously separate in few seconds. Discard the upper phase (that contains fatty acid methyl esters dissolved in n-heptane) and repeat the extraction with n-heptane.

During the second extraction, it is important to remove the upper organic phase completely.

8.1.6 Add 250 µl of phenylboronic acid solution (5.4.6), shake for 10 s using a vortex mixer (6.1) and incubate the mixture for 5 min in an ultrasonic bath (6.3) at room temperature.

8.1.7 Extract the phenylboronic derivatives of 2- and 3-MCPD as well as 3-MBPD by adding 1 ml of n-heptane (5.3.3), shaking for 10 s using a vortex mixer (6.1) and transferring the upper phase to an empty

test tube. Repeat the extraction with 1 ml of n-heptane and combine the two extracts. Evaporate the extracts to dryness under a nitrogen stream. Dissolve the residue in 400 µl of n-heptane by shaking the mixture for 10 s using a vortex mixer (6.1) and transfer the supernatant to an empty GC vial (a glass insert of about 250 µl of volume is typically used).

The evaporation of the extracts under nitrogen stream can be carried out at approximately 35 °C to 40 °C to facilitate the evaporation of the n-heptane, but it shall be stopped as soon as the solvent is evaporated in order to avoid losses due to the high volatility of the phenylboronic derivatives.

If the evaporation of the extracts is carried out at elevated temperature (at a maximum of 40 °C), the test tubes shall be allowed to cool down to room temperature before dissolving the residue.

NOTE During the evaporation of the extracts, the excess of phenylboronic acid that did not react with 2- and 3-MCPD, as well as 3-MBPD, crystallizes by forming a white precipitate on the walls of the test tube. The addition of 400 µl of n-heptane (final step of the sample preparation) does not lead to its complete dissolution that would otherwise interfere with the analysis.

8.2 Preparation of the calibration curve

8.2.1 Prepare nine calibration samples by pipetting 50 µl of both internal standard working solutions [5.2.3 g) and h)] and the volume of calibration solutions [5.2.3 a) to f)] indicated in Table A.1. Add 2 ml of tetrahydrofuran (5.3.1) and shake vigorously for 10 s using a vortex mixer (6.1).

NOTE The addition of 100 mg to 110 mg of blank oil (containing no 2-MCPD/3-MCPD esters and glycidyl esters) to the calibration samples can improve the robustness of the method, since the oil matrix acts as a keeper for the analytes during the evaporation step.

8.2.2 Treat the calibration samples according to the procedure used for the test samples (see 8.1).

8.3 Gas chromatography/mass spectrometry references

8.3.1 Injection volume: 1,0 µl.

8.3.2 Injection mode: pulsed splitless.

8.3.3 Injection temperature: 250 °C.

8.3.4 Carrier gas: helium, flow rate: 0,8 ml/min.

8.3.5 GC oven temperature programme: 80 °C (isothermal for 1 min), from 80 °C to 170 °C at 10 °C/min, from 170 °C to 200 °C at 3 °C/min, from 200 °C to 300 °C at 15 °C/min, 300 °C (isothermal for 15 min).

8.3.6 Mass spectrometric detector: electron-impact (EI), transfer line temperature: 300 °C, ion source temperature: 230 °C, quadrupole temperature: 150 °C, SIM mode – acquisition time window: 5 min to 20 min.

NOTE Molecular ions (*m/z* 196 and 201 for 3-MCPD and 3-MCPD-d5, respectively, and *m/z* 240 and 245 for 3-MBPD and 3-MBPD-d5, respectively) can also be used for quantification purposes; but they are not preferred because of the lower intensity of their signal, which can compromise the sensitivity.

8.3.7 Parameters for SIM mode:

a) phenylboronic derivative of 3-MCPD (*m/z*) 147 (quantifier ion), 196, 198 (qualifier ions);

b) phenylboronic derivative of 2-MCPD (*m/z*) 196 (quantifier ion), 198 (qualifier ion);

c) phenylboronic derivative of 3-MCPD-d5 (*m/z*) 150 (quantifier ion for 3-MCPD), 201 (quantifier ion for 2-MCPD), 203 (qualifier ion);

d) phenylboronic derivative of 3-MBPD (*m/z*) 147 (quantifier ion), 240 (qualifier ion); e) phenylboronic derivative of 3-MBPD-d5 (*m/z*) 150 (quantifier ion), 245 (qualifier ion).

9 Expression of results

9.1 Quantification of 3-MCPD esters

9.1.1 Prepare a calibration curve by plotting the ratio of the amount of standard (expressed as free 3-MCPD equivalent) to the amount of internal standard (expressed as free 3-MCPD-d5 equivalent) on the x-axis, against the ratio of the corresponding peak areas on the y-axis (see Figure A.1). Ions at *m/z* 147 (3-MCPD) and 150 (3-MCPD-d5) are used for quantification. Calculate the regression line as shown by Formula (1):

$$y = ax + b \tag{1}$$

where

a is the slope;

b is the y-intercept.

Confirm that the linearity is good ($R^2 > 0{,}99$) and the y-intercept preferably < |0,02| in order to achieve a good accuracy for samples at very low concentration of 3-MCPD esters.

9.1.2 Determine the concentration of 3-MCPD esters in the test sample (mg/kg) by applying Formula (2):

$$c = \frac{\left(\frac{A_a}{A_b} - b\right) \cdot m_{IS}}{a \cdot W} \tag{2}$$

where

c is the concentration of 3-MCPD esters in the test sample (expressed as bound 3-MCPD, mg/kg oil);

A_a is the area of the peak corresponding to the 3-MCPD derivative (*m/z* 147);

m_{IS} is the absolute amount (in µg, adjusted for the purity) of internal standard added to the test sample;

A_b is the area of the peak corresponding to the 3-MCPD-d5 derivative (*m/z* 150);

a is the slope of the calibration curve;

W is the weight of the sample, in g;

b is the y-intercept of the calibration curve.

9.2 Quantification of 2-MCPD esters

9.2.1 Prepare a calibration curve by plotting the ratio of the amount of standard (expressed as free 2-MCPD equivalent) to the amount of internal standard (expressed as free 3-MCPD-d5 equivalent) on the x-axis, against the ratio of the corresponding peak areas on the y-axis. Ions at *m/z* 196 (2-MCPD) and 201 (3-MCPD-d5) are used for quantification. Calculate the regression line as shown by Formula (3):

$$y = ax + b \tag{3}$$

where

a is the slope;

b is the y-intercept.

Confirm that the linearity is good (R^2 > 0,99) and the y-intercept preferably < |0,05| in order to achieve a good accuracy for samples at very low concentration of 2-MCPD esters.

9.2.2 Determine the concentration of 2-MCPD esters in the test sample (mg/kg) by applying Formula (4):

$$c = \frac{\left(\frac{A_a}{A_b} - b\right) \cdot m_{IS}}{a \cdot W} \quad (4)$$

where

c is the concentration of 2-MCPD esters in the test sample (expressed as bound 2-MCPD, mg/kg oil);

A_a is the area of the peak corresponding to the 2-MCPD derivative (m/z 196);

m_{IS} is the absolute amount (in µg, adjusted for the purity) of internal standard added to the test sample;

A_b is the area of the peak corresponding to the 3-MCPD-d5 derivative (m/z 201);

a is the slope of the calibration curve;

W is the weight of the sample, in g;

b is the y-intercept of the calibration curve.

9.3 Quantification of glycidyl esters

9.3.1 Prepare a calibration curve by plotting the ratio of the amount of standard (expressed as glycidol equivalent) to the amount of internal standard (expressed as deuterated glycidol equivalent) on the x-axis, against the ratio of the corresponding peak areas on the y-axis (see Figure A.2). Ions at m/z 147 (3-MBPD) and 150 (3-MBPD-d5) are used for the quantification. Calculate the regression line as shown by Formula (5):

$$y = ax + b \quad (5)$$

where

a is the slope;

b is the y-intercept.

Confirm that the linearity is good (R^2 > 0,99) and the y-intercept preferably < |0,02| in order to achieve a good accuracy for samples at very low concentration of glycidyl esters.

9.3.2 Determine the concentration of glycidyl esters in the test sample (mg/kg) by applying Formula (6):

$$c = \frac{\left(\frac{A_a}{A_b} - b\right) \cdot m_{IS}}{a \cdot W} \quad (6)$$

where

c is the concentration of glycidyl esters in the test sample (expressed as bound glycidol, mg/kg oil);

A_a is the area of the peak corresponding to the 3-MBPD derivative (m/z 147);

m_{IS} is the absolute amount (in µg, adjusted for the purity) of internal standard added to the test sample;

A_b is the area of the peak corresponding to the 3-MBPD-d5 derivative (m/z 150);

a is the slope of the calibration curve;

W is the weight of the sample, in g;

b is the y-intercept of the calibration curve.

NOTE Typical chromatograms of a real sample are given in Figures A.3 and A.4.

10 Precision

10.1 General

The method was evaluated in the international collaborative study conducted by AOCS. Owing to the satisfactory results provided by participating laboratories it was recognized as AOCS Official Method Cd 29a-13[5]. Details of the interlaboratory test on the precision of the method are summarized in Annex B. The values derived from this interlaboratory test may not be applicable to concentration ranges and matrices other than those given in Annex B.

10.2 Repeatability

The difference between two independent single test results, obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within the same day should not exceed 3 % s_r.[8]

10.3 Between-day reproducibility

The difference between two independent single test results, obtained with 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 (e.g. five days) should not exceed 5 % s_R. [8]

11 Test report

The test report shall specify at least the following:

a) all information necessary for the complete identification of the sample;

b) test method used and the element to be determined, with reference to this document, i.e. ISO 18363-3;

c) test results obtained using Clause 9 and the units in which they are specified;

d) date of sampling and sampling procedure (if known);

e) date when the analysis was finished;

f) all operating details not specified in this document, or regarded as optional, together with details of any incidents occurred when performing the method which can have influenced the test result(s).

Annex A
(informative)

Construction of the calibration curves

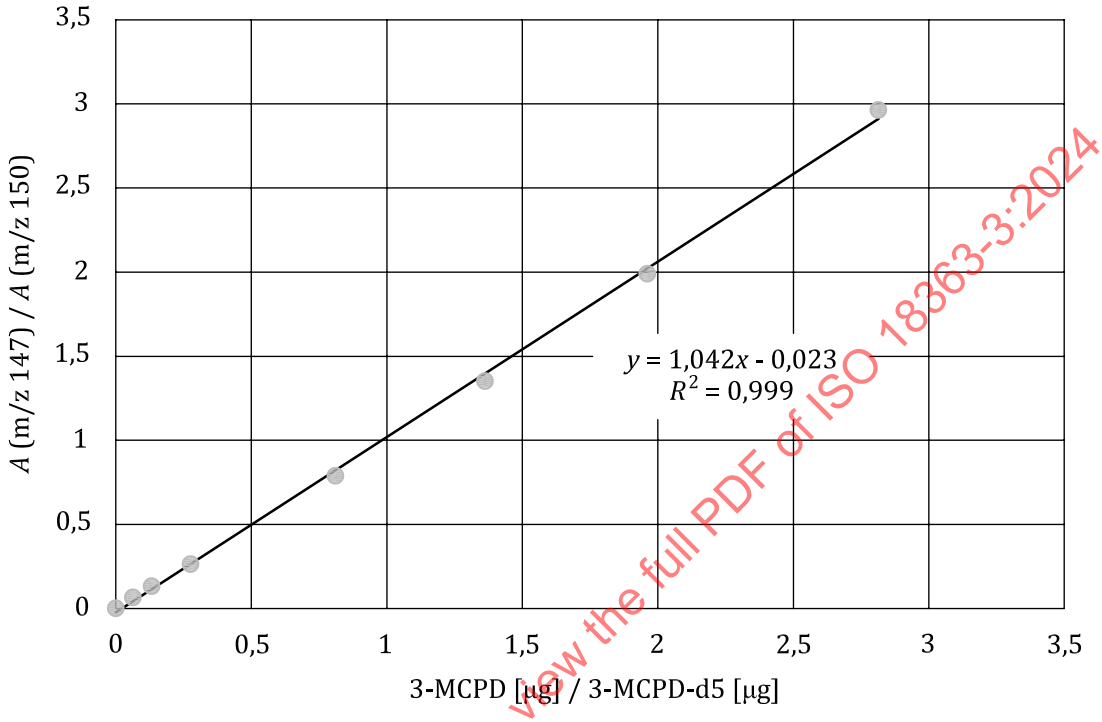


Figure A.1 — Calibration curve of PP-3-MCPD

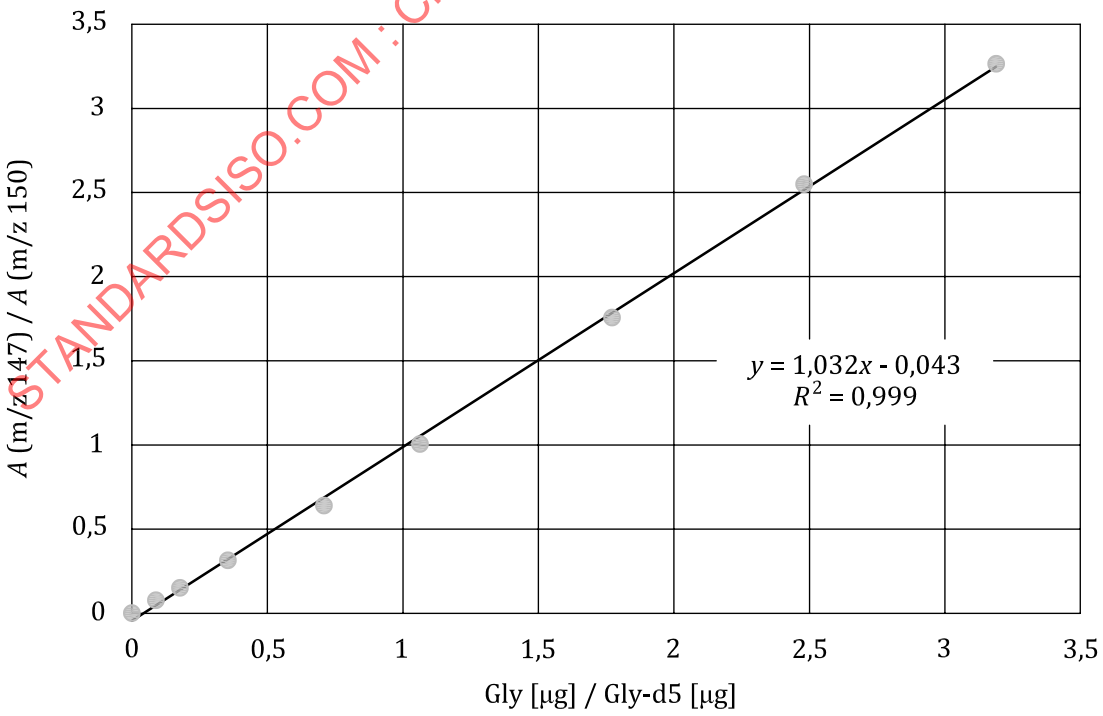
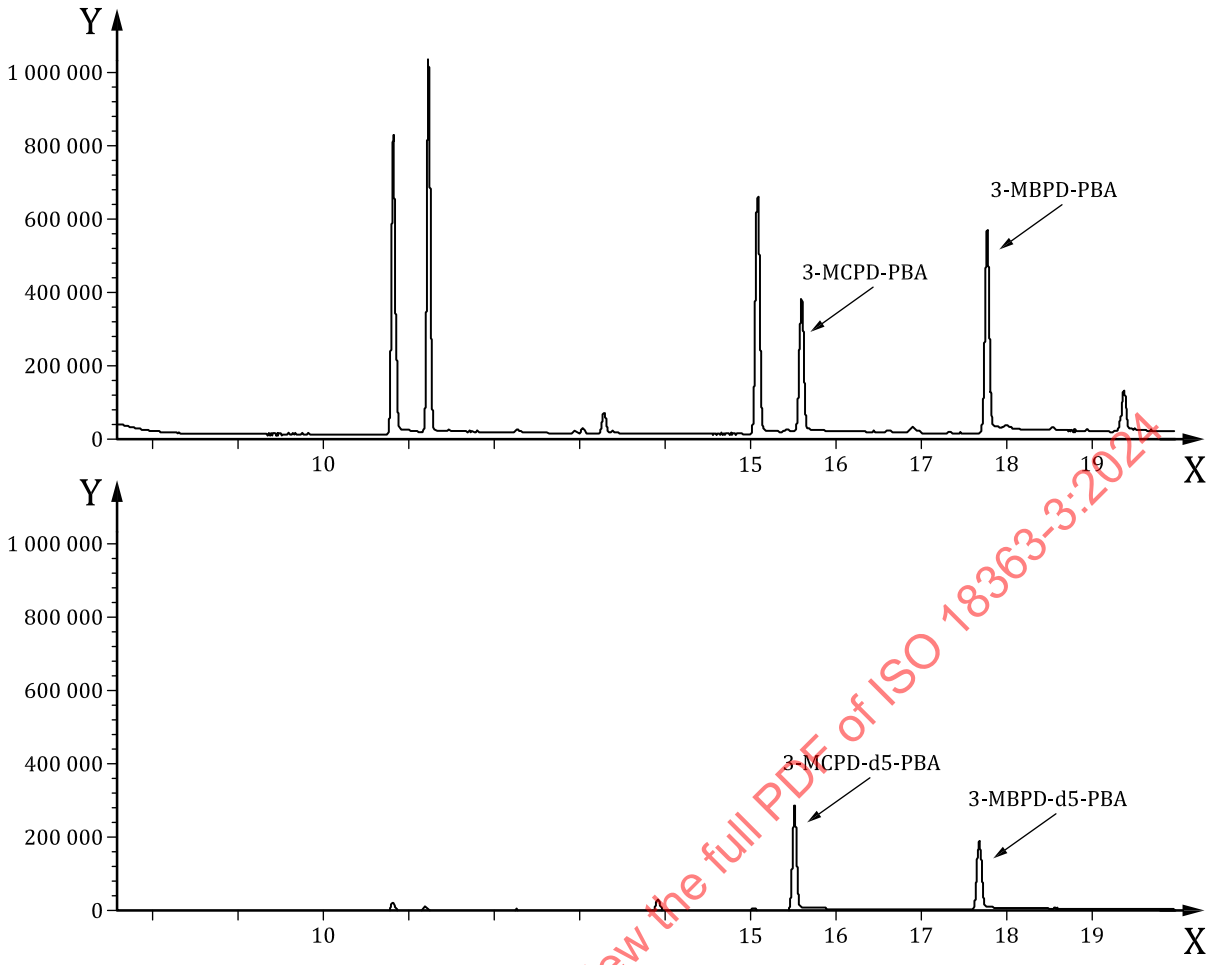


Figure A.2 — Calibration curve of Gly-P

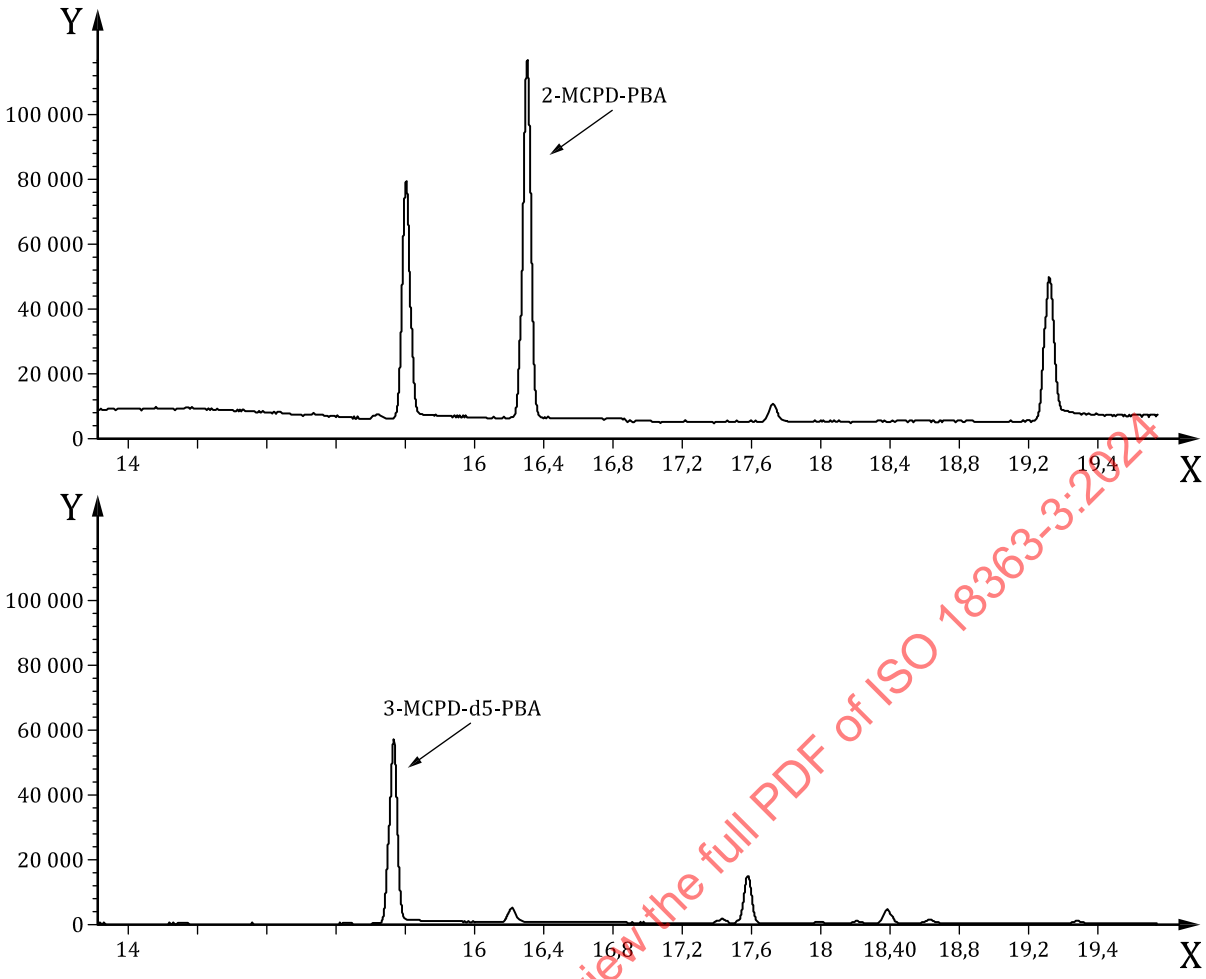


Key

X time

Y abundance

Figure A.3 — SIM chromatogram of a sample of fully refined palm oil (extracted ions *m/z* 147 and 150)



Key

X time

Y abundance

Figure A.4 — SIM chromatogram of a sample of fully refined palm oil (extracted ions *m/z* 196 and 201)