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

Part 2:
Method using slow alkaline transesterification and measurement ~~of~~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 2: Méthode par transestérification alcaline lente et mesure pour le 2-MCPD, le 3-MCPD et le glycidol

FDIS stage

ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: + 41 22 749 01 11
E-mail: copyright@iso.org
Website: www.iso.org

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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 ~~documents~~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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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 18363-2:2018), of which it constitutes a minor revision~~. The main changes are as follows: — the text of~~ to align 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[2] is a differential method equivalent to the DGF standard C-VI 18 (10)[3] and identical to AOCS Official Method Cd 29c-13[4]. 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,[5] but again, a validation study ~~has to~~must be undertaken before the analysis of this analyte.

This document (i.e. ISO 18363-2) represents AOCS Official Method Cd 29b-13.[6][6][7] For information on corresponding validation data, see Annex B. 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. This document 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 isotope-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. 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 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.

ISO 18363-3[8] represents AOCS Official Method Cd 29a-13.[9][10] 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 ~~for~~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 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[11] 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 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, 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 2:
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1 Scope

This document specifies a procedure for the parallel determination of glycidol together with 2-MCPD and 3-MCPD present in bound or free form in oils and fats. The method is based on alkaline-catalysed ester cleavage, transformation of the released glycidol into monobromopropanediol (MBPD) and derivatisation of the derived free diols (MCPD and MBPD) with phenylboronic acid (PBA). Though free MCPD and glycidol are supposed to be present in fats and oils in low to negligible quantities only, in the event that free analytes are present, they would contribute proportionately to the results. The results always being the sum of the free and the bound form of a single analyte.

This method is applicable to solid and liquid fats and oils. This document can also apply to animal fats and used frying oils and fats, but a validation study is undertaken before the analysis of these matrices.

Milk and milk products (or fat coming from milk and milk products) 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 https://www.electropedia.org/

3.1
bound 2-MCPD
sum of all 2-MCPD-derivatives that are cleaved by alkaline-catalysed alcoholysis

Note 1 to entry: The content of bound 2-MCPD is reported in milligrams per kilogram (mg/kg).

3.2
bound 3-MCPD
sum of all 3-MCPD-derivatives that are cleaved by alkaline-catalysed alcoholysis

Note 1 to entry: The content of bound 3-MCPD is reported in milligrams per kilogram (mg/kg).

3.3
bound glycidol
sum of all glycidyl derivatives that are cleaved by alkaline-catalysed alcoholysis

Note 1 to entry: The content of bound glycidol is reported in milligrams per kilogram (mg/kg).

4 Principle

For the determination of bound 2-MCPD, bound 3-MCPD and bound glycidol as free 2-MCPD, free 3-MCPD and free 3-MBPD (3-Monobromopropanediol), two aliquots (A and B) of the sample are spiked with surrogate standards (d_5-2-MCPD, d_5-3-MCPD, d_5-glycidylester in assay A and d_5-2-MCPD-1,3-diester, d_5-3-MCPD-1,2-diester in assay B) and dissolved in diethyl ether. Both assays are processed in parallel. The addition of a diluted solution of sodium hydroxide or sodium methoxide in methanol in the cold will release free 2-MCPD, free 3-MCPD and free glycidol over a period of 8 h to 12 h. This reaction is stopped by the addition of an excess amount of sodium bromide in acidic solution. Under acidic conditions, free glycidol reacts with inorganic bromide to form 3-MBPD and a small amount of 2-MBPD. Undesired non-polar compounds in the sample are removed by double extraction of the aqueous phase with isohexane. The analytes, together with the surrogate standards, are transferred into an organic phase by multiple extraction of the aqueous phase with diethyl ether, ethyl acetate or a mixture of both solvents. Derivatization takes place in the organic phase by reaction with PBA. In order to remove excess amounts of PBA, the analytes are concentrated and transferred into an inert organic solvent. The sample extract is then placed over a small amount of anhydrous sodium sulfate and evaporated to dryness under a stream of nitrogen before being finally redissolved in *iso*-octane for the measurement by GC/MS.

The alkaline catalysed transesterification in the cold minimizes the undesired transformation of 3-MCPD into glycidol that proceeds to a significant extend when the reaction is carried out at room temperature. Nevertheless, in case of large amounts of 3-MCPD being present, even a minor transformation into glycidol might increase the glycidol results from assay A artificially. In order to achieve the correct glycidol results, even in the presence of high 3-MCPD content, assay B serves for the determination of the undesired 3-MCPD–glycidol transformation by determining the amount of d_5-glycidol that has been generated from d_5-3-MCPD-diester by the sample preparation. The corresponding transformation ratio is used for correcting the glycidol value derived from assay A. Another point to be taken into account is that 3-MCPD is converted approximately 1,2-fold faster via glycidol into 3-MBPD than 3-MCPD-d_5 via glycidol-d_5 into 3-MBPD-d_5. Consequently, the isotopic factor I = 1,2 has to be considered for the quantitative determination of the amount of glycidol that has been generated accidentally from the non-labelled 3-MCPD by alkaline treatment in assay A.

Quantification of the analytes is carried out by internal one-point-calibration using the corresponding d_5-esters as surrogate standards. Therefore, no external calibration is necessary. Likewise, no analyte recoveries have to be considered. However, the cleaving rates of MCPD mono- and diesters might be different and as only d_5-MCPD-diesters serve as internal standards, the degree of ester cleavage should have proceeded on a large scale. Therefore, the degree of variations in ester cleavage is monitored by calculating the differences in 3-MCPD results between assay A and B. For information on deriving quantitative data from corresponding chromatograms, see expression of results[9] and Annex A.

As 3-MCPD can occur in certain polymers used for wet strengthening resins and for other purposes, it might also occur from the use of consumables, e.g. screw lid vials or filter. Baking the glassware at 400 °C to 500 °C can reduce this problem. A better solution is the use of non-contaminated materials.

5 Reagents

5.1 General

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 comply with grade 3 of ISO 3696.

5.2 Solvents and chemicals

5.2.1 Toluene.

5.2.2 *tertiary*-Butyl methyl ether (*t*BME), (2-Methoxy-2-methylpropane).

5.2.3 Methanol.

5.2.4 *iso*-Hexane (2-methyl pentane).

5.2.5 Ethyl acetate.

5.2.6 Diethyl ether.

5.2.7 *iso*-Octane.

5.2.8 Sodium sulfate anhydrous, granular.

5.3 Standard and reference compounds

5.3.1 2-MCPD.

5.3.2 2-MCPD-d_5.

5.3.3 2-MCPD-1,3-*bis*-stearoylester *.

5.3.4 2-MCPD-d_5-1,3-*bis*-stearoylester *.

5.3.5 3-MCPD.

5.3.6 3-MCPD-d_5.

5.3.7 3-MCPD-1,2-*bis*-palmitoylester *.

5.3.8 3-MCPD-d_5-1,2-*bis*-palmitoylester *.

5.3.9 Glycidyl oleate*.

5.3.10 Glycidyl-d_5 oleate*.

*Other commercially available fatty acid esters of the analytes may be substituted.

5.4 Working solutions**

5.4.1 2-MCPD: 10,0 µg/ml in methanol.

5.4.2 2-MCPD-d_5: 10,0 µg/ml in methanol.

5.4.3 3-MCPD: 10,0 µg/ml in methanol.

5.4.4 3-MCPD-d_5: 10,0 µg/ml in methanol.

5.4.5 2-MCPD-1,3-*bis*-stearoyl ester: 29,1 µg/ml in toluene; equivalent to 5,0 µg/ml free 2-MCPD.

5.4.6 2-MCPD-d_5-1,3-*bis*-stearoyl ester: 29,3 µg/ml in toluene; equivalent to 5,0 µg/ml free 2-MCPD.

5.4.7 3-MCPD-1,2-*bis*-palmitoyl ester: 26,6 µg/ml in toluene; equivalent to 5,0 µg/ml free 2-MCPD.

5.4.8 3-MCPD-d_5-1,2-*bis*-palmitoyl ester: 26,8 µg/ml in toluene; equivalent to 5,0 µg/ml free 2-MCPD.

5.4.9 Glycidyl oleate: 22,8 µg/ml in toluene; equivalent to 5,0 µg/ml free glycidol.

5.4.10 Glycidyl-d_5 oleate: 23,2 µg/ml in toluene; equivalent to 5,0 µg/ml free glycidol.

**Other concentrations of working solutions may be substituted.

5.5 Other solutions

5.5.1 Sodium hydroxide solution. Weigh 0,25 g freshly ground sodium hydroxide in a 100 ml plastic bottle. Add 100 ml methanol and tightly seal the bottle. Shake vigorously (vortex) until the sodium hydroxide has been dissolved completely. Store in a freezer at –22 °C to –25 °C (see 10.1).

5.5.2 Acidified sodium bromide solution. Weigh 600 g sodium bromide in a 1 l screw cap glass volumetric flask, add deionised water up to the 1 l mark. Acidify the mixture with 3 ml of *ortho*-phosphoric acid (85 %), seal tightly and shake (vortex) until the solution is clear. 600 µl of this solution must neutralize

350 µl of sodium hydroxide solution (5.5.1). Adjust the pH-value to the acidic range (pH 3 to pH 1). Store the solution in a freezer at –22 °C to –25 °C (see 10.1).

5.5.3 Saturated solution of phenylboronic acid (PBA) in diethyl ether. Add approximately 200 mg PBA to 10 ml diethyl ether in a screw cap vial. Shake well, allow non-dissolved PBA to settle and remain as precipitate. For derivatization purposes, use only the clear supernatant.

6 Apparatus

6.1 Eppendorf pipettes (e.g. 10 µl to 100 µl, 10 µl to 200 µl, 100 µl to 1 000 µl).

6.2 Piston stroke and volumetric pipettes, various sizes.

6.3 Volumetric flasks, various sizes.

6.4 Analytical balance, readability 0,000 1 g, weighing precision 0,001 g.

6.5 Screw cap vials (approximately 2 ml in capacity) and screw caps with polytetrafluoroethylene (PTFE)-coated septa.

6.6 Pasteur pipettes and pipette bulbs.

6.7 Micro inserts (approximately 200 µl in capacity) for screw cap vials (approximately 2 ml in capacity).

6.8 Nitrogen blow-off equipment.

6.9 GC/MS-system with temperature programmable injector.

6.10 Fused-silica-GC-column, stationary phase 50 % diphenyl/50 % dimethyl polysiloxane, length 30 m, ID 0,25 mm, film thickness 0,25 µm, low bleed for MS purpose, with pre-column.

The precolumn, that should be exchanged periodically, retards non-volatile components and thereby serves to prolong the lifetime of the main column.

7 Sample

7.1 Sampling

Sampling is not part of this method. A recommended sampling method is given in ISO 5555[12].

7.2 Preparation of the test sample

Aliquot liquid samples directly. Melt solid or semi-solid fats at approximately 80 °C in a drying oven or water bath. For high-melting point fats, the temperature can be increased in 10 °C steps until the melting process starts. Aliquot solid samples that contain higher amounts of water without melting to avoid phase separation.

8 Procedure

NOTE See 10.2.

8.1 Spiking with surrogate standard and homogenization

Weigh two (100 ± 0,5) mg aliquots of the sample into two screw cap vials, approximately 2 ml capacity, or weigh accurately approximatively two 100 mg aliquots of the sample and adopt measures to ensure the correct mass balance for quantification. To assay A, add 50 µl each 2- and 3-MCPD-d_5 standard working