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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 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

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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 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*.

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 three documents currently published or proposed 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[1] is a differential method equivalent to the DGF standard C-VI 18 (10)[2] and identical to AOCS Official Method Cd 29c-13[3]. Briefly, 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. Thereby, 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 has to 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[4], but again, a validation study has to be undertaken before the analysis of this analyte.

This document represents AOCS Official Method Cd 29b-13[5][6]. For information on corresponding validation data, see Annex B. Briefly, 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 will be 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 will be 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 isotope-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 will have to 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 vegetable oils and fats.

ISO 18363-3[7] represents AOCS Official Method Cd 29a-13[8][9]. Briefly, 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 can be applied for the determination of ester-bound 2-MCPD, 3-MCPD, and glycidol in refined and non-refined vegetable oils and fats. It can also apply to animal fats and used frying oils and fats, but a validation study has to 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 would be included in the results and the amount of free analytes can be calculated as a 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 vegetable oils and fats.

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 for 2-MCPD, 3-MCPD and glycidol

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 http://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[10].

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 solutions (5.4.2 and 5.4.4). To assay A, pipette 100 µl glycidyl-d_5 ester standard working solution (5.4.10). To assay B, transfer 100 µL 2-MCPD-d_5-*bis*-ester standard working solution (5.4.6) and 100 µl 3-MCPD-d_5-*bis*-ester standard working solution (5.4.8). To each sample, add 600 µl diethyl ether and shake (vortex) the mixtures until completely dissolved. In the case of high melting sample material, the reaction vessels may require gentle warming.

8.2 Ester cleavage and glycidol transformation

Place both assays for a minimum of 15 min in a freezer to cool down to –22 °C to –25 °C (precipitation of sample material at this stage of sample preparation is not a problem). To each assay, add 350 µl methanolic sodium hydroxide solution (5.5.1). To complete the ester cleavage, seal the vials, briefly shake (vortex) and keep for at least 16 h at –22 °C to –25 °C. Stop the reactions with 600 µl acidified sodium bromide solution (5.5.2) kept at –22 °C to –25 °C. Briefly shake (vortex) the mixtures and place under a gentle stream of nitrogen to reduce the volume of the organic phase to approximately 100 µl. To both assays, add 600 µl *iso*-hexane, seal the vials and shake vigorously (vortex). Leave the mixtures

at room temperature for approximately 5 min to 10 min to complete the transformation of glycidol into MBPD (see 10.3).

NOTE Other temperature ranges for the procedure of ester-cleavage can be applied if a method validation has been carried out. It is important to consider that working at temperature ranges above –22 °C causes an increasing conversion of 3-MCPD into glycidol. As a consequence the uncertainty of glycidol determination can increase.

8.3 Matrix removal

Remove the organic phase by Pasteur pipette. Wash the aqueous phase in each assay with a second 600 µl of *iso*-hexane.

8.4 Derivatization

Using new Pasteur pipettes, extract each essay three times with 600 ul of 3:2 (v:v) mixture of diethyl ether/ethyl acetate. Take care that the pipette does not touch the aqueous phase. For each assay A and B, combine the organic extracts in a new screw cap vial containing a small amount of anhydrous sodium sulfate. If the drying agent gets sticky, transfer the solution into a new screw cap vial with fresh sodium sulfate. Add 20 µl of the derivatization reagent (5.5.3) to both organic extracts to achieve derivatization. If the derivatization reaction results in a small analyte signal response, increase the amount of derivatization agent until the signal to noise ratio in the corresponding chromatogram does not increase. The maximum amount of derivatization reagent that can be used is limited by the individual capacity of the gas chromatographic–mass spectrometric system to exclude excess PBA. To complete the derivatization reaction and to remove excess reagent, evaporate to dryness both assays using a gentle stream of nitrogen. Re-dissolve soluble fractions in approximately 300 µl to 500 µl *iso*-octane and vigorously shake (vortex) the mixture for a few seconds. For GC/MS measurement, transfer a portion of each solution into a 200 µl micro-insert.

8.5 Gas chromatography/mass spectrometry references

8.5.1 Gas chromatography: injection volume: 1 µl to 2 µl.

8.5.2 Carrier gas: helium 4.6 (99,996 %) or helium 5.0 (99,999 %), const. flow 1 ml/min to 1,5 ml/min.

8.5.3 Programmed temperature vaporizer (PTV) temperature programme: e.g. 85 °C, increase temperature at 300 °C/min to 165 °C, hold 10 min isothermal, increase at 300 °C/min to 320 °C, hold 8 min isothermal.

8.5.4 Injector: e.g. split-less, purge flow 50 ml/min after 0,5 min to 1 min, septum purge 3 ml/min.

8.5.5 GC oven temperature programme: e.g. 85 °C, isothermal 0,5 min, increase temperature at 6 °C/min to 150 °C, then 12 °C/min to 180 °C, then 25 °C/min to 280 °C, hold isothermal 8 min.

8.5.6 Mass spectrometry: electron-impact (EI), selected ion monitoring (SIM).

8.5.7 Detected ion traces related to the mass to charge ratios (m/z) from the phenylboronic derivatives of the analytes are summarized in Table 1.

Table 1 — Summary of detected ion traces related to the mass to charge ratios (m/z) from the phenylboronic derivatives of the analytes

	m/z	m/z	m/z	m/z
3-MCPD-d_5	149	150	201	203
3-MCPD	146	147	196	198
2-MCPD-d_5	201	203		
2-MCPD	196	198		
3-MBPD-d_5 (glycidol-d_5 transformation product)	245	247		
3-MBPD (glycidol transformation product)	240	242		

Target ions of labelled and unlabelled analytes should correspond for quantification purposes, e.g. m/z = 150 versus 147 (3-MCPD-d_5 versus 3-MCPD), m/z = 201 versus 196 (2-MCPD-d_5 versus 2-MCPD), m/z = 245 versus 240 (3-MBPD-d_5 versus 3-MBPD).

8.5.8 Suitable software with automatic data analysis is recommended for quantification.

9 Expression of results

NOTE See 10.4.

9.1 Determination of bound glycidol

9.1.1 Bound glycidol in assay A is quantified in 9.1.2 without correction, when 3-MCPD is low in proportion to the glycidol content. In the presence of larger amounts of 3-MCPD, 3-MCPD conversion into glycidol may be incomplete leading to overestimation of glycidol. To compensate for this, the conversion ratio of 3-MCPD-d_5-diester into 3-MBPD-d_5 in assay B is determined and a factor is applied to quantify the potential conversion of 3-MCPD into 3-MBPD in assay A. This procedure is described in 9.1.3 to 9.1.5.

9.1.2 For the quantitative determination of bound glycidol, the signal area of the phenylboronic derivative of 3-MBPD in assay A is multiplied by the spiking level of the internal standard glycidol-d_5 (introduced as glycidyl-d_5 ester) related to its concentration in the sample and divided by the signal area of the phenylboronic derivative of 3-MBPD-d_5, as shown by Formula (1):

$$w_{\text{glycidol uncorrected(A)}} = \frac{SA_{\text{3-MBPD(A)}} \times w_{\text{glycidol-d5(A)}}}{SA_{\text{3-MBPD-d5(A)}}} \quad (1)$$

where

$w_{\text{glycidol uncorrected(A)}}$ is the uncorrected mass fraction of glycidol in assay A, in mg/kg;

$w_{\text{glycidol-d5(A)}}$ is the mass fraction of glycidol-d_5 in assay A, in mg/kg;

$SA_{\text{3-MBPD(A)}}$ is the signal area of the phenylboronic derivative of 3-MBPD in assay A;

$SA_{\text{3-MBPD-d5(A)}}$ is the signal area of the phenylboronic derivative of 3-MBPD-d_5 in assay A.

If the sample contains significant amounts of 3-MCPD, the uncorrected glycidol value can be adjusted by a factor as follows.

9.1.3 To determine the undesired transformation of 3-MCPD via glycidol into 3-MBPD during sample preparation, the corresponding transformation ratio 3-MCPD-d_5 → 3-MBPD-d_5 in assay B is calculated

by dividing the signal area of the phenylboronic derivative of 3-MBPD-d_5 by the signal area of the phenylboronic derivative of 3-MCPD-d_5 in assay B, as shown by Formula (2):

$$t_{3\text{MCPD}} = \frac{SA_{3\text{-MBPD-d5(B)}}}{SA_{3\text{-MCPD-d5(B)}}} \tag{2}$$

where

$t_{3\text{-MCPD}}$ is the transformation ratio 3-MCPD-d_5 → 3-MBPD-d_5;

$SA_{3\text{-MBPD-d5(B)}}$ is the signal area of the phenylboronic derivative of 3-MBPD-d_5 in assay B;

$SA_{3\text{-MCPD-d5(B)}}$ is the signal area of the phenylboronic derivative of 3-MCPD-d_5 in assay B.

9.1.4 After sample preparation and GC/MS analysis in accordance with the procedure and equipment sections, the ratio of detected 3-MBPD and 3-MBPD-d_5 correlates to the isotopic factor (see 10.5), as shown by Formula (3):

$$I = \frac{SA_{\text{3-MBPD}}}{SA_{\text{3-MBPD-d5}}} \tag{3}$$

where

I is the isotopic factor;

$SA_{3\text{-MBPD}}$ is the signal area of the phenylboronic derivative of 3-MBPD;

$SA_{3\text{-MBPD-d5}}$ is the signal area of the phenylboronic derivative of 3-MBPD-d_5.

9.1.5 Subsequently the corrected amount of glycidol can be determined as shown by Formula (4):

$$w_{\text{glycidol corrected(A)}} = \frac{\left[SA_{\text{3-MBPD(A)}} - (t_{\text{3-MCPD}} \times SA_{\text{3-MCPD(A)}} \times I)\right] w_{\text{glycidol-d5(A)}}}{SA_{\text{3-MBPD-d5(A)}}} \tag{4}$$

where

$w_{\text{glycidol corrected(A)}}$ is the corrected mass fraction of glycidol in assay A, in mg/kg;

$t_{3\text{-MCPD}}$ is the transformation ratio 3-MCPD → 3-MBPD (9.1.3);

I is the isotopic factor (9.1.4.);

$w_{\text{glycidol-d5(A)}}$ is the mass fraction of glycidol-d_5 in assay A, in mg/kg;

$SA_{3\text{-MBPD(A)}}$ is the signal area of the phenylboronic derivative of 3-MBPD in assay A;

$SA_{3\text{-MCPD(A)}}$ is the signal area of the phenylboronic derivative of 3-MCPD in assay A;

$SA_{3\text{-MBPD-d5(A)}}$ is the signal area of the phenylboronic derivative of 3-MBPD-d_5 in assay A.

9.2 Determination of bound 2-MCPD

For the quantitative determination of bound 2-MCPD, the signal area of the phenylboronic derivative of 2-MCPD in assay B is multiplied by the spiking level of the internal standard 2-MCPD-d_5 (introduced

as 2-MCPD-d_5 ester) related to its concentration in the sample and divided by the signal area of the phenylboronic derivative of 2-MCPD-d_5, as shown by Formula (5):

$$w_{2\text{-MCPD(B)}} = \frac{SA_{2\text{-MCPD(B)}} \times w_{2\text{-MCPd-d5(B)}}}{SA_{2\text{-MCPD-d5(B)}}} \quad (5)$$

where

$w_{2\text{-MCPD(B)}}$ is the mass fraction of 2-MCPD in assay B, in mg/kg;

$w_{2\text{-MCPD-d5(B)}}$ is the mass fraction of 2-MCPD-d_5 in assay B, in mg/kg;

$SA_{2\text{-MCPD(B)}}$ is the signal area of the phenylboronic derivative of 2-MCPD in assay B;

$SA_{2\text{-MCPD-d5(B)}}$ is the signal area of the phenylboronic derivative of 2-MCPD-d_5 in assay B.

9.3 Determination of bound 3-MCPD

For the quantitative determination of bound 3-MCPD, the signal area of the phenylboronic derivative of 3-MCPD in assay B is multiplied by the spiking level of the internal standard 3-MCPD-d_5 (introduced as 3-MCPD-d_5 ester) related to its concentration in the sample and divided by the signal area of the phenylboronic derivative of 3-MCPD-d_5, as shown by Formula (6):

$$w_{3\text{-MCPD(B)}} = \frac{SA_{3\text{-MCPD(B)}} \times w_{3\text{-MCPd-d5(B)}}}{SA_{3\text{-MCPD-d5(B)}}} \quad (6)$$

where

$w_{3\text{-MCPD(B)}}$ is the mass fraction of 3-MCPD in assay B, in mg/kg;

$w_{3\text{-MCPD-d5(B)}}$ is the mass fraction of 3-MCPD-d_5 in assay B, in mg/kg;

$SA_{3\text{-MCPD(B)}}$ is the signal area of the phenylboronic derivative of 3-MCPD in assay B;

$SA_{3\text{-MCPD-d5(B)}}$ is the signal area of the phenylboronic derivative of 3-MCPD-d_5 in assay B.

9.4 Determination of the degree of diester cleavage

NOTE See 10.6.

For the quantitative determination of the ester cleavage, bound 3-MCPD is quantified in assay A via free 3-MCPD-d_5. Therefore, the signal area of the phenylboronic derivative of 3-MCPD is multiplied by the spiking level of 3-MCPD-d_5 and divided by the signal area of the phenylboronic derivative of 3-MCPD-d_5, as shown by Formula (7):

$$w_{3\text{-MCPD(A)}} = \frac{SA_{3\text{-MCPD(A)}} \times w_{3\text{-MCPD-d5(A)}}}{SA_{3\text{-MCPD-d5(A)}}} \quad (7)$$

where

$w_{3\text{-MCPD(A)}}$ is the mass fraction of 3-MCPD in assay A, in mg/kg;

$w_{3\text{-MCPD-d5(A)}}$ is the mass fraction of 3-MCPD-d_5 in assay A, in mg/kg;

$SA_{3\text{-MCPD(A)}}$ is the signal area of the phenylboronic derivative of 3-MCPD in assay A;

$SA_{3\text{-MCPD-d5(A)}}$ is the signal area of the phenylboronic derivative of 3-MCPD-d_5 in assay A.

The degree of 3-MCPD-d_5-diester cleavage is subsequently calculated as shown by Formula (8) (see 10.7):

$$ec_{\text{3-MCPD-diester}} = \frac{100 \times w_{\text{3-MCPD(B)}}}{w_{\text{3-MCPD(A)}}} \quad (8)$$

where

$w_{\text{3-MCPD(A)}}$ is the mass fraction of 3-MCPD in assay A, in mg/kg;

$w_{\text{3-MCPD(B)}}$ is the mass fraction of 3-MCPD in assay B, in mg/kg;

$ec_{\text{3-MCPD-diester}}$ is the degree of 3-MCPD-diester cleavage, in %.

9.5 Quality control

9.5.1 Method validation should be carried out in accordance with international analytical method validation guidelines.

9.5.2 To control specificity and trueness of the method, analyse a reference material daily and derive a quality control chart for all analytes from the corresponding data.

9.5.3 For quality control purposes, it is recommended to analyse a blank oil sample (e.g. a virgin vegetable or animal oil or fat) daily. Virgin vegetable oils do not contain bound glycidol or 2- and 3-MCPD in detectable amounts (< 10 µg/kg).

10 Notes

CAUTION — Some consumables, such as membrane filters or glassware, can cause contamination. If there is some indication of contamination by consumables, glassware should be heated at 400 °C to 500 °C. Replace non thermo-resistant material or clean by rinsing with sufficient amounts of organic solvents (e.g. acetone and hexane).

10.1 For method establishment purposes, it can be useful to work with double- or triple-concentrated solutions of sodium hydroxide in methanol (0,5 g/100 ml or 0,75 g/100 ml). Adjust the acidity of the sodium bromide solution (5.5.2) similarly.

10.2 Divide every sample into two aliquots (A and B). Spike aliquot A with an isotope-labelled glycidyl ester and isotopic labelled free 2- and 3-MCPD as standards. Spike aliquot B with isotopic labelled 2- and 3-MCPD esters as standards. Add diethyl ether to both assays followed by a dilute solution of sodium hydroxide in methanol in the cold to release free MCPD and free glycidol. Under these controlled conditions, the undesired conversion of free 3-MCPD to induced glycidol, a well-known side reaction by alkaline treatment of 3-MCPD at room temperature, will be minimized. The cleaving reaction is stopped in the cold by the addition of an excess amount of a cold solution of sodium bromide in diluted phosphoric acid. Under acidic conditions the unstable free glycidol reacts rapidly with inorganic bromide to form stable MBPD (3-MBPD > 2-MBPD). The analytes MCPD and MBPD are water soluble so that the undesired lipophilic fractions of the sample aliquots can be removed by double extraction of the aqueous phase with *iso*-hexane. The analytes, together with the standards, are recovered into an organic solvent by triple extraction of the aqueous phase with diethyl ether, ethyl acetate or a mixture of both solvents. The diol-selective derivatization is performed in the extraction solvent by reaction with PBA at room temperature. The absence of the aqueous phase as well as the avoidance of heat during the derivatization reaction minimizes the risk of undesired side reactions. In order to remove excess amounts of PBA, concentrate the analytes and transfer them into a more inert organic solvent. The reaction mixture is then placed over a small amount of anhydrous sodium sulfate and evaporated to dryness under a stream of nitrogen before being re-dissolved in *iso*-octane for the measurement by GC/MS. Differences in the results for 2- and 3-MCPD between the assays A and B serve for the quantitative control of ester cleavage. To determine the undesired reaction of 3-MCPD to glycidol during alkaline treatment, the transformation

rate of isotopic labelled 3-MCPD into isotopic labelled 3-MBPD in assay B is monitored and adapted to the "naturally" occurring analytes in assay A. 2-MCPD is more resistant than 3-MCPD against alkaline-catalysed transformation into glycidol and does not act as a considerable glycidol precursor under the conditions used within this method.

10.3 It is important that the samples are not allowed to warm up at any stage of alkaline treatment. Furthermore, it is recommended to occasionally test the acidity of the aqueous phase of the mixture after the addition of sodium bromide solution (5.5.2).

10.4 Assay A determines the bound glycidol while assay B determines the bound MCPD. In parallel, the transformation of isotopic labelled 3-MCPD ester into 3-MBPD-d_5 can be determined via assay B. It is assumed that the undesired transformation of bound 3-MCPD to induced glycidol (determined as 3-MBPD) in assay A during sample preparation follows similar kinetics and can be determined quantitatively when the ratio itself is known. Finally, the combination of both assays A and B serves for the evaluation of how complete ester cleavage has taken place. All quantifications refer to the sample aliquots spiked with isotopic labelled internal standards (8.1) in purpose of internal one-point-calibration. External calibrations are not intended to serve for analyte quantification but for the determination of the linearity of the method.

10.5 Slight differences in the physical and chemical properties of d_5-labelled and unlabelled 3-MCPD have indicated a little faster conversion of the unlabelled analyte in comparison to the labelled 3-MCPD-d_5. This assumed "isotopic effect" has been experimentally determined for the alkaline treatment (8.2). Under these conditions, 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. If the conditions of alkaline-catalysed ester cleavage are modified, the isotopic factor has to be newly determined, e.g. by spiking a blank oil (only one assay necessary in this case) with the same amount of isotopic labelled and non-labelled 3-MCPD-1,2-*bis*-palmitoylester (in a sufficient concentration not less than 100 mg/kg each). It is not yet known if impurities of the reference compounds (3-MCPD-*bis*-ester and 3-MCPD-d_5-*bis*-ester) may have an influence on the observed effect. In most cases, the corrected value for bound glycidol (9.1.5) does not differ significantly from the uncorrected value (9.1.2) so that the impact of the isotopic factor is negligible.

10.6 One basic approach of this method is a slow and mild alkaline-catalysed ester cleavage that minimizes the undesired transformation of 3-MCPD into glycidol. Under these conditions, the ester cleavage might not be complete in any case. As quantification of the analytes is based on the use of esterified isotopic labelled internal standards, an incomplete ester cleavage should not have a major impact on the results as long as the cleavability of the selected internal standards is representative to one of the analytes. In particular matrices, this might not be the case as the cleavability, especially of 3-MCPD monoesters and diesters, probably is different. 3-MCPD diesters are supposed to be more resistant to alkaline catalysed ester cleavage than 3-MCPD mono-esters, 2-MCPD mono- and diesters, as well as glycidyl esters. Therefore, the determination of 3-MCPD diester cleavage can be a useful tool to overcome this potential source of error. To determine the degree of 3-MCPD diester cleavage, the released 3-MCPD in both assays A and B serves as a reference. The spiked free 3-MCPD-d_5 in assay A stands for complete hydrolysis; whereas, the spiked 3-MCPD-d_5-*bis*-ester in assay B refers as a model substance that will be cleaved the least. If ester cleavage of the 3-MCPD-d_5-*bis*-ester in assay B is absolute, both assays are assumed to give the same value for 3-MCPD within the deviation of measurement uncertainty. If the 3-MCPD-d_5-*bis*-ester cleavage is incomplete, the 3-MCPD value will increase proportionally.

10.7 If *ec* is < 90 %, it is recommended to repeat analysis with a higher concentrated solution of sodium hydroxide or under extended reaction time for alkaline hydrolysis. Therefore, the use of double or triple concentrated solutions of sodium hydroxide (0,5 % or 0,75 %) in methanol combined with equally higher acidified solutions of sodium bromide (5,4 ml/l or 8,1 ml/l *ortho*-phosphoric acid) has shown to be suitable.

Annex A
(informative)

Examples of relevant chromatograms and data evaluation using “low-MCPD” palm oil

A.1 Examples of relevant selected ion monitoring (SIM) chromatograms

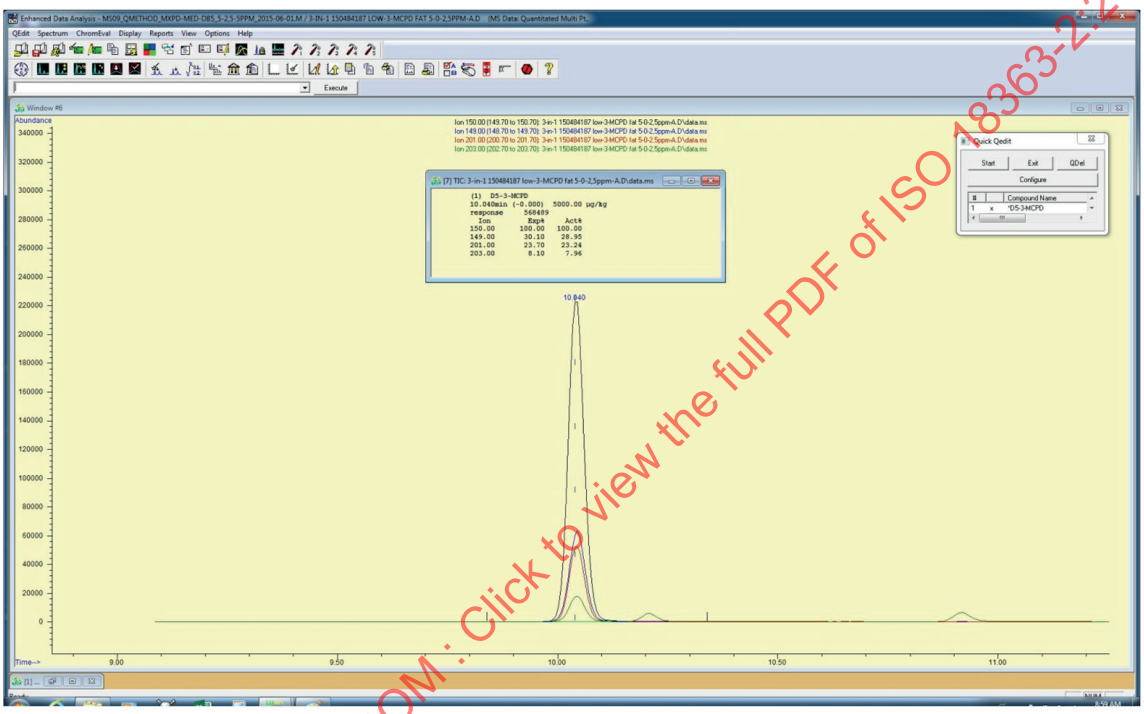

Figure A.1 — SIM — Chromatogram of 3-MCPD-d_5 (5 mg/kg) in assay A

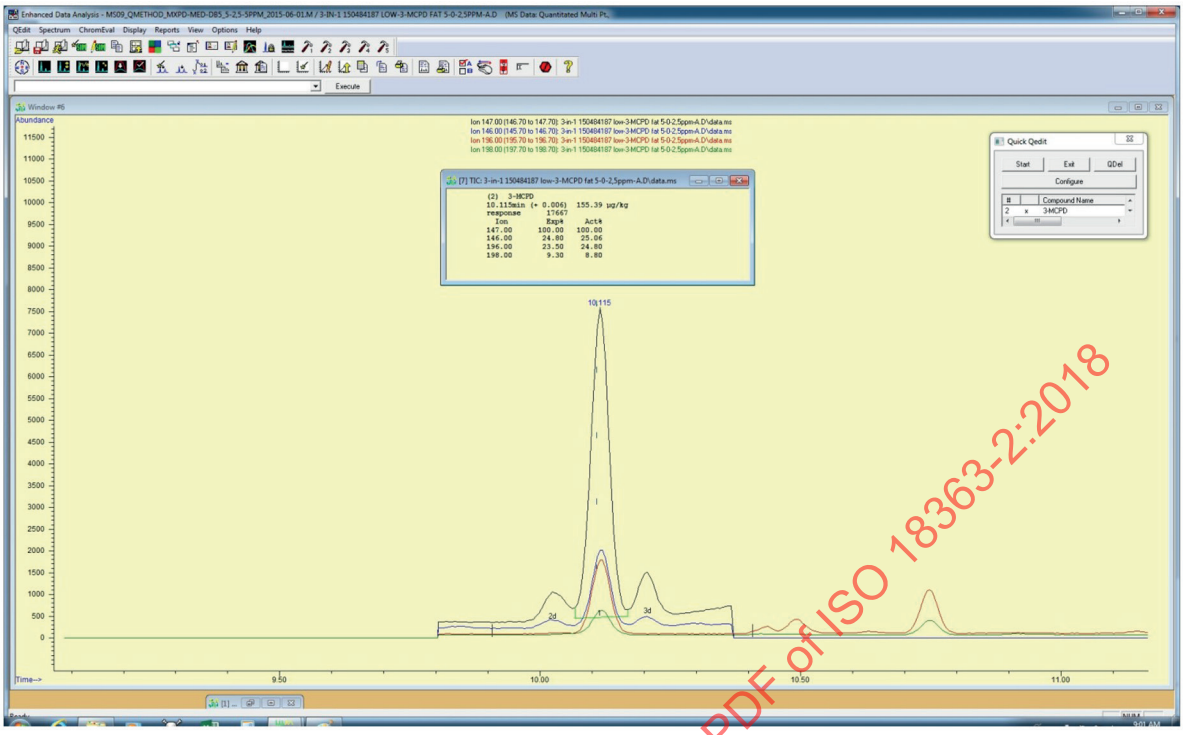

Figure A.2 — SIM — Chromatogram of 3-MCPD (0,155 mg/kg) in assay A

Figure A.3 — SIM — Chromatogram of glycidol-d_5 (5 mg/kg) in assay A

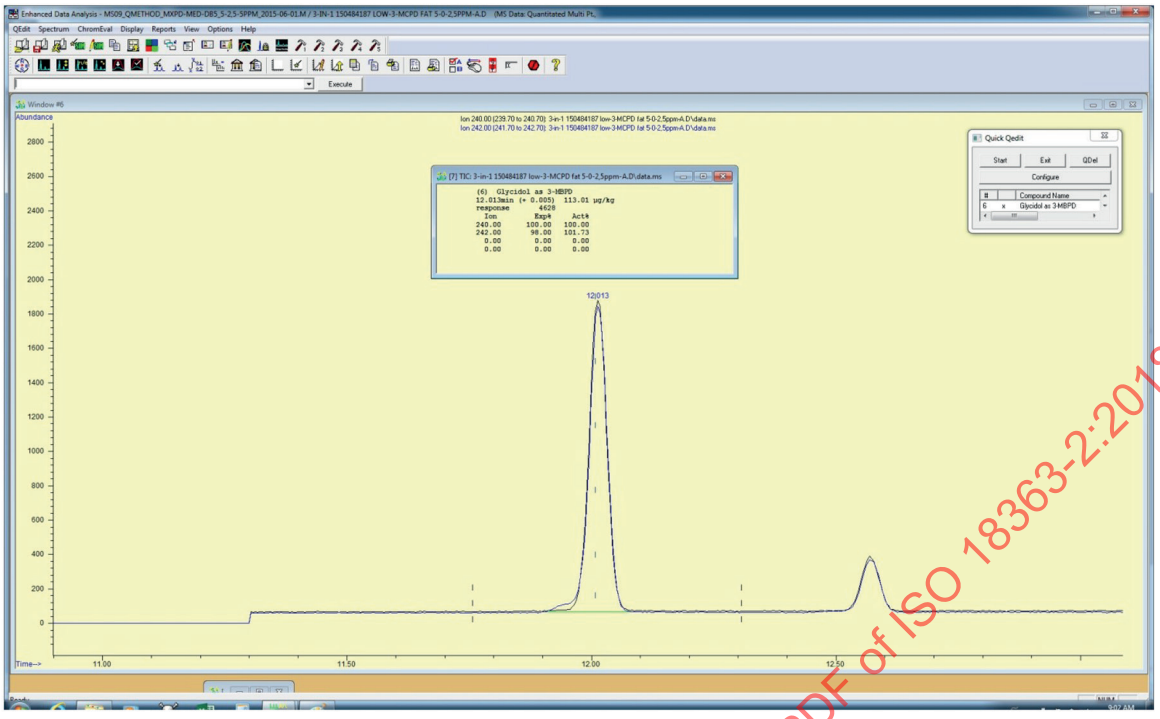

Figure A.4 — SIM — Chromatogram of glycidol (0,113 mg/kg) in assay A

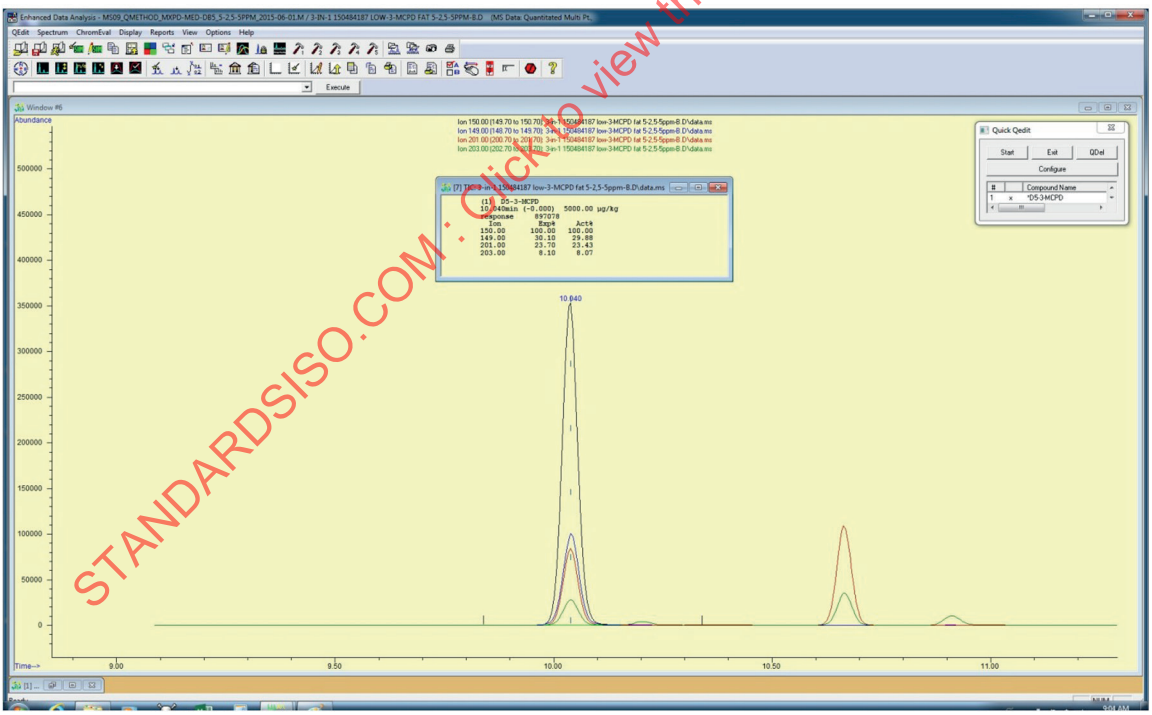

Figure A.5 — SIM — Chromatogram of 3-MCPD-d_5 (5 mg/kg) in assay B

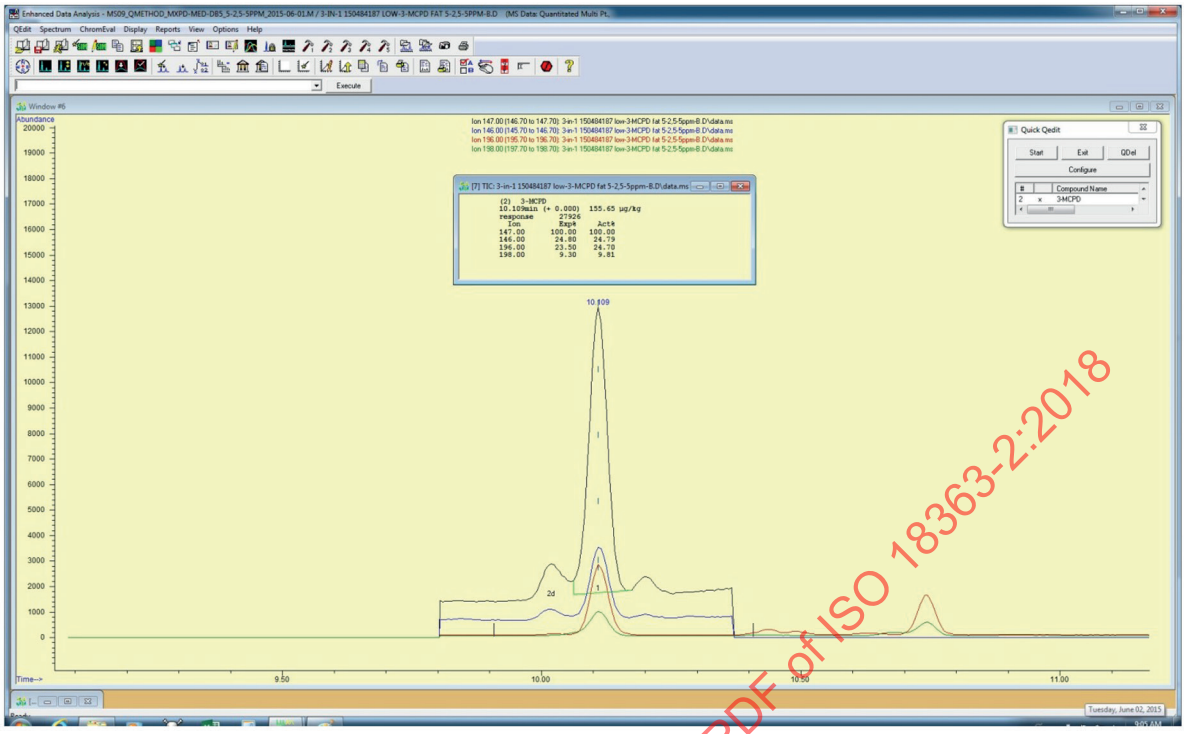

Figure A.6 — SIM — Chromatogram of 3-MCPD (0,156 mg/kg) in assay B

Figure A.7 — SIM – Chromatogram of 2-MCPD-d_5 (2,5 mg/kg) in assay B

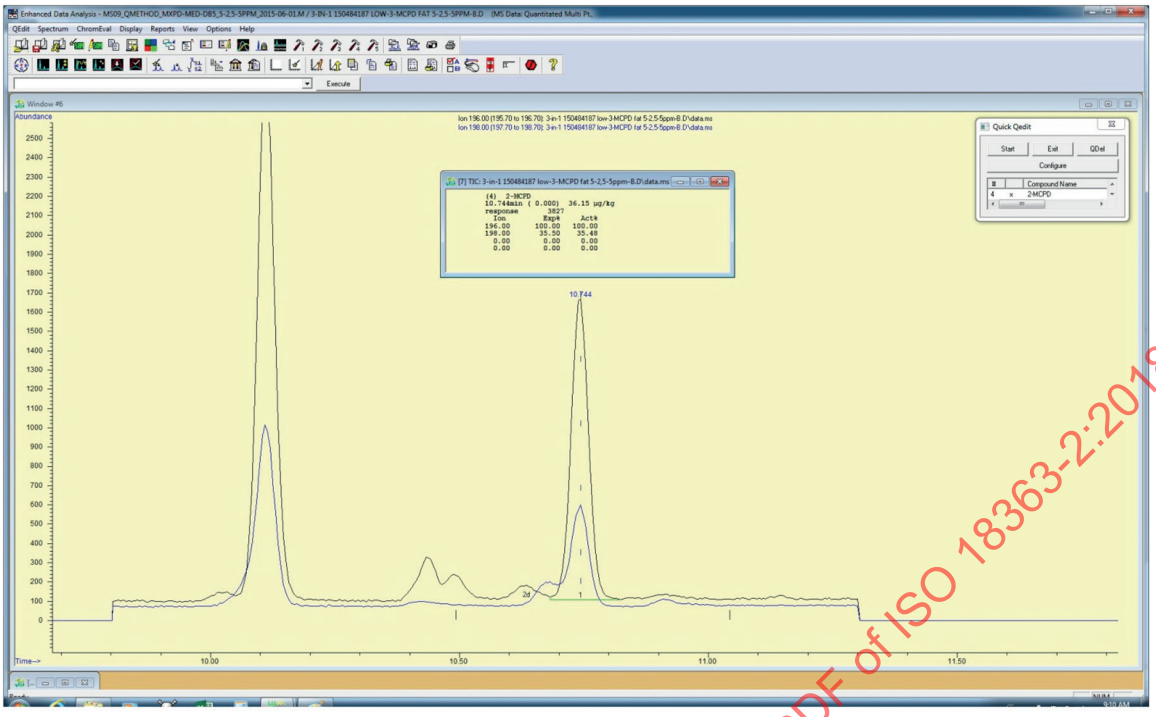

Figure A.8 — SIM — Chromatogram of 2-MCPD (0,036 mg/kg) in assay B

Figure A.9 — SIM — Chromatogram of glycidol-d_5 (for response determination) in assay B