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

Part 4: Method using fast alkaline transesterification and measurement for 2-MCPD, 3-MCPD and glycidol by GC-MS/MS

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

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

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

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

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

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

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

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

Introduction

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

ISO 18363-1 is a differential method equivalent to the DGF standard C-VI 18 (10)[9] and identical to AOCS Official Method Cd 29c-13[6]. In brief, it is based on a fast alkaline catalysed release of 3-MCPD and glycidol from the ester derivatives. Glycidol is subsequently converted into induced 3-MCPD. It consists of two parts. The first part (A) allows the determination of the sum of ester-bound 3-MCPD and ester-bound glycidol, whereas the second part (B) determines ester-bound 3-MCPD only. Both assays are based on the release of the target analytes 3-MCPD and glycidol from the ester-bound form by an alkaline-catalysed alcoholysis carried out at room temperature. In part A, an acidified sodium chloride solution is used to stop the reaction and subsequently convert the glycidol into induced 3-MCPD. Thus, 3-MCPD and glycidol become indistinguishable in part A. In part B, the reaction stop is achieved by the addition of an acidified chloride-free salt solution which also prevents the conversion of glycidol into induced MCPD. Consequently, part B allows the determination of the genuine 3-MCPD content. Finally, the glycidol content of the sample is proportional to the difference of both assays (A – B) and can be calculated when the transformation ratio from glycidol to 3-MCPD has been determined. ISO 18363-1 is applicable to the fast determination of ester-bound 3-MCPD and glycidol in refined and non-refined vegetable oils and fats. ISO 18363-1 can also apply to animal fats and used frying oils and fats, but a validation study must be undertaken before the analysis of these matrices. Any free analytes within the sample would be included in the results, but the document does not allow the distinction between free and bound analytes. However, as of publication, research has not shown any evidence of a free analyte content as high as the esterified analyte content in refined vegetable oils and fats. In principle, ISO 18363-1 can also be modified in such a way that the determination of 2-MCPD is feasible, but again a validation study must be undertaken before the analysis of this analyte.

ISO 18363-2 represents the AOCS Official Method Cd 29b-13[5]. In brief, it is based on a slow alkaline release of MCPD and glycidol from the ester derivatives. Glycidol is subsequently converted into 3-MBPD. ISO 18363-2 consists of two sample preparations that differ in the use of internal standards. Both preparations are used for the determination of ester-bound 2-MCPD and 3-MCPD. In part A, a preliminary result for ester-bound glycidol is determined. Because the 3-MCPD present in the sample is converted to some minor extent into induced glycidol by the sample preparation, part B serves to quantify this amount of induced glycidol that is subsequently subtracted from the preliminary glycidol result of part A. By the use of isotopically labelled free MCPD isomers in assay A and isotopically labelled ester-bound 2-MCPD and 3-MCPD in part B, the efficiency of ester cleavage can be monitored. Both assays, A and B, are based on the release of the target analytes 2-MCPD, 3-MCPD, and glycidol from the ester-bound form by a slow alkaline catalysed alcoholysis in the cold. In both sample preparations, the reaction is stopped by the addition of an acidified concentrated sodium bromide solution so as to convert the unstable and volatile glycidol into 3-MBPD, which shows comparable properties to 3-MCPD with regard to its stability and chromatographic performance. Moreover, the major excess of bromide ions prevents the undesired formation of 3-MCPD from glycidol in the case of samples which contain naturally occurring amounts of chloride. ISO 18363-2 is applicable to the determination of ester-bound 3-MCPD, 2-MCPD and glycidol in refined and unrefined vegetable oils and fats. It also applies to animal fats and used frying oils and fats, but a validation study must be undertaken before the analysis of these matrices. Any free analytes within the sample are included in the results, but the document does not allow a distinction between free and bound analytes. However, as of publication of this document, research has not shown any evidence of a free analyte content as high as the esterified analyte content in vegetable oils and fats.

ISO 18363-3 represents AOCS Official Method Cd 29a-13[4]. In brief, it is based on the conversion of glycidyl esters into 3-MBPD esters and a slow acidic catalysed release of MCPD and MBPD from the ester derivatives. ISO 18363-3 is based on a single sample preparation in which glycidyl esters are converted into MBPD monoesters and, subsequently, the free analytes 2-MCPD, 3-MCPD and 3-MBPD are released by a slow acid-catalysed alcoholysis. The 3-MBPD represents the genuine content of bound glycidol. ISO 18363-3 is applicable to the determination of ester-bound 2-MCPD, 3-MCPD and glycidol in refined

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

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

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

Part 4: Method using fast alkaline transesterification and measurement for 2-MCPD, 3-MCPD and glycidol by GC-MS/MS

1 Scope

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

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

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

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 3696, *Water for analytical laboratory use — Specification and test methods*

3 Terms and definitions

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

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

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

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

3.1
bound 2-MCPD
amount of 2-MCPD cleaved from its esterified (bound) forms by alkaline-catalysed transesterification according to the reference method

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

3.2
bound 3-MCPD
amount of 3-MCPD cleaved from its esterified (bound) forms by alkaline-catalysed transesterification according to the reference method

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

3.3
bound glycidol
amount of glycidol cleaved from its esterified (bound) forms by alkaline-catalysed transesterification according to the reference method

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

4 Principle

The oil or fat sample is dissolved in toluene and tert-butyl-methyl-ether, and the internal standards (3-MCPD-$^{13}C_3$ diester and pentadeuterated glycidyl ester) are added. The sample is then cooled down to 10 °C before the alkaline transesterification is initiated by the addition of a sodium methoxide solution in methanol. After 12 min incubation at 10 °C, the sample mixture is acidified with an acidic solution of sodium bromide to convert the released glycidol to 3-MBPD. The fatty acid methyl esters generated during the transesterification are removed by duplicate extraction of the organic layer. Finally, the purified sample – containing cleaved (free) analytes – is derivatized with phenylboronic acid prior to GC-MS/MS analysis.

The quantification of ester bound 2-MCPD and 3-MCPD is based on the 2-MCPD/3-MCPD-$^{13}C_3$ and 3-MCPD/3-MCPD-$^{13}C_3$ signal ratio, respectively. The quantification of ester-bound glycidol is based on the 3-MBPD/3-MBPD-d5 signal ratio. The amount of 3-MBPD-$^{13}C_3$ formed after the transesterification reaction signifies the amount of released 3-MCPD-$^{13}C_3$ that has degraded to glycidol due to the conditions of the alkaline transesterification. Because no difference in degradation speed between 3-MCPD and 3-MCPD-$^{13}C_3$ has been observed, it is then used to correct for overestimation of the glycidyl ester induced glycidol caused by this degradation of 3-MCPD. Under the conditions used the 2-MCPD is considered stable and thus will not significantly contribute to possible glycidol overestimation[7][8].

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

5 Reagents

5.1 General

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

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

5.2 Standard and reference compounds

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

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

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

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

5.2.3 Carbon-13 labelled 1,2-dipalmitoyl-3-chloropropanediol (PP-3-MCPD-$^{13}C_3$), purity ≥ 95 %.

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

5.2.4 Glycidyl stearate (Gly-S), purity ≥ 98 %.

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

5.2.5 Pentadeuterated glycidyl stearate (Gly-S-d5), purity ≥ 98 %.

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

5.3 Standard solutions

5.3.1 General

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

5.3.2 Stock solutions

NOTE Stock solutions are stable for at least 12 months when stored at −18 °C. Using an ultrasonic bath can help to ensure all standards are completely dissolved.

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

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

5.3.2.3 PP-3-MCPD-$^{13}C_3$ stock (3-MCPD-$^{13}C_3$: 38,5 µg/ml). Weigh 20 mg of PP-3-MCPD-$^{13}C_3$ (5.2.3) in a 100 ml volumetric flask. Fill up to the mark, making sure that the standard is completely dissolved in the solvent.

5.3.2.4 Gly-S-d5 stock (Glycidol-d5: 45,8 µg/ml). Weigh 10 mg of Gly-S-d5 (5.2.5) in a 50 ml volumetric flask. Fill up to the mark, making sure that the standard is completely dissolved in the solvent.

5.3.3 Working solutions

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

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

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

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

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

5.3.3.3 Calibration working solution III (3-MCPD: 0,16 µg/ml, glycidol: 0,16 µg/ml, 2-MCPD: 0,14 µg/ml). Pipette 40 µl of calibration working solution I (5.3.3.1) into a 2,5 ml GC vial containing 1 960 µl of toluene (5.4.4) and homogenize using a vortex mixer.

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

5.3.3.5 Internal standard solution (3-MCPD-$^{13}C_3$: 1,54 µg/ml, glycidol-d5: 0,92 µg/ml). Pipette 5,0 ml of the Gly-S-d5 (5.3.2.4) and 10,0 ml of PP-3-MCPD-$^{13}C_3$ (5.3.2.3) into a 250 ml volumetric flask and fill up to the mark with the solvent.

5.4 Other reagents

5.4.1 Methanol, analytical grade.

5.4.2 Iso-octane, analytical grade.

5.4.3 Acetone, analytical grade.

5.4.4 Toluene, analytical grade.

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

5.4.6 Water, ultra-pure.

5.4.7 Sulfuric acid (purity ≥ 95 %).

5.4.8 Phenylboronic acid (purity ≥ 97 %).

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

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

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

5.5 Reagent solutions

5.5.1 Aqueous sulfuric acid solution (25 %). Transfer 25 ml of sulfuric acid (5.4.7) to a 100 ml volumetric flask containing 50 ml of H_2O (5.4.6). Fill up to the mark with H_2O (5.4.6) and homogenize.

5.5.2 Acid aqueous solution of sodium bromide (sodium bromide 600 mg/ml, sulfuric acid volume fraction of 0,9 %). Dissolve 600 g of sodium bromide (5.4.9) into 700 ml of ultrapure water (5.4.6). Transfer the sodium bromide solution to a 1 000 ml volumetric flask containing 36 ml of sulfuric acid solution (5.5.1). Fill up to the mark with H_2O (5.4.6) and homogenize.

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

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

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

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

6 Apparatus

6.1 Vortex mixer.

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

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

6.4 Ultrasonic bath.

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

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

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

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

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

7 Sample and storage

7.1 Sampling

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

7.2 Preparation of the test sample

Liquid samples shall be used without additional treatment. Solid or turbid fats shall be carefully melted at approximately 60 °C in a drying oven or water bath. For high-melting fats, the temperature shall be carefully increased in 10 °C steps until the melting process starts. Samples containing high amounts of water shall be dried (e.g. by anhydrous Na_2SO_4) before sampling.

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

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

7.3 Storage conditions

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

8 Procedure

8.1 Test sample preparation

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

8.1.1 Weigh 100 mg to 120 mg of oil or fat sample (to a precision of 0,01 mg) in a 2,5 ml GC vial. For fats or oil matrices with melting points > 60 °C, a maximum of 100 mg of fat is weighed to prevent solidification of the reaction mixture during transesterification (see 10.1).

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

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

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

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

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

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

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

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

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

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

8.1.11 Add 100 µl of PBA solution (5.5.4) and homogenize using a vortex mixer for 10 s.

8.1.12 Add 600 µl of fresh iso-octane (5.4.2) and homogenize using a vortex mixer for 10 s to extract the derivatives and analyse using a GC-MS/MS system by injection 2,0 µl of the organic phase.

8.2 Preparation of the calibration curve

8.2.1 Weigh 100 mg to 120 mg of blank oil (5.4.11) into 10 separate 2,5 ml GC vials. Add toluene (5.4.4), tBME (5.4.5), internal standard solution (5.3.3.5) and the corresponding calibration solution (5.3.3.1 to 5.3.3.3) as indicated in Table A.1.

8.2.2 Treat the calibration samples according to the procedure used for the test samples, starting with the dissolution of the sample (see 8.1.3).

All ratios of solvent and reagents should be equal for all calibration samples and unknown samples as described in 8.1.

8.3 Gas chromatography and mass spectrometry settings

8.3.1 Injection volume: 2,0 µl.

8.3.2 Injection mode: splitless, splitless time = 2,0 min.

8.3.3 Injection temperature: 350 °C.

8.3.4 Carrier gas: helium, flow rate: 1,7 ml/min, constant septum purge flow: 5,0 ml/min, split flow = 100 ml/min.

8.3.5 Backflush initiation time: 6,0 min. The backflush initiation setting shall be redetermined after each change to the analytical or pre-column.

8.3.6 GC oven temperature programme: 70 °C (isothermal for 1 min), from 70 °C to 120 °C at 15 °C/min (isothermal for 0,5 min), from 120 °C to 350 °C at 40 °C/min (isothermal for 2,5 min).

8.3.7 Tandem mass spectrometric detector: electron-impact (EI), selected reaction monitoring (SRM) transfer line temperature: 315 °C, ion source temperature: 290 °C.

8.3.8 Example retention time ± acquisition window: 3-MCPD (6,70 ± 0,3) min, 2-MCPD (6,80 ± 0,3) min and 3-MBPD (7,00 ± 0,3) min. Actual retention times can differ between columns.

Parameters for MS/MS mass transitions are given in Table 1. Example chromatograms are provided in Annex C.

Table 1 — Overview of tandem mass spectrometric detector settings for mass transitions of each ion pair

Component name	Parent ion Q1 m/z	Collision cell Q2 eV[a]	Daughter ion Q3 m/z	Purpose of mass transition
3-MCPD	196	8	147	Quantification 3-MCPD
3-MCPD	198	8	147	Qualification 3-MCPD
3-MCPD-$^{13}C_3$	199	8	149	Quantification Internal standard 3-MCPD/2-MCPD
3-MCPD-$^{13}C_3$	201	8	149	Qualification Internal standard 3-MCPD/2-MCPD
2-MCPD	196	14	104	Quantification 2-MCPD
2-MCPD	198	14	104	Qualification 2-MCPD
3-MBPD	240	8	147	Quantification glycidol
3-MBPD	242	8	147	Qualification glycidol
3-MBPD-$^{13}C_3$[b]	243	8	149	Quantification Glycidol overestimation from 3-MCPD
3-MBPD-d5	245	8	150	Quantification Internal standard glycidol
3-MBPD-d5	247	8	150	Qualification Internal standard glycidol

[a] Collision cell eV settings can differ between types of tandem mass spectrometers, systems of different manufacturers or both, and require optimization for each individual system.

[b] A qualification mass transition for 3-MBPD-$^{13}C_3$ is not possible due to interference effect of natural ^{13}C isotope of 3-MBPD (m/z 242), which co-elutes under the described conditions.

9 Expression of results

9.1 General

The calculation of the results has to happen in a specific order so that the glycidol overestimation can be quantified and corrected. The 3-MCPD is the source of the in vitro formed glycidol during the transesterification reaction (see 8.1.5) and the amount of glycidol formed is directly proportional to the 3-MCPD ester concentration[8]. Therefore, the 2-MCPD- and 3-MCPD esters are quantified first with the formulae given in 9.2.

Subclause 9.3 provides the calculations for the glycidyl esters. To start with, the total amount of glycidol is quantified. Because the 3-MCPD-$^{13}C_3$ is present in a known amount, it serves as a model compound to mimic 3-MCPD degradation during transesterification. As both labelled and unlabelled 3-MCPD converts to their glycidol counterpart with the same speed and independent of the 3-MCPD concentration, the amount of glycidol-$^{13}C_3$ (see 9.3.3) serves as a one-point calibration to calculate the amount of glycidol induced by the 3-MCPD esters (see 9.3.4)[8]. The conversion of 3-MCPD into glycidol is matrix-independent. Therefore, all of the formulae described in 9.2 and 9.3 shall be applied to all samples, including the spiked samples for recovery determination, without exception.

9.2 Quantification of 2-MCPD- and 3-MCPD esters

9.2.1 Prepare a calibration curve by plotting the ratio of the amount of standard (expressed as free x-MCPD equivalent) to the amount of internal standard (expressed as free 3-MCPD-$^{13}C_3$ equivalent) on

the x-axis against the ratio of the corresponding peak areas on the y-axis. Ions at m/z 104 (2-MCPD) or m/z 147 (3-MCPD) and 149 (3-MCPD-$^{13}C_3$) are used for quantification. Calculate the regression line as shown by Formula (1):

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

where

y is the measured ratio of 2-/3-MCPD and 3-MCPD-$^{13}C_3$ signals;

x is the theoretical ratio of 2-/3-MCPD (µg) and 3-MCPD-$^{13}C_3$ (µg) in the calibration sample;

a is the slope of the calibration curve;

b is the y-intercept of the calibration curve.

Confirm that the linearity is good ($R^2 > 0{,}99$) and the y-intercept preferably < |0,01| in order to achieve a good accuracy for samples at very low concentration of 2-/3-MCPD esters. See 10.4.

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

$$w_{\text{uncorrected}} = \frac{\left(\frac{A_{\text{analyte}}}{A_{\text{IS}}}\right) - b}{a} \cdot m_{\text{IS}} \cdot \frac{1\,000}{m_{\text{sample}}} \tag{2}$$

where

$w_{\text{uncorrected}}$ is the uncorrected concentration of 2-/3-MCPD esters in the test sample (expressed as free 2-/3-MCPD, mg/kg oil);

A_{analyte} is the area of the unlabelled analyte (2-MCPD, m/z 104 or 3-MCPD, m/z 147);

m_{IS} is the mass (in µg) of 3-MCPD-$^{13}C_3$ added to the test sample;

A_{IS} is the area of the 3-MCPD-$^{13}C_3$ derivative peak (m/z 149);

a is the slope of the calibration curve;

b is the y-intercept of the calibration curve;

m_{sample} is the mass of the sample (in mg).

9.2.3 Determine the recovery of 2-/3-MCPD esters in the spiked blank oil samples (%) by applying Formula (3):

$$R = \frac{w_{\text{spiked blank oil}}}{w_{\text{theoretical}}} \cdot 100 \tag{3}$$

where

R is the recovery (%) for 2-/3-MCPD esters in the batch of tested samples;

$w_{\text{spiked blank oil}}$ is the averaged uncorrected concentration of 2-/3-MCPD esters result of 9.2.2 for the spiked blank oils;

$w_{\text{theoretical}}$ is the calculated theoretical concentration of 2-/3-MCPD esters in the spiked blank oils (mg/kg) based on the sample mass and the spike solution concentration (5.3.3.4).

9.2.4 Determine the final result for the 2-/3-MCPD esters in the test sample (mg/kg) by applying Formula (4):

$$w_{\text{final}} = w_{\text{uncorrected}} \cdot \frac{100}{R} \tag{4}$$

where

w_{final} is the final corrected concentration of ester bound 2-/3-MCPD esters in the test sample (expressed as free 2-/3-MCPD, mg/kg oil);

$w_{\text{uncorrected}}$ is the uncorrected concentration of 2-/3-MCPD esters (expressed as free 2-/3-MCPD, mg/kg oil) in the test sample acquired by 9.2.2;

R is the calculated recovery (%) as acquired by 9.2.3.

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. 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):

$$y = ax + b \tag{5}$$

where

y is the measured ratio of 3-MBPD and 3-MBPD-d5 signals;

x is the theoretical ratio of 3-MBPD (µg) and 3-MBPD-d5 (µg) in the calibration sample;

a is the slope of the calibration curve;

b is the y-intercept of the calibration curve.

Confirm that the linearity is good ($R^2 > 0{,}99$) and the y-intercept preferably < |0,01| in order to achieve a good accuracy for samples at very low concentration of glycidyl esters. See 10.4.

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

$$w_{\text{uncorrected}} = \frac{\left(\frac{A_{\text{analyte}}}{A_{\text{IS}}}\right) - b}{a} \cdot m_{\text{IS}} \cdot \frac{1\,000}{m_{\text{sample}}} \tag{6}$$

where

$w_{\text{uncorrected}}$ is the total concentration of glycidol in the test sample (expressed as free glycidol, mg/kg oil);

A_{analyte} is the area of the 3-MBPD derivative peak (m/z = 147);

m_{IS} is the mass (in µg) of glycidol-d5 added to the test sample;

A_{IS} is the area of the 3-MBPD-d5 derivative peak (m/z = 150);

a is the slope of the calibration curve;

b is the y-intercept of the calibration curve;

m_{sample} is the mass of the sample (in mg).

9.3.3 Determine the amount of glycidol-$^{13}C_3$ that formed during the alkaline transesterification (mg/kg) by applying Formula (7), see 10.3:

$$w_{gly13C3} = \left(\frac{A_{analyte}}{A_{IS}} \right) \cdot m_{IS} \cdot \frac{1\ 000}{m_{sample}} \quad (7)$$

where

$w_{gly13C3}$ is the concentration of glycidol-$^{13}C_3$ in the test sample (expressed as free glycidol-$^{13}C_3$, mg/kg oil);

$A_{analyte}$ is the area of the 3-MBPD-$^{13}C_3$ derivative peak (m/z = 149);

m_{IS} is the mass (in µg) of glycidol-d5 added to the test sample;

A_{IS} is the area of the 3-MBPD-d5 derivative peak (m/z = 150);

m_{sample} is the mass of the sample (in mg).

9.3.4 Determine the concentration of glycidol induced by 3-MCPD caused by alkaline transesterification (mg/kg) by applying Formula (8):

$$w_{glyMCPD} = \left(\frac{R_{3-MCPDe}}{w_{3-MCPD-^{13}C_3}} \right) \cdot w_{gly13C3} \quad (8)$$

where

$w_{glyMCPD}$ is the concentration of 3-MCPD induced glycidol in the test sample (expressed as free glycidol, mg/kg oil);

$R_{3\text{-}MCPDe}$ is the concentration of 3-MCPD esters in the test sample (see 9.2.4);

$w_{3\text{-}MCPD\text{-}13C3}$ is the concentration (mg/kg, oil) of 3-MCPD-$^{13}C_3$ added to the test sample;

$w_{gly13C3}$ is the concentration (mg/kg, oil) of glycidol-$^{13}C_3$ in the sample (see 9.3.3).

9.3.5 Determine the concentration of glycidyl esters induced glycidol in the test sample (mg/kg) by applying Formula (9):

$$w_{GE} = w_{uncorrected} - w_{glyMCPD} \quad (9)$$

where

w_{GE} is the concentration of glycidyl esters induced glycidol in the test sample (expressed as free glycidol, mg/kg oil);

$w_{uncorrected}$ is the uncorrected concentration of glycidol in the test sample (see 9.3.2);

$w_{glyMCPD}$ is the concentration (mg/kg, oil) of 3-MCPD induced glycidol in the test sample (see 9.3.4).

9.3.6 Calculate the recovery for glycidyl esters induced glycidol (%) by applying Formula (10):

$$R = \frac{w_{\text{spiked blank oil}}}{w_{\text{theoretical}}} \cdot 100 \quad (10)$$

where

R is the recovery (%) for glycidyl esters in the batch of tested samples;

$w_{\text{spiked blank oil}}$ is the averaged result of 9.3.5 of the spiked blank oils;

$w_{\text{theoretical}}$ is the calculated theoretical concentration of glycidyl esters in the spiked blank oils (mg/kg) based on the sample mass and the spike solution concentration (5.3.3.4).

9.3.7 Determine the final result for the glycidyl esters in the test sample (mg/kg) by applying Formula (11):

$$w_{\text{final}} = w \cdot \frac{100}{R} \quad (11)$$

where

w_{final} is the final corrected concentration of ester-bound glycidol in the test sample (expressed as free glycidol, mg/kg oil);

w is the glycidyl ester concentration in the test sample (expressed as free glycidol, mg/kg oil) obtained in 9.3.5;

R is the calculated recovery (%) as acquired in 9.3.6.

9.4 Quality control

Method validation should be carried out according to international analytical method validation guidelines.

The recovery factors (see 9.2.3 and 9.3.6) shall be determined at least once every 24 h, in duplicate, by spiking blank, non-thermally treated vegetable oil with the spike solution (5.3.3.4).

For the determination of the recovery, replace the 100 µl of toluene in step 8.1.2 with 100 µl of spike solution (5.3.3.4). The rest of the procedure is identical to the test samples.

Examples of suitable non-thermally treated vegetable oils include extra virgin olive oil and crude rapeseed oil. Virgin and crude vegetable oils do not contain bound glycidol or 2-/3-MCPD in detectable amounts.

Spiking large quantities of blank fats or oils to be used over a long period of time is to be avoided. Some blank oils contain trace amounts of unknown components which can react with glycidyl esters, causing unstable and decreasing concentrations over time. Therefore, fresh spiking followed by direct analysis as described in 8.1 is strongly recommended.

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.

10 Notes

10.1 For some (crude) oil or fat matrices the organic layer can be (partially) solidified or jellified[7]. This will not influence the quantification of the components if step 8.1.8 is carried out correctly. If samples with melting points of > 60 °C show signs of solidification during transesterification or step 8.1.8 and the peak area of internal standards < 50 % of typical internal standard peak areas for the spike samples used for recovery determination (see 7.2), the sample preparation needs to be repeated with less sample mass (< 100 mg). Matrices with this particular behaviour have not been included in this method's validation.

10.2 All (internal) standard solutions used are prepared using ester-bound reference compounds. For all calculations in 9.2 and 9.3, the equivalent concentration of free compound is used. The concentrations listed for the standard solutions in 5.3 are also expressed as free compound equivalent concentrations. These concentrations of free equivalents of 2-/3-MCPD and glycidol in µg/ml are based on the mass of the ester-bound components listed in 5.2. Other ester-bound components can be substituted as standards, but the mass of the ester bound compound shall be adjusted accordingly to achieve similar free equivalent concentrations. Formula (12) can be used to calculate the required mass of the ester-bound standard to acquire a solution with the desired concentration of free equivalent in µg/ml.

$$m_{\text{std}} = \frac{m_{\text{mol}}}{m_{\text{free eq}}} \cdot w_{\text{std sol}} \cdot \frac{V_{\text{std sol}}}{1\ 000} \tag{12}$$

where

m_{std} is the mass of the standard to weigh (mg);

m_{mol} is the molar mass of the used standard of MCPD or glycidylester (g/mol);

$m_{\text{free eq}}$ is the molar mass of the free equivalent component; MCPD (= 110,539 g/mol) or glycidol (74,079 g/mol);

$w_{\text{std sol}}$ is the desired concentration (mg/l) of the free equivalent in the standard solution;

$V_{\text{std sol}}$ is the desired volume of the standard solution.

10.3 Applying the transesterification conditions described in Clause 8 of this procedure, the conversion of 3-MCPD into glycidol is expected to be between 5 % and 10 % for a typical blank oil. This conversion rate is stable under reproducible conditions but is strongly influenced by the $NaOCH_3$ concentration, reaction temperature and time. Thus, the conversion rate for the recovery samples can serve as an extra check on the method stability and control point for possible reagent degradation or preparation errors. Diversion from this conversion rate is a typical result from a deviating transesterification temperature (inefficient cooling) or $NaOCH_3$ concentration with regard to the procedure described in 8.1, or miscalculation of internal standard concentrations.

EXAMPLE For the recovery determination, a concentration of 1,5 mg/kg of 3-MCPD-$^{13}C_3$ in the sample is expected to generate 0,08 mg/kg to 0,15 mg/kg of glycidol-$^{13}C_3$ concentration as calculated with 9.3.4.

Additionally, under the described conditions, conversion of 2-MCPD into glycidol will be < 1 %. Therefore, the correction does not account for 2-MCPD induced glycidol as this can be ignored with regard to overestimating the glycidyl ester content.

10.4 Calibration curve slope values are to be expected between 0,80 and 1,00 and between 0,95 and 1,05 for 2-MCPD and 3-MCPD, respectively. For 3-MBPD, the calibration curve slope can be expected to be situated between 1,00 and 1,25. Calibration curves are not required to be collected on a day-to-day basis

and can be applied for several weeks when using dedicated instruments, so long as recovery standards and quality control samples are included to check the calibration curve validity.

It is imperative that calibration samples and unknown samples are treated equally to acquire representative calibration curves. Any deviations in transesterification conditions, such as ratio of sample to solvent and types of solvent, will result in different slopes. The slopes can thus be used as another quality control point of the method to monitor method performance.

Other possible causes for slope deviations are problems with the calibration stock production or erroneous calculation of the theoretical ratios between the target analyte and internal standard.

10.5 Maintenance of the SSL injector (6.5) has proved critical to guarantee method accuracy, especially for the quantification of glycidyl esters. If the injector is not cleaned regularly with isopropanol and the liner replaced, glycidyl ester results can develop an increasingly positive bias. The recommended interval for injector maintenance is once or twice per week, depending on the number of samples analysed. Furthermore, it is strongly recommended that the SSL injector housing is thoroughly cleaned at least every six months with methanol, isopropanol and hexane[7].

10.6 Recovery values of 80 % to 120 % can be expected in a normal situation. Values that fall outside this range often indicate degradation of the spike solution (5.3.3.4) or internal standard solution (5.3.3.5). Other causes often originate from inconsistency during sample preparation, e.g. differences in the transesterification conditions between the calibration and sample batches.

11 Precision

11.1 General

The method was evaluated in an international collaborative study organized by ISO and AOCS, conducted between February and May 2020 in accordance with ISO 5725-5. Outlier analysis was conducted according to Cochran's test (intralaboratory variance) and Grubb's test (interlaboratory variance). The study included eight different oils and fats of vegetable origin (see Tables B.1 to B.3).

11.2 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 12 % RSD (2-MCPD), 9 % (3-MCPD) or 11 % (glycidol).

11.3 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 27 % (2-MCPD), 15 % (3-MCPD) or 38 % (glycidol) RSD.

Annex A
(normative)

Supporting tables

Table A.1 — Scheme for the construction of the calibration curve for SS-2-MCPD, PP-3-MCPD and Gly-S

	Calibration sol. (5.3.3.1 to 5.3.3.3)	Toluene (5.4.4)	tBME (5.4.5)	2-MCPD µg	3-MCPD µg	Gly µg	Internal standard sol. (5.3.3.5)	3-MCPD-$^{13}C_3$ µg	Gly-d5 µg
Cal 1	—	100 µl	200 µl	0,000	0,000	0,000	100 µl	0,154	0,092
Cal 2	Cal III (5.3.3.3) 20 µl	80 µl	200 µl	0,003	0,003	0,003	100 µl	0,154	0,092
Cal 3	Cal III (5.3.3.3) 50 µl	50 µl	200 µl	0,007	0,008	0,008	100 µl	0,154	0,092
Cal 4	Cal III (5.3.3.3) 100 µl	—	200 µl	0,014	0,016	0,016	100 µl	0,154	0,092
Cal 5	Cal II (5.3.3.2) 20 µl	80 µl	200 µl	0,057	0,063	0,063	100 µl	0,154	0,092
Cal 6	Cal II (5.3.3.2) 70 µl	30 µl	200 µl	0,202	0,221	0,219	100 µl	0,154	0,092
Cal 7	Cal II (5.3.3.2) 100 µl	—	200 µl	0,289	0,316	0,313	100 µl	0,154	0,092
Cal 8	Cal I (5.3.3.1) 60 µl	40 µl	200 µl	0,433	0,474	0,470	100 µl	0,154	0,092
Cal 9	Cal I (5.3.3.1) 75 µl	25 µl	200 µl	0,541	0,593	0,587	100 µl	0,154	0,092
Cal 10	Cal I (5.3.3.1) 100 µl	—	200 µl	0,722	0,790	0,783	100 µl	0,154	0,092

NOTE 1 All samples are prepared with 100 mg to 110 mg of blank oil (5.4.11).

NOTE 2 For calibration samples, the blank toluene added in step 8.1.2 is replaced by the calibration solution I, II or III according to this table. If the calibration solution does not equal 100 µl, the difference is completed with blank toluene according to this table.

Table A.2 — Time scheme for the parallel ester cleavage for a batch of 20 samples for steps 8.1.5 and 8.1.6

Sample	Sodium methoxide addition (8.1.5) min	Acidified NaBr solution addition (8.1.6) min
1	00:00	12:00
2	00:30	12:30
3	01:00	13:00
4	01:30	13:30
5	02:00	14:00
6	02:30	14:30
7	03:00	15:00
8	03:30	15:30
9	04:00	16:00
10	04:30	16:30
11	05:00	17:00
12	05:30	17:30
13	06:00	18:00
14	06:30	18:30
15	07:00	19:00
16	07:30	19:30
17	08:00	20:00
18	08:30	20:30
19	09:00	21:00
20	09:30	21:30
The time interval between samples is purely indicative and may be adjusted to suit the user as long as the total reaction time of 12:00 min for each individual sample is strictly adhered to.		

Annex B
(informative)

Statistical results of the ISO collaborative study

Table B.1 — Statistical results for 3-MCPD ester in mg/kg (expressed as free 3-MCPD)

3-MCPD ester		S4	S5	S6	S7	S8	S9	S10	S11
Description	**Symbol**	**Soy bean oil**	**Palm oil**	**Rapeseed oil**	**Coconut oil**	**Palm oil stearin, Dfr**	**Palm oil olein, Dfr**	**Corn oil**	**Refined sunflower oil**
Number of laboratories after outlier elimination	p	17	17	17	16	15	15	16	17
Global mean	M	**0,458**	**1,376**	**0,271**	**0,352**	**0,352**	**1,420**	**0,242**	**0,137**
Repeatability standard deviation	s_r	0,013	0,075	0,013	0,021	0,031	0,042	0,009	0,010
Relative standard deviation	$C_{V,r}$ (s_r /M, %)	3 %	5 %	5 %	6 %	9 %	3 %	4 %	7 %
Limit of repeatability	r	0,036	0,208	0,036	0,059	0,085	0,117	0,026	0,027
Reproducibility standard deviation	S_R	0,035	0,108	0,031	0,033	0,044	0,137	0,031	0,021
Relative standard deviation	$C_{V,R}$ (S_R /M, %)	**8 %**	**8 %**	**11 %**	**9 %**	**13 %**	**10 %**	**13 %**	**15 %**
Limit of reproducibility	R	0,098	0,300	0,086	0,091	0,123	0,378	0,086	0,058
HorRat value		0,4	0,5	0,6	0,7	0,5	0,7	0,6	0,7
Key									
Dfr = double fractionated									