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Determination of the MCPD fatty acid esters and glycidyl fatty acid esters in food

Report on the collaborative trial organised by the EURL-PAH and Process Contaminants

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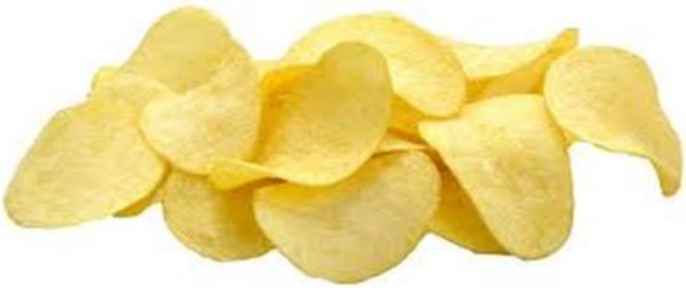


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Executive summary

The European Union Reference Laboratory for Polycyclic Aromatic Hydrocarbons and Process Contaminants, hosted by the Joint Research Centre of the European Commission, organised on request of the Directorate-General SANTE a method validation study via collaborative trial to evaluate the performance of a method for the determination of fatty acid esters of 3-monochloropropane-1,2-diol (3-MCPD-ester), 2-monochloropropane-1,2-diol (2-MCPD-ester) and glycidyl esters (GEs) in food samples.

The method validation study was conducted according to the International Union for Pure and Applied Chemistry harmonised protocol and the Collaborative Study Guidelines of AOAC International for blind (unpaired) replicates. The method was applied for the determination of 3-MCPD-, 2-MCPD-esters and GEs in naturally contaminated oil, fat, waffles, potato chips (crisps) and crackers.

The standard operating procedure was based on the extraction of the test materials by pressurised liquid extraction, followed by acid transesterification and derivatisation of the released free (non-esterified) form with phenylboronic acid. The determination was carried out by gas chromatography-mass spectrometry with electron ionisation in selected ion monitoring mode.

The trial involved 10 participants, representing a cross section of research, private and official control laboratories from 4 EU Member States (Germany, UK, Ireland and The Netherlands), USA and Japan. The selection of collaborators was based on the performance in a pre-trial, organised prior to the collaborative trial with the participation of 12 laboratories.

The relative standard deviation for repeatability (RSDr) ranged from 1.3 to 21%.
The relative standard deviation for reproducibility (RSDR) ranged from 6.5 to 49.0%, reflecting HorRat values from 0.5 to 2.2 according to the Horwitz function modified by Thompson. However the precision was not very satisfactory (> 22% RSDR) for mass fraction levels around and lower of 100 µg/kg on fat basis.

For an analyte content higher than 100 µg/kg, excellent precision was achieved with HorRat values in the range of 0.4-0.7.

Table of content

Acknowledgements

The laboratories participating in this exercise, listed in **Table 1**, are sincerely acknowledged. The authors would like to thank the JRC colleagues involved in the project for their support.

Table 1: The following laboratories participated in the pre-trial and the trial.

Organisation	**City**	**Country**
Federal Institute for Risk Assessment (BfR)	Berlin	Germany
LAVES (Lower Saxony State Office for Consumer Protection and Food Safety)	Braunschweig	Germany
Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit	Erlangen	Germany
LAV Sachsen-Anhalt	Halle/Saale	Germany
Eurofins WEJ Contaminants	Hamburg	Germany
Chemisches Veterinäruntersuchungsamt Rheinland	Hürth	Germany
Nestlé	Polling	Germany
Dublin Public Analyst Laboratory	Dublin	Ireland
Japan Food Research Laboratories	Ibaraki-shi, Osaka	Japan
Dr. A. Verwey B.V.	Rotterdam	The Netherlands
Premier Analytical Services	High Wycombe	UK
United States Food and Drug Administration (U.S. FDA)	College Park	USA

1. Introduction

Fatty acid esters of 3-monochloropropanediol (3-MCPDEs), 2-monochloropropanediol (2-MCPDEs) and glycidol (GEs) are substances that are mainly generated during the refining of edible fats and oils, or in a domestic cooking environment [1]. They were detected in a variety of different foodstuffs, especially in products containing higher amounts of vegetable oils. High levels above 4 mg kg^{-1} have been reported in refined vegetable oils and various heat processed foods [2]. Although the information currently available on their toxicological properties and bioavailability is not sufficient to draw firm conclusions, great concern has been raised due to the carcinogenicity of their hydrolysable moieties [3]. The Scientific Panel on Contaminants in the Food Chain (CONTAM) of the European Food Safety Authority (EFSA) assumed a 100 % release of the MCPD moiety from its esters in humans through the action of gut lipases. Free 3-MCPD is listed as a threshold genotoxic carcinogen and glycidol as probably carcinogenic to humans [4, 5].

Concerns surrounding the presence of MCPDEs and GEs in many types of thermally processed foods have necessitated the development of analytical methods suitable for their determination in diverse matrices with the scope of improving overall confidence in analytical results. For years, the scientific community has debated whether the information on the MCPDEs and GEs content in oils/fats is dependent on the method used to perform the analysis [2]. Unfortunately, the analytical community still lacks the certified reference materials with defined analyte concentrations to perform trueness check. For this reason, analysts compare their results to data gained by other analytical methods. So far, it is not yet apparent which methods and conditions provide results closer to the true value.

The EFSA report from 2013 [3] highlighted weaknesses in the analytical methods for the determination of 3-MCPD in different food groups and recommended to further develop and establish standard analytical methods for analysing 3-MCPD in its different forms with adequate performance parameters, in order to reduce the uncertainty in occurrence and exposure estimates.

Between 2012 and 2013 three improved analytical methods for the analysis of MCPD esters and glycidyl esters in fats and oils have been developed. In August 2013, after a collaborative study with 20 participants from eight countries, the American Oil Chemists' Society (AOCS) has validated and adopted the improved methods as official AOCS methods [6, 7, 8]. The methods are suitable for the analysis of fats and oils, and provide a valuable starting point for the development of methods covering the analysis of other food matrices.

A method comparison study organized in 2012 by the Joint Research Centre (JRC) of the European Commission on the determination of 3- and 2- MCPD esters and glycidyl esters in edible oils confirmed the comparability of results obtained with different indirect analytical methods [9].

In order to gather reliable occurrence data to support the exposure assessment for the EFSA opinion, improved and validated analytical methods are required for the analysis of 3- and 2-MCPD esters and glycidyl esters in the relevant food matrices.

Consequently, EFSA requested the JRC to develop and validate in-house a robust indirect analytical method for determining 3- and 2-MCPD- fatty acid esters and GEs in a wide variety of food matrices with adequate performance parameters. It should allow the analysis of more than 600 food samples, covering all categories of food commodities. Methods specified by the AOCS for oil and fats were used as a basis for the method development and optimisation. The method that includes acidic transesterification of the ester forms [6] was applied with slight modifications as building block for the design of the Standard Operating Procedure (SOP) of the analytical method described here.

The project included the performance of an *ad hoc* survey on specific food groups to test the analytical methods and to provide a minimum database on levels of 3- and 2-MCPD and glycidol in those food groups. The food groups addressed by the survey included in the JRC Project were:

- ✓ Bread and rolls

- ✓ Fine bakery wares
- ✓ Smoked fish products
- ✓ Smoked meat products
- ✓ Fried or roast meat (all possible types, including grilled and griddled)
- ✓ Chips, crisps, fries and dough-based analogues (both, potato- or cereal-based)
- ✓ Margarine
- ✓ Infant and follow-on formulae

The analyses of 3- and 2-MCPD covered both, those originally present in food in free form and those from esters of fatty acids. The analyses of glycidol were for glycidol derived from esters only.

A variety of solvents were used for the extraction of 3- and 2-MCPD fatty acid esters (with or without GE) from food samples for indirect determination. The solvents generally need to be more polar than simple alkanes such as hexane for complete recovery, particularly for 3-MCPD monoesters. The solvents commonly used are t-butyl methyl ether or mixtures of *t*-butylmethyl ether with hexane or petroleum ether, or mixtures of hexane with diethyl ether [10, 11]. The inclusion of acetone is particularly useful for the extraction of MCPD fatty acid esters from dry infant formula [12].

In a collaborative trial dedicated only to the determination of 3- and 2-MCPD fatty esters, the BfR compared extraction methods for MCPD fatty acid esters in infant formula, sweet spread and chocolate cream, each containing vegetable fats, plant-based onion lard, and mayonnaise [12]. The collaborative trial used an extraction step based on accelerated solvent extraction (ASE, also known as pressurised liquid extraction, PLE) with a solvent mixture of petroleum ether/*iso*-hexane/acetone (2/2/1 v/v) using two extraction cycles at a temperature of 125 °C. The ASE method had very good repeatability within the laboratories and sufficient reproducibility between the laboratories for all of the matrices for 3- and 2-MCPD fatty esters. The ASE method was compared with Soxhlet extraction, which was found to be less reproducible, possibly due to variations in the procedures used. The ASE method was also compared to several cold solvent extraction methods, which exhibited poorer recovery. The trueness and precision were better for hot extraction methods than for cold ones, and varied with the type of food product analysed. Analysis of the glycidyl esters contents, however, was not covered by the scope of that study.

An SOP that included the determination of glycidyl esters was elaborated by the European Union Reference Laboratory for Polycyclic Aromatic Hydrocarbons (EURL PAH) of the JRC, based on the BfR method [12] with a small deviation during the derivatisation step. It has been compared in a collaborative study with four external expert laboratories in the field (Nestle, Federal Institute for Risk Assessment /BfR/, SGS-Germany, Hamburg University) to assess the performance of different analytical procedures in various food commodities. The test items used in this exercise consisted of five naturally contaminated samples such as smoked fish, waffles, infant formula, puff pastry, andolive oil. The participants were asked to use the method of their choice and no further requirements were imposed regarding methodology.

In total, eight datasets for each matrix were reported to the JRC as organiser of the study. Their evaluation supported the assumption of an agreement of the results from the determination of MCPD esters and GEs in most of the tested food matrices, independent of the conditions applied during extraction and transesterification. The only problematic matrix appeared to be fish, as fish contains free 3-MCPD. This collaborative trial served as an external control for the reliability of the SOP, developed by JRC, in the situation of a lack of a reference method and reference substances for trueness evaluation.

The SOP was applied for the analysis of more than 600 samples of breads and bread rolls, fine bakery wares, smoked fish and meat products, fried and roasted meat, potato-based snacks and fried potato products, cereal-based snacks, and margarines. All the results were reported to EFSA in 2014.

In 2016 an assessment of critical steps in the above mentioned method was performed for the simultaneous determination of MCPDEs and GEs in the fat phase obtained from bakery and potato products, smoked and fried fish and meat, and other cereal products [13]. It was

concluded that the trueness of the measurement results is affected by the additional formation of 3-MBPD esters from monoacylglycerols (MAGs), which are frequently present in food. The overestimation of GE contents for some samples was confirmed by the comparison of results with data obtained by an independent analytical method (direct analysis of GE by HPLC-MS/MS). An additional sample pre-treatment by SPE was introduced to remove MAGs from fat prior to the GEs conversion, while the overall method sensitivity was not significantly affected. Trueness of the determination of GEs by the modified analytical procedure was confirmed by comparison with a direct analysis of GEs.

The derivatization of the free forms of MCPD and MBPD with PBA was evaluated in aqueous or organic phases, as well as the assessment of the potential impact on the accuracy of results of the final sample preparation step of the analytical procedure. Different commercial batches of PBA showed differences in solubility in a non-polar organic solvent. The PBA derivatization in an organic solvent did not affect the precision and the trueness of the method due to the isotopic standard dilution. However, the method sensitivity might be significantly compromised [13].

After submission of the results from the survey to EFSA, DG SANTE requested a collaborative trial for the validation of the SOP. As such, a collaborative trail has never been conducted before for glycidyl esters.

This method validation study (MVS) is aimed at evaluating the precision characteristics of an analytical method [Annex 10] for the determination of fatty acid esters of 3-monochloropropanediol (3-MCPDEs), of 2-monochloropropanediol (2-MCPDEs) and of glycidol (GEs) in a wide variety of food products, with a fat content of more than 5 %.

2. Method description

After extraction by pressurised liquid extraction (PLE), acid transesterification and derivatisation of the released free (non-esterified) form with phenylboronic acid (PBA), the PBA derivatives are consecutively measured by gas chromatography-mass spectrometry (GC-MS) with electron ionisation (EI) in selected ion monitoring mode (SIM). Quantification of the analytes is carried out using 3-MCPD-ester-d5 (rac 1,2-bis-palmitoyl-3-chloropropanediol-d5), 2-MCPD-ester-d5 (1,3-Distearoyl-2-chloro-propanediol-d5) and Gly-O-d5 (pentadeuterated glycidyl oleate) as internal standards. Results are expressed as free forms of 2-MCPD, 3-MCPD and as glycidol. The working range of the method is 25 µg kg^{-1} – 4 mg kg^{-1} for free forms of 2-MCPD and 3-MCPD and 12.5 µg kg^{-1} – 2 mg kg^{-1} for the free form of glycidol.

The study was designed and evaluated according to the International Union for Pure and Applied Chemistry (IUPAC) Harmonised Protocol [14]. Statistical analyses were performed along the lines of ISO 5725 [12] using the ProLab software [15].

3. Design of the interlaboratory validation study

3.1 Time frame

Table 1 provides information on the timing of the study.

Call for expression of interest in participation	13 December 2016
Public consultations/ derivatisation step selection	until 16 January 2017
Informing the registered participant for the final SOP to be validated	16 January 2017
Sample dispatch for the pre-trial	08 February 2017
Deadline for reporting results	20 March 2017
Participants selection for the trial	04 May 2017
Sample dispatch for the co-lab study	23 June 2017
Deadline for collection of the results	15 August 2017
Announcement of the results to the participants	November 2017

Table 1: Timing of the study

3.2. Invitation for participation and public consultations

The SOP of the method to be validated was published for public consultations simultaneously with the call for the expression of interest. E-mails with the information and registration link were sent to more than 150 contact points in Europe, Asia and America via EURL-NRL-PAH network, as well as to the participants in the previous collaborative trials in that field [9, 12].

Having in mind the variations in different steps of the available methods for determination of MCPDEs and GEs in foodstuff, two options for the final derivatisation step of the procedure were proposed - in an aqueous (as it is in AOCS method) and in an organic phase (modified to prevent the problems encountered by some laboratories from the high PBA content in the aqueous phase). Depending on the number of potential participants, interested in one or another procedure, the launch of the respective SOP for validation by laboratories would be chosen after the registration phase.

The outcome from the registration showed a slight preference to the SOP applying a derivatisation in organic phase (PBA in diethyl ether). As the number of registered participants would have not been sufficient to validate both SOPs, we asked the applicants that chose the SOP with aqueous derivatisation whether they are going to participate in a collaborative trial for the validation of a SOP with derivatisation in an organic phase. Participants were invited, on a voluntary basis, to provide an additional set of results obtained by applying the derivatisation in an aqueous phase with the intention to validate both SOPs (if feasible).

Two comments were received from applicants, pointing out problems with PLE for the reproducible extraction of fat from powder IF. As the SOP has been applied successfully to determine the target analyte from more than 600 food sample including 25 powder infant formula samples, and due to the time constrains, the organisers decided to go on with the SOP without making any changes.

In total 16 applicants from NRLs, OCLs and industry in six member states agreed to participate in the pre-trail and to follow the SOP, suggested by the JRC (Figure 1). Fourteen had already previous experience with the determination of 3-MCPD- and 2-MCPD-esters in oil and fat, nine of them applied a similar method in routine for other food commodities as bakery products or confectionary, while 5 more had limited experience with additional food commodities than oil and fat (Figure 2). Fewer participants declared mastering of the glycidyl esters' analysis as concerns for the occurrence of glycidol and glycidyl esters in food and their analysis emerged more recently (Figure 3).

Fig.1 Number of registered participants per country

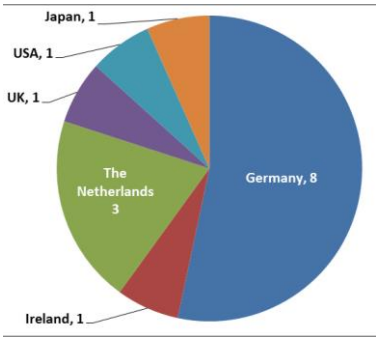


Fig.2 Previous experience with similar analysis for 3- and 2-MCPD esters

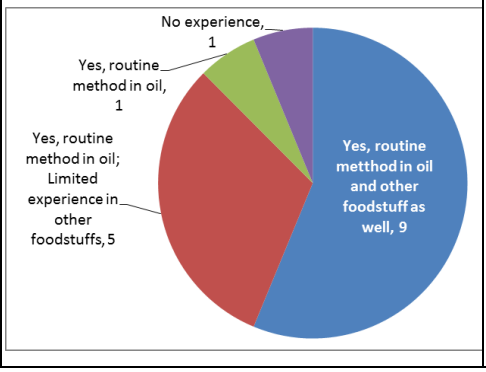
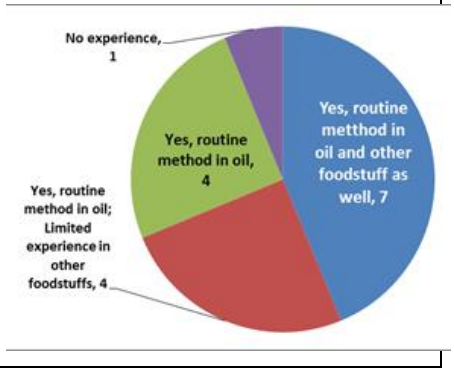


Fig.3 Previous experience with similar analysis for glycidyl esters



3.3 Pre-trial

Although almost all of the participants declared previous experience with similar SOPs for the determination of 3-MCPD- and 2-MCPD-esters and a bit less with glycidyl esters, a preliminary trial was conducted with the aim to familiarise the laboratories with this specific SOP method, to optimise instrument parameters where needed and, most importantly, to check the detection capability of laboratories' instruments, given the anticipated working range.

The pre-trial was conducted in the period January-April 2017.

Two test samples were prepared in-house for the pre-trial - Sample A - edible oil (1 ml) and Sample B - infant formula (10 g). The choice of foodstuff was made based on the proposal of the European Commission for a Regulation of the levels of 3-MCPD esters and glycidyl esters only for oils and fats and infant formulae. The samples, together with 1 ml labelled standard mix to be used as internal standard, were dispatched in dry ice to 16 participants in the end of January with 6 weeks reporting deadline.

In the course of the pre-trial other participants informed us that they were not able to obtain satisfactory fat recovery from their quality control samples of powdered infant formula when applying PLE. Upon detailed communication with those participants and exchange of samples, we jointly concluded that there are Infant Formulae on the market for which PLE is not suitable for the extraction of the entire fat.

After the deadline only 12 datasets were submitted, 10 of which were accepted (Figure 4). One participant informed us about some internal problems with implementation of the methods and withdrew their result until further confirmation. Confirmation has not be achieved as of yet. Another laboratory was not able to fulfil the requirement to use PLE for the extraction of the fat from the different types of the food commodities and provided dataset of results obtained after Soxhlet extraction. Those two datasets were rejected. The rest of the results were subjected to robust statistics, and the resulting parameters for reproducibility are shown in Table 2.

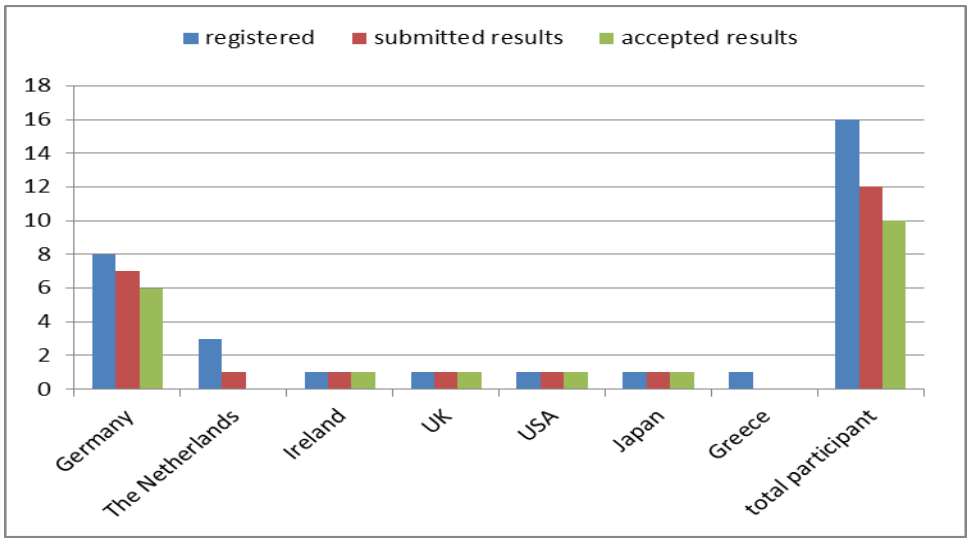


Figure 4. Participants per country for the pre-trail of the MVS.

No problems were observed in the analyses of the oil test sample. Relative reproducibility standard deviations (RSD_R) were far below truncated Horwitz, resulting in very satisfactory HorRat values in the range of 0.3-0.7. Similar were RSD_R for 3-MCPD- and 2-MCPD-esters from infant formula on expressed fat base. Due to the relatively lower content and the complexity of the analytical procedure, RSD_R for GEs was much higher.

	IF_Product base			IF_FAT base				Oil		
Lab. Code	3MCPDE	2MCPDE	GES	3MCPDE	2MCPDE	GES	IF1 FAT	3MCPDE	2MCPDE	GES
	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	g/100g	µg/kg	µg/kg	µg/kg
06	151.7	84.4	41.6	624.9	347.8	171.5	24.28	548.9	296.1	943.2
11				730	340	530	10.5	540	270	1080
03	170	79	13	610	283	46	27.9	557	270	1033
08	101	46	7.2	718	328	51	14	544	282	1093
13	216.1	99.3	26.5	777.5	357.2	95.2	27.8	659.9	360.2	867
07	35.7	13.9	3.2	713	278	64	5	563	252	981
09	163.4	87.9	48.4	649.2	349.2	192.2	25.2	513.6	272.8	937.6
14	188	88.1	84.2	709	333	318	26.4	586	283	1140
16	225	101	56	747	337	185	30.1	563	288	1140
17	166.4	71.5	75.1	650.7	279.7	293.8	25.7	533.3	323.2	911.5
	– –	– –	– –	– –	– –	– –	– –	– –	– –	–
Mean	163	78	39	693	323	180	22	554	286	1012.63
Target s.d.	54	24	33	63	35	136	9	28	27	111.33
Rel. SDPA	32.9%	31.1%	84.1%	9.1%	10.8%	75.4%	40.4%	5.0%	9.3%	11.0%
Rel. reproducibility s.d.	32.9%	31.1%	84.1%	9.1%	10.8%	75.4%	40.4%	5.0%	9.3%	11.0%
HORRAT	1.6	1.3	3.2	0.5	0.6	3.6	16.1	0.3	0.5	0.7
Relative classical Horwitz s.d.	21.0%	23.5%	26.0%	16.9%	19.0%	20.7%	2.5%	17.5%	19.3%	15.97%
Standard error	17.84	8.05	11.06	19.98	11.07	42.93	2.82	8.72	8.44	35.21
No. of laboratories after elimination of outliers type A-L except E	9	9	9	10	10	10	10	10	10	10
No. of measurement values and states	10	10	10	10	10	10	10	10	10	10

Table 2. Results from the pre-trial

3.4 Participating Laboratories

All 10 participants, whose results have been accepted for statistical treatment during the pre-trail phase, were invited to participate in the collaborative trial.

3.5. Test materials

The initial plans were to include in the collaborative trial food samples representing all the categories addressed by the Commission Recommendation 2014/661/EU.

Unfortunately, during the preparatory stage of the collaborative trial, the EURL-PAH experienced difficulties in finding suitable naturally contaminated fish/meat products on the market. Consequently this category of food samples was not included in the trial.

Meanwhile, at the end of March 2017, the AOAC published a call for establishing a Working Group to begin standard development activities for 2- and 3-MCPD and GE in finished infant formula (IF) products, which triggered the decision not to include IF as matrix for the collaborative trial at that stage until a more robust method is suggested for powder IF.

Recently AOAC INTERNATIONAL announced the availability of the draft standard method performance requirements (SMPR®) for 2- and 3-MCPD esters & Glycidyl esters (GE); invited method developers to submit methods for consideration and possible evaluation through the AOAC Official Method SM program and launched a call for experts in 2- and 3-MCPD esters & Glycidyl esters (GE) analysis to participate on the Expert Review Panel (ERP). The ERP will review and evaluate candidate methods for First and/or Final Action Official Methods of Analysis SM status, which is expected for be finalised during the first half of 2018.

Table 3 summarises the test samples included in the pre-trial/trial, their sample coding and their corresponding food categories.

Table 3: Test items used in the pre-trial and the trial.

Food category	Food item	Sample code
(a) vegetable oils and fats and derived products such as margarine and similar products,	Oil Oil Fat	Pre-trial SAMPLE **1** SAMPLE **B** and **O - S001** SAMPLE **C** and **M - S002**
(b) foods for particular nutritional uses as defined in Directive 2009/39/EC of the European Parliament and of the Council (1) and **intended for infants and young children, including infant- and follow on formulae**	Infant formula	Pre-trial SAMPLE **2**
(c) fine bakery wares, bread and rolls	waffle 1 waffle 2 crackers	SAMPLE **E** and **K - S004** SAMPLE **F** and **I - S005** SAMPLE **G** and **L - S006**
(d) canned meat (smoked) and canned fish (smoked),	Not available	Not available
(e) potato- or cereal-based snacks, other fried potato-based products,	potato chips	SAMPLE **D** and **J - S003**
(f) vegetable oil containing foods and foods prepared/ produced with vegetable oils.	-	-

3.5.1 Preparation

The test items were prepared at JRC Geel from starting materials, acquired at a local supermarket. The content of MCPD esters and glycidyl esters was tested on a small subsample. Afterwards the materials, except the fat and oil sample, were frozen in liquid nitrogen and ground. The materials were sieved through a 1 mm metal sieve and homogenized. Aliquots of about 10 g were packed in plastic screw cap tubes and stored at –18 °C.

The oil sample was homogenized and filled in amber glass ampoules. The fat sample was melted, homogenized and filled in amber crimp cap vials.

3.5.2 Homogeneity and stability

The food test samples were tested for significant inhomogeneity, according to the IUPAC International Harmonized Protocol for the Proficiency Testing of Analytical Chemistry Laboratories [14].

Homogeneity experiments included samples analysis of 10 samples, randomly selected along the filling sequence among the amber glass vials prepared for dispatch. The duplicate analyses were performed in random order. The test material was rated sufficiently homogenous and no trend was observed. Details of the homogeneity tests are given in ANNEX 6. Homogeneity for the samples C and M (fat) was not tested in this study, as it had been proven in a previous collaborative trial [9].

The samples were dispatched in polystyrene containers with dry ice to maintain the samples frozen during shipping. Laboratories were requested to store the test materials at -18 °C upon arrival until analysis.

The stability of the analytes in the test materials from all different food categories was investigated in a detailed isochronous study, conducted by the EURL-PAHs in 2015 [16].

Nevertheless, a short stability study was performed with the test materials included in this collaborative trial. Randomly selected test samples were stored at two different conditions over the period from dispatch of the material to the end of the submission of the results.

The first set of samples was stored in a freezer at recommended conditions (~ -18 °C). The second set of samples was stored for the whole period of the study in a deep freezer at the reference conditions (~ - 80 °C). After the deadline for reporting of the results had expired, all samples were analysed in duplicate under repeatability conditions .

No significant difference of the analyte contents among the test samples was found (t-tests). Hence stability of the samples over the whole period can be assumed under the recommended conditions.

4. Evaluation of the submitted results

4.1 General

Six test materials in blind duplicates, in total 12 coded samples, were dispatched to the 10 participants, plus 1 ml labelled standard mix to be used as internal standard. The detailed SOP (Annex 8), instructions on handling of the samples and reporting (Annex 3), together with a "sample receipt form" (Annex 4) were included in the parcel as well.

After the deadline for reporting, nine participants (from the initial ten) returned their results. Unfortunately one participant reported severe technical problems with the instrumentation for a long period, therefore not being able to provide results even after the deadline for reporting.

The results are listed in ANNEX 7.

Statistical evaluation of the results was carried out with a ProLab software [15], according to ISO 5725-2 [18] and the Collaborative Study Guidelines of AOAC for blind (unpaired) replicates [19].

These guidelines recommend assessing the results in the following manner:

(i) visual inspection of the data for identification of irregular data (and removal of such data);

(ii) identification of outliers using numerical statistical tests.

Irregular or outlying data were discarded, if applicable. After evaluation of the data, the HorRat (Horwitz ratio) is calculated. HorRat is described for evaluation of collaborative studies. The HorRat is the ratio of the reproducibility relative standard deviation, expressed in percent (RSDR %) and the predicted reproducibility relative standard deviation, expressed in percent (PRSDR %) derived from the Horwitz function modified by Thompson.

The following guidelines should be used to evaluate the assay precision [19]:

HorRat ≤ 0.5— Method reproducibility may be in question due to lack of study independence, unreported averaging, or consultations.

0.5 < HorRat ≤ 1.5 Method reproducibility as normally would be expected.

HorRat > 1.5— Method reproducibility worse than normally expected: possible reasons should be critically looked at (e.g., were test samples sufficiently homogeneous, indefinite analyte or property?) and discussed this in the collaborative study report.

HorRat > 2.0— Method reproducibility is problematic. A high HorRat may result in rejection of a method because it may indicate unacceptable weaknesses in the method or the study.

4.2 Evaluation of the chromatograms

Participants were requested to send chromatograms for the analysed samples. The study organiser checked them for sufficient resolution between the analyte peaks and neighbouring peaks. Moreover, chromatograms were checked for consistency in the retention time of the 3-MCPD, 2-MCPD and 3-MBPD as analytes and for sufficient peak intensity.

4.3 Evaluation for deviations from the method and outlier check

The first step of the data evaluation was the identification of laboratories that deviated significantly from the analytical protocol either intentionally or unintentionally. A dedicated questionnaire had to be filled in by the participants in order to enable the organisers of the study to identify major deviations from the analytical procedure (Annex 5). Data obtained by the application of such procedures would be considered incompatible with data generated by the tested procedure. Such discordant data have to be removed from the data set according to ISO 5725-2. Thanks to the pre-trail step, such deviating datasets were not detected.

Evaluating the answers from the questionnaire, few other slight deviations were identified. However the organisers do not consider them as influencing the outcome:
- in 11.2 from SOP - use of polyacrylic acid (<1000 µm) and sand (48 - 150 mesh);
- in 8.10 from SOP - glass fibre filters instead of cellulose;
- in 11.3 from SOP - evaporation of solvent after PLE over night at 30 °C;
- in 11.4 point 8 from SOP - during the extraction with ethyl acetate, the phase separation was difficult to be seen; consequently addition of a small amount of a dye (10 µl Cochenille red A - E124) to each sample/standard.

The results from 9 participants underwent statistical data analysis (Grubbs tests applied to single suspects mean measurement values and Cochran test applied to any suspect repeatability variances). Due to the low number of datasets (9), maximum 1 outlier per test item could have been removed. Eight individual Cochran outliers were removed from 8 datasets, before calculation of the performance parameters.

4.4 Evaluation of the results - precision parameters

Statistical analyses were performed along the lines of ISO 5725-2 [18] with the help of the PROLab® software [15] and the outcome is presented on Table 4.

The precision data for most of the analyte-matrix combinations (15/18 on fat basis and 12/14 on product basis) were excellent.

With only one exception (GE in sample S005) the repeatability wasin all the cases satisfactory (1.3-10%) at the validated mass fraction levels.

The reproducibility standard deviations were lower than the truncated Horwitz prediction, resulting in outstanding HorRat values around 0.4-0.7. Only for the glycidyl esters' content in two test materials (S004 and S005) expressed on fat basis as well as on product basis, the HorRat values were close to 2 with relative standard deviations of 42-49% (Figure 4).

The mean values for the analyte (3-MBPD) in those two test materials were 79.1 µg kg^{-1} and 110.3 µg kg^{-1} respectively, falling well in the range of LOQs reported by most of the participants (Figure 5). We attribute this finding to the fact that some of the participants did not tune their instruments for higher sensitivity, as 100 µg kg^{-1} is the requested LOQ laid down in the Commission Recommendation for monitoring [20]. The assumption is supported by the detailed evaluation of the chromatograms for sufficient peak intensity, where we observed rather low intensity of the peaks for 3-MBPD in samples E, F, K and I.

To our knowledge this is the first collaborative trial for the determination of the 3-MCPD- and 2-MCPD esters as well as glycidyl esters, including test materials with very low contents of the analytes. The outcome revealed that content in the range of 100 µg kg^{-1} and lower in the fat remains challenging to be well reproducible in different laboratories.

Table 4a. Performance parameter for precision on fat base

	2-MCPDEs fat base						3-MCPDEs fat base						GEs fat base					
	S001	S002	S003	S004	S005	S006	S001	S002	S003	S004	S005	S006	S001	S002	S003	S004	S005	S006
Unit	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg
Method	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2
No. of labs submitted results	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
Type Grubb outliers																		
Type Cochran outliers		1					1					1	1	1	1			
No. of lab after elimination	9	8	9	9	9	9	8	9	9	9	9	8	8	8	8	9	9	9
Mean	283.2	285.3	1677.7	438.5	530	1176.9	569.5	724.7	4187.1	971.8	1095.3	2542.6	994.8	1455.2	265.8	79.1	110.3	2277.9
Target s.d.	54.8	55.1	248.3	79.4	93.3	183.7	99.2	121.7	540	156.1	172.8	353.4	159.3	220	51.9	17.4	24.3	321.9
Reproducibility s.d.	32.6	68.5	122.7	43	62.6	103.3	50.2	62.5	288.7	81.2	121.4	166.1	75.2	104.7	56.7	33.7	51.5	156.4
Repeatability s.d.	32.6	9.8	73.8	17.2	44.4	29.1	12.7	18.5	149.8	40.2	90.6	33.8	42.6	104.7	26.6	6.6	19.3	113.2
tr-Horwitz	19.3%	19.3%	14.8%	18.1%	17.6%	15.6%	17.4%	16.8%	12.9%	16.1%	15.8%	13.9%	16.0%	15.1%	19.5%	22.0%	22.0%	14.1%
Relative reproducibility s.d.	11.5%	14.4%	7.3%	9.8%	11.8%	8.8%	8.8%	8.6%	6.9%	8.4%	11.1%	6.5%	7.6%	8.6%	21.3%	42.7%	46.7%	6.9%
Rel. repeatability s.d.	11.5%	7.8%	4.4%	3.9%	8.4%	2.5%	2.2%	2.6%	3.6%	4.1%	8.3%	1.3%	4.3%	6.7%	10.0%	8.3%	17.5%	5.0%
Horrat	0.6	0.7	0.5	0.5	0.7	0.6	0.5	0.5	0.5	0.5	0.7	0.5	0.5	0.6	1.1	1.9	2.1	0.5

Table 4b. Performance parameter for precision on product base

	2-MCPDEs product base				3-MCPDEs product base				GEs product base			
	S003	S004	S005	S006	S003	S004	S005	S006	S003	S004	S005	S006
Unit	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg
Method	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2
No. of labs submitted results	9	9	9	9	9	9	9	9	9	9	9	9
Type Grubb outliers												
Type Cochran outliers	1		1				1		1			
No. of lab after elimination	8	9	8	9	9	9	8	9	8	9	9	9
Mean	456.2	113.9	141.1	135.2	1133.9	253	292.7	289.9	71.9	20.5	28.2	261.6
Target s.d.	82.1	25.1	30.3	29.2	178	49.8	56.3	55.9	15.8	4.5	6.2	51.2
Reproducibility s.d.	35.8	10.9	13.4	12.8	80.4	23.5	22.7	22	15	8.7	13.8	20
Repeatability s.d.	9.5	4.1	2.9	2.6	34.3	9.1	4.8	5.9	7.4	1.5	6.1	9.5
tr-Horwitz	18.0%	22.0%	21.5%	21.6%	15.7%	19.7%	19.3%	19.3%	22.0%	22.0%	22.0%	19.6%
Relative reproducibility s.d.	7.9%	9.5%	11.2%	9.5%	7.1%	9.3%	9.2%	7.6%	20.8%	42.5%	49.0%	7.6%
Rel. repeatability s.d.	2.1%	3.6%	6.2%	1.9%	3.0%	3.6%	4.7%	2.0%	10.3%	7.5%	21.6%	3.6%
Horrat	0.4	0.4	0.5	0.4	0.5	0.5	0.5	0.4	0.9	1.9	2.2	0.4

Figure 4. RSDr (black line), RSDR (blue line) and tr-Horwitz (red line) depending on the level of content of the analyte, expressed on fat base

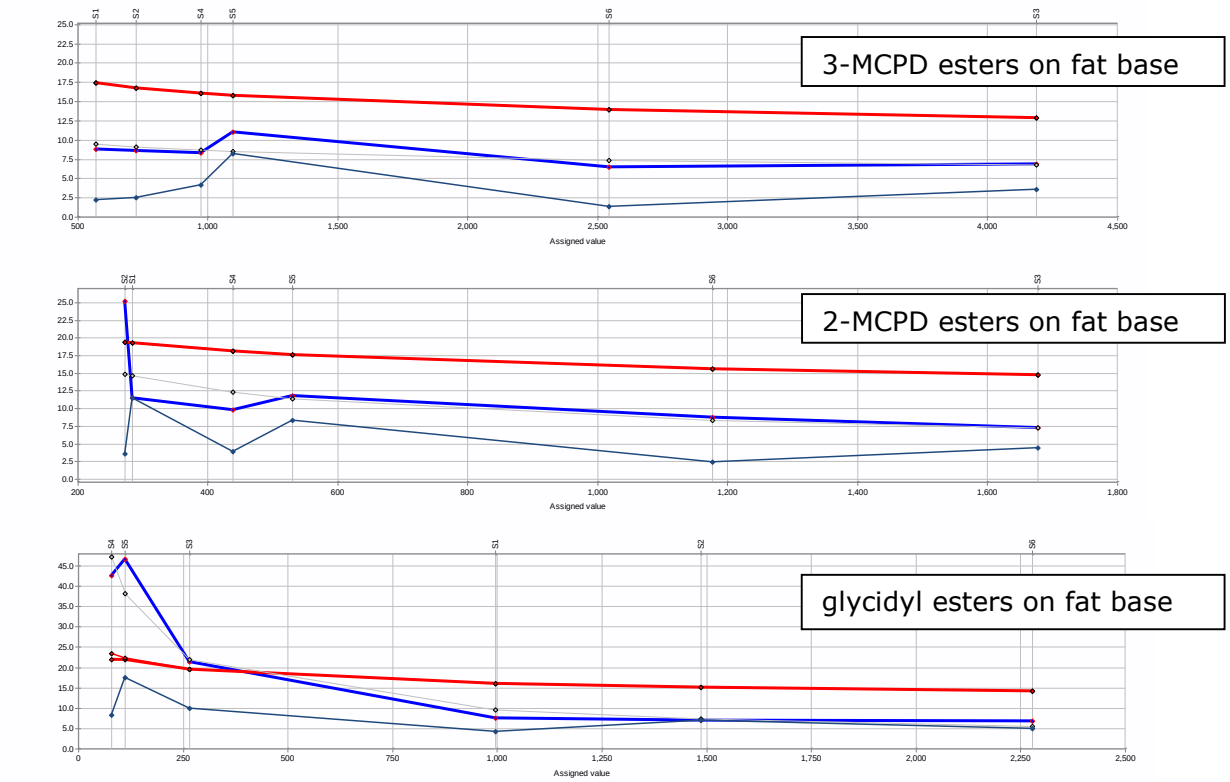
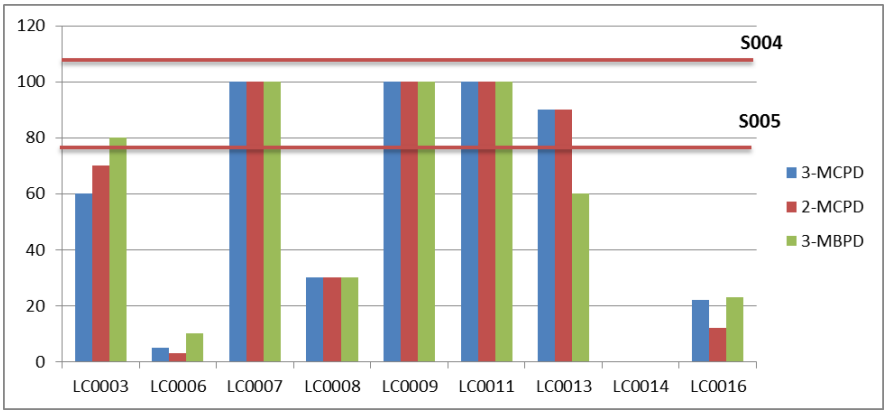


Figure 5. LOQs reported by the participants and 3-MBPD content in samples **S004** and **S005** (**red lines**).



5. Conclusions

Within this collaborative study for 3-MCPD-, 2-MCPD esters and glycidyl esters in thermally processed food, a standard operation procedure, prescribed by the EURL-PAH and Process Contaminants, based on acid transesterification and derivatization in organic phase, was tested by several laboratories from Europe, Asia and USA. The following is concluded:

- The method is suitable for the determination of 3-MCPD-, 2-MCPD esters and glycidyl esters in oil and fat, waffles, chips and crackers in the range of 250-2500 µg kg^{-1} fat corresponding to 100-1100 µg kg^{-1} product with HorRat values of 0.5-1.0;
- The determination of content lower and around 100 µg kg^{-1} fat still remains challenging with HorRat values of 1.9-2.2. This range may be tolerated as the range is close to the LOQ for most of the laboratories.

References

1 R. H Stadler, Monochloropropane-1,2-diol esters (MCPDEs) and glycidyl esters (GEs): an update, Current Opinion in Food Science (2015), 6:12–18

2 Crews C, Chiodini A, Granvogl M, Hamlet C, Hrnčiřík K, Kuhlmann J, Lampen A, Scholz G, Weisshaar R, Wenzl T, Jasti PR, Seefelder W, 2013. Analytical approaches for MCPD and glycidyl esters in food and biological samples: a review and future perspectives. Food Additives and Contaminants A, 30, 11-45.

3 EFSA, Analysis of occurrence of 3-monochloropropane-1,2-diol (3-MCPD) in food in Europe in the years 2009-2011 and preliminary exposure assessment, EFSA Journal 11 (2013) 3381.

4 IARC (International Agency for Research on Cancer), 2000. Monographs on the Evaluation of Carcinogenic Risks to Humans. Volume 77 Some Industrial Chemicals. Summary of Data Reported and Evaluation, Lyon, France, 469-486, Available online: http://monographs.iarc.fr/ENG/Monographs/vol77/ .

5 IARC (International Agency for Research on Cancer), 2012. Some Chemicals Present in Industrialand Consumer Products, Food and Drinking–Water. Volume 101. Lyon, France,:349-374. Available online: http://monographs.iarc.fr/ENG/Monographs/vol101/.

6 AOCS (American Oil Chemists Society), 2013a. 2- and 3-MCPD Fatty Acid Esters and Glycidol Fatty Acid Esters in Edible Oils and Fats by Acid Transesterification, AOCS Official Method CD 29a-13, Available online: https://aocs.personifycloud.com/PersonifyEBusiness/Default.aspx?TabID=251&productId=11827 2

7 AOCS (American Oil Chemists Society), 2013b. Determination of Bound Monochloropropanediol-(MCPD-) and Bound 2,3-epoxy-1-propanol (glycidol-) by Gas Chromatography/Mass Spectrometry (GC/MS), AOCS Official Method CD 29b-13. Available online: https://aocs.personifycloud.com/PersonifyEbusiness/Store/ProductDetails.aspx?productId=11827 3.

8 AOCS (American Oil Chemists Society), 2013c. Fatty-acid-bound 3-chloropropane-1,2-diol (3-MCPD) and 2,3-epoxi-propane-1-ol (glycidol), Determination in Oils and Fats by GC/MS (Differential Measurement), AOCS Official Method CD 29c-13, Available online: https://aocs.personifycloud.com/PersonifyEbusiness/Store/ProductDetails.aspx?productId=11827 5.

9 Karasek, L., Wenzl, T. & Ulberth, F, 2013,Report on the Interlaboratory comparison on the determination of MCPD and glycidyl esters in edible oils, European Commission, JRC, Publication office, Luxembourg

10 Hamlet, C. G., Asuncion, L., Velíšek, J., Doležal, M., Zelinková, Z. and Crews, C. (2011), Formation and occurrence of esters of 3-chloropropane-1,2-diol (3-CPD) in foods: What we know and what we assume. Eur. J. Lipid Sci. Technol., 113: 279–303. doi:10.1002/ejlt.201000480

11 Wenzl, T., Samaras, V., Giri, A., Buttinger G., Karasek, K. & Zelinkova Z. (2015). Development and validation of analytical methods for the analysis of 3-MCPD (both in free and ester form) and glycidyl esters in various food matrices and performance of an ad-hoc survey on specific food groups in support to a scientific opinion on comprehensive risk assessment on the presence of 3-MCPD and glycidyl esters in food. EFSA supporting publication 2015: EN-779, 78p. Available online: www.efsa.europa.eu/publications

12 Fry, H., Schödel, C., These, A. & Preiß-Weigert, A. (2013). Collaborative study for the determination of 3-MCPD- and 2-MCPD-fatty acid esters in fat containing foods – First collaborative study part II; Method validation and proficiency test. BfR, Wissenschaft, Berlin, Germany, 131p.

13 Z. Zelinkova, Anupam Giri, ThomasWenzl "Assessment of critical steps of a GC/MS based indirect analytical method for the determination of fatty acid esters of monochloropropanediols (MCPDEs) and of glycidol (GEs)" Food Control 77 (2017) 65-75, doi:10.1016/j.foodcont.2017.01.024

14 Thompson, M., Ellison, S.L.R., and Wood, R. 2006. The International Harmonised Protocol for the Proficiency Testing of Analytical Chemistry Laboratories. Pure Appl. Chem. 78(1):145–196. http://media.iupac.org/publications/pac/2006/pdf/7801x0145.pdf

15 Software for PT programs and collaborative studies, ProLab; http://quodata.de/en/software/for-interlaboratory-tests.html

16 Samaras V. and T. Wenzl*, Isochrones stability of MCPD- and glycidyl fatty acid esters in food. European Commission, Joint Research Centre, Health, Consumer & Reference Materials, Geel, Belgium (submitted for publication)

17 Thompson, M. 2000. Recent trends in inter-laboratory precision at ppb and sub-ppb concentrations in relation to fitness for purpose criteria in proficiency testing. Analyst 125:385-386.

18 Practical guide to ISO 5725-2:1994 — Accuracy (trueness and precision) of measurement methods and results — Part 2: Basic method for the determination of repeatability and reproducibility of a

standard measurement method. Geneva, Switzerland
19 Appendix L: AOAC Recommended Guidelines for Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) Single-Laboratory Validation, Official Methods of Analysis of AOAC INTERNATIONAL (current edition), AOAC INTERNATIONAL, Rockville, MD, USA. (http://www.eoma.aoac.org/app_l.pdf)
20 EC, Commission Recommendation 2014/661/EU of 10 September 2014 on the monitoring iof the presence of 2 and 3-monochloropropane-1,2-diol (2 and 3-MCPD), 2- and 3-MCPD fatty acid esters and glycidyl esters in food, OJEU L271 (2014) 93.

List of abbreviations and definitions

DG SANTE	Directorate General Health and Food Safety
EC	European Commission
EU	European Union
EFSA	European Food Safety Autority
EURL-PAHs	European Union Reference Laboratory for Polycyclic Aromatic Hydrocarbons
GC-MS/MS	Gas chromatography with tandem mass spectrometry
ILC	Interlaboratory comparison
ISO	International Organization for Standardization
JRC	Joint Research Centre
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantification
MRL	Maximum residue level
NRL	National Reference Laboratory
OCL	Official food control laboratory
SOP	Standard Operating Procedure
3-MCPDEs	fatty acid esters of 3-monochloropropane-1,2-diol
2-MCPDEs	fatty acid esters of 2-monochloropropane-1,2-diol
GEs	fatty acid esters of glycidol

ANNEX 1: Call for expression of interest

Ref. Ares(2016)6934666 - 13/12/2016

EUROPEAN COMMISSION
DIRECTORATE-GENERAL
JOINT RESEARCH CENTRE
Directorate F - Health, Consumers and Reference Materials
European Union Reference Laboratory for Polycyclic Aromatic Hydrocarbons

Geel, 13 December 2016
JRC.F.5/SB/acs/ARES(2016)

CALL FOR EXPRESSION OF INTEREST

MVS by collaborative trial
for determination of MCPD esters and GEs in thermally processed fatty food

Dear Madam, dear Sir,

In order to support the current regulatory follow up on 3-MCPD fatty acid esters (3-MCPDEs) and glycidyl fatty acid esters (GEs), discussed between the Commission and Member States, EURL-PAHs&process contaminants has been requested to organize of a method validation study (MVS) by collaborative trial of an indirect analysis method for the determination of MCPDEs and glycidyl esters in fatty food.

In the period of 2014-2015, EURL-PAH&process contaminants successfully developed/modified an analytical procedure for determination of MCPDEs and GEs in thermally processed fatty food. The method was internally evaluated via comparison with a direct method and some improvements have been suggested. Additionally the method was assessed via comparison with the methods used by other four expert laboratories (seven SOP in total).

The JRC methods was successfully applied to more than 600 different food samples covering breads and rolls, fine bakery wares, smoked fish and smoked meat, pan fried and roasted meat, potato based snacks and fried potato products, cereal based products, and margarines. The produced analysis results were reported to EFSA

EURL-PAH&process contaminants would like to lunch a call for expression of interest in participation of your laboratory in a collaborative trial for method validation, planned for 2017. We strongly encourage you to forward this call to other interested parties from official control laboratories, universities and industry. In order to increase the probability for success, we could like to create a pool of laboratories, with expertise in similar analytical procedures. For that purpose incentives of 1000-2000 Euro would be foreseen.

Having in mind the variations in different steps of the available methods for determination of MCPDEs and GEs in foodstuff, we would like to propose two options for the final

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Web site: https://ec.europa.eu/jrc/en/eurl/pahs

derivatisation step of the procedure - in aqueous (as it is in AOCS method) and in organic phase (modified to prevent the problems encountered by some laboratories from the high PBA content in the aqueous phase). Depending on the number of potential participants interested in one or another procedure, we will launch the respective SOP for validation by laboratories chosen after a pre-trial.

The pre-trial study will be organised in the beginning of 2017 (dispatch in January) and data collection until the end of February on 2 test samples - oil and infant formula. They are chosen based on the proposed EU regulation for those food commodities. For the pre-trial as well as for the trial, we will provide the participants with labled standard solutions. After evaluation of the results, a pool of expert laboratories will be chosen for participation in the collaborative trial for method validation (max 20).

Method performance specifications as laid down in Commission Recommendation 2014/661/EU will serve as target for the MVS. During the collaborative trial, planned for the period April-Jully 2017 we foresee to focus on six food matrices (16 food samples in total). Participants in the collaborative trial will be contacted.

In case you are interested for participation in the pre-trial, please fill in the following survey, which will not take you more than 5 minute.

https://ec.europa.eu/eusurvey/runner/MVS_MCPDE

Sincerely yours,

Stefanka Bratinova
EURL-PAHs and other process contaminants

Cc: H. Emons (JRC), F.Verstraete (SANTE)

ANNEX 2: Public consultation survey

MVS by collaborative trial for determination of MCPD esters and glycidyl esters in food

Fields marked with * are mandatory.

2017 MVS - MCPD esters and glycidyl esters in food - Call for expression of interest

EURL-PAH&process contaminants lunches a call for expression of interest in participation of your laboratory in a collaborative trial for method validation for determination of 3-MCPD fatty acid esters (3-MCPDEs), 2-MCPD fatty acid esters (2-MCPDEs) and glycidyl fatty acid esters (GEs). We strongly encourage you to forward this call to other interested parties from official control laboratories, universities and industry.

Having in mind the variations in different steps of the available methods for determination of MCPDEs and GEs in foodstuff, we would like to propose two options for the final derivatisation step of the procedure - in aqueous (as it is in AOCS method) and in organic phase (modified to prevent the problems encountered by some laboratories from the high PBA content in the aqueous phase). Depending on the number of potential participants interested in one or another procedure, we will launch the respective SOP for validation by laboratories chosen after a pre-trial.

The pre-trial study will be organised in the beginning of 2017 (dispatch in January) and data collection until the end of February on 2 test samples - oil and infant formula. They are chosen based on the proposed EU regulation for those food commodities. For the pre-trial as well as for the trial, we will provide the participants with labled standard solutions. After evaluation of the results, a pool of expert laboratories will be chosen for participation in the collaborative trial for method validation (max 20).

Method performance specifications as laid down in Commission Recommendation 2014/661/EU will serve as target for the MVS. During the collaborative trial, planned for the period April-Jully 2017 we foresee to focus on six food matrices (16 food samples in total). Participants in the collaborative trial will be contacted.

In case you are interested for participation in the pre-trial, please fill in the following survey:

SOPs, proposed for validation. The differences are only in the derivatisation part.

Standard_Operating_Procedure_MCPDEs_GEs_rev._organic_new_rev.pdf
Standard_Operating_Procedure_MCPDEs_GEs_aqua_new_rev.pdf

* 1. Are you interested in participation in a collaborative trial for validation of a method for determination of 2- and 3- monochlor-propane-diol fatty acid esters (MCPDEs) and glycidyl fatty acid esters (GEs) in foodstuff based on PLE with t-MBE and transesterification in acidic media

- YES
- NO

* Organisation

Department

* Address (for DHL shipment)

* City

* Postal code

* Country

* Name of the contact person

*Email

* Telephone (DHL requirement)

*2. Do you have previous experience with analysis for determination of 3- and 2-MCPD esters in foodstuffs?

- ☐ Yes, routine method in oil
- ☐ Yes, routine method in other foodstuff as well - specify matrices
- ☐ Limited experience in oil
- ☐ Limited experience in other foodstuff as well - specify matrices
- ☐ No experience

Specify matrices

*3. Do you have previous experience with analysis for determination of glycidyl esters in foodstuffs?

- ☐ Yes, routine method in oil
- ☐ Yes, routine method in other foodstuff as well - specify matrices
- ☐ Limited experience in oil
- ☐ Limited experience in other foodstuff as well - specify matrices
- ☐ No experience

Specify matrices

*4. What is the preferred media for derivatisation with PBA to be proposed for validation?

- ○ PBA in diethylether
- ○ PBA in acetone/water

*5. Are you going to participate in a collaboratibe trial (six matrices, 16 samples foreseen) with PBA derivatisation in

- ○ PBA in diethylether (organic phase)
- ○ PBA in acetone/water (aqueous phase)
- ○ Both

*6. Do you have the equipment/chemicals required to perform the analysis and report the results for the pre-trial? We are planning to dispatch standard solutions of mixed labled standards.

- ○ Yes
- ○ No

* 7. if NO, what else do you need??

8. Any comments, sugestions ?

ANNEX 3: Instructions to participants

EUROPEAN COMMISSION
DIRECTORATE-GENERAL
JOINT RESEARCH CENTRE

Directorate F - Health, Consumers & Reference Materials
European Union Reference Laboratory for Polycyclic Aromatic Hydrocarbons

Geel, 23th June 2017

Dear Participant,

On behalf of the EU-RL for PAH and other process contaminants, I would like to announce the opening of the collaborative trial study for the validation of a method for the determination of MCPDEs and GEs in food.

I thank you for joining the study and ask you, in order to obtain consistent results, to please follow all instructions included in the documents you received.

Outline of the study.

- The target analytes are 2-MCPD and 3-MCPD from MCPD ester; 3-MBPD from glycidyl ester. The participants are requested to report results on all of them.
- Each participant is provided with
  - Eight 50 ml plastic tubes (labeled **D, E, F, G, I, J, K, L**) containing about 10 g of - fried potato products, fine bakery ware, bread and crisps product test sample with fat content > 10g fat/100g product,
  - Two 5 ml amber glass ampules (**B, O**) containing 1 ml oil test sample
  - Two 5 ml amber glass crimp cap vials (**C, M**) containing 3 ml fat test sample
  - One 5 ml amber glass ampules containing 4 ml blank oil sample
  - One 5 ml amber glass ampules containing 1 ml labelled standard mix to be used as internal standard;
- Please check that the content of the parcel is complete and undamaged (and fill out and **e-mail** the enclosed receipt form).
- Please store goods at appropriate conditions (+4 °C) until the analysis. Let materials reach ambient temperature before use.
- Read **all** accompanying documents prior starting with the analysis. **THE METHOD PROTOCOL MUST BE FOLLOWED.** In case you would like to perform GC-MS/MS analysis, separate set of results should be sent in addition to the set obtained as requested by this SOP. The same refers to the derivatisation step. In case you would like to perform derivatisation in aqueous solution, separate set of results should be provided in addition to the set obtained following this SOP.
- Please make sure that all required instruments and consumables are at hand before starting the analysis.

Retieseweg 111, B-2440 Geel - Belgium. Telephone: (32-14) 571 211. http://.jrc.ec.europa.eu
Telephone: direct line (32-14) 571 229. Fax: (32-14) 571 783.

E-mail: jrc-eurl-pah@ec.europa.eu

- **Analyse each sample only once**. In case you encounter any problem during the analysis, please contact us for a replacement of the lost sample
- You will receive by mail some files for reporting results. Please follow the instructions below
- Please also send us the **chromatogram** for the samples and standards. They can be sent by e-mail (jrc-eurl-pah@ec.europa.eu)

The deadline for this collaborative trial is **15/08/2017** which gives a time period of **six weeks** for the analysis. We are looking forward to your feedback and hope that the method suits your needs for future use.

The method protocol (SOP) is included in the sample parcel.

Below you find further details on how to report your results. Anyhow we would like to encourage you to contact us in case you need further clarifications (see contact details at the end of this document).

Reporting the results

Data generated by the participants will be collected by using the software RingDat, supplementary to the ProLab software that has been used for professional data handling and statistical analyses of interlaboratory test results. You will receive by email two files for reporting the results. You should follow the instructions below:

- Download a simple data entry program (called RingDat) free from the QuoData web page using following link: http://quodata.de/ringdat_en.php

  User: *ringdat*
  Password: *prolabdata*

- Save the two lab specific files with the extension "***.Lab**" and "***.LA2**", generated by the ProLab software and provided to each individual laboratory (personal files attached to this email) to the same folder as RingData.exe.
- Start the RingDat.exe program and open "***.LAB**" file for reporting the results. A table will appear with cells for every measurand/sample combination:
  - The "***.LA2**" file contains information about the participant – laboratory name and laboratory number.
  - The "***.LAB**" file is unique to each laboratory (personal) and contains information about the samples and measurand that have to be analysed and reported.
  - The first tab contains detailed information for the laboratory.
  - The second tab contains a table for entering the results.
  - The third tab contains a general questionnaire.
- Fill in the results table with your data. Please find below some captures illustrating what has been explained above (Fig.1).

Please report the samples results in µg kg^{-1}, accompanied by its expanded uncertainty and date of analysis. You have the option to write comments for any analyte/matrix combination.

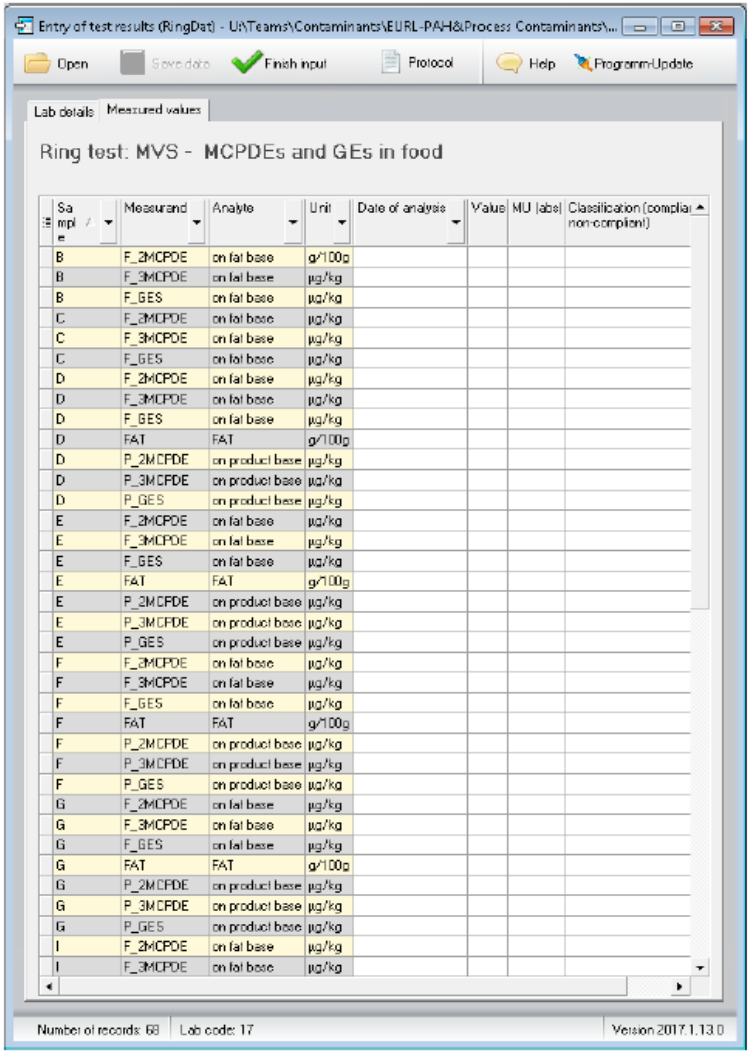

Figure 1 - Example of the "Measured Values" screen capture

Please report the fat content of the products in g/100g. For sample B, E, F, G, I, J, K, L analyte content expressed on both fat and product base should be reported in µg/kg

- Afterwards, please fill in the questionnaire on the next tab.
- After finishing the input, save **the file** using the button on the top menu of the window. You can change the inputs after saving the file as long as you haven't pushed the "Finish input" button. At the end finalise the data entry by pressing the "Finish input" button.
- Send both the "*.**LAB**" and "*.**LA**" files back to us by e-mail to our functional mail box - JRC-EURL-PAH@ec.europa.eu not later than **15th August 2017**
- **Should you want to correct some of your entries after finishing the input, you must use the original *.LAB file** downloaded from the email and introduce all the information again.

In case you need assistance, please do not hesitate to contact us as soon as possible.

Good luck with the analysis and thank you again for your participation.

With kind regards,

Stefanka Bratinova

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Directorate F – Health, Consumers and Reference Material
Retieseweg 111, 2440 Geel, Belgium
+32 (0)14 571 800
Stefanka-Petkova.BRATINOVA@ec.europa.eu

ANNEX 4. "Sample Receipt" Form

EUROPEAN COMMISSION
DIRECTORATE-GENERAL

JOINT RESEARCH CENTRE
Directorate F - Health, Consumers & Reference Materials
European Union Reference Laboratory for Polycyclic Aromatic Hydrocarbons

MATERIAL RECEIPT FORM

2017 MVS - MCPDEs and GEs in food

Contact person	
Affiliation	
City, Country	

Content of the parcel

1. Eight 50 ml plastic tubes (labeled **D, E, F, G, I, J, K, L**) containing about 10 g of - fried potato products, fine bakery ware, bread and crisps product test samples,
2. Two 5 ml amber glass ampules (**B, O**) containing 1 ml oil test sample
3. Two 5 ml amber glass crimp cap vials (**C, M**) containing 3 ml fat test sample
4. One 5 ml amber glass ampules containing 4 ml blank oil sample
5. One 5 ml amber glass ampules containing 1 ml labelled standard mix to be used as internal standard;
6. Labelled standard solution mixtures specification sheets;
7. Solvent safety data sheet;
8. One sample receipt form (= this form), which is e-mailed as well to be filed and send electronically
9. One standard operation procedure (SOP)

IF NOT ANALYSED IMMEDIATLY AFTER RECEIVING THE PARCEL, PLEASE PUT THE TEST SAMPLES IN THE FRIDGE (at +4 °C).

Please ensure that the items listed below have been received undamaged, and then describe the relevant statement:

Date of the receipt of the test materials	
All items have been received undamaged	YES / NO
If NO, please list damaged items according to the letters associated at each item in the list above Please write one item per row	
Items are missing	YES / NO
If YES, please list missing items according to the letters associated at each item in the list above	

Codes of the samples you received	**B**	__ __ __
	C	__ __ __
	D	__ __ __
	E	__ __ __
	F	__ __ __
	G	__ __ __
	I	__ __ __
	J	__ __ __
	K	__ __ __
	L	__ __ __
	M	__ __ __
	O	__ __ __
	Blank oil	__ __ __
	labelled standard mix	__ __ __

Please return the completed form by mail to

Stefanka Bratinova

Retiesseweg 111, B-2440 Geel - Belgium. Telephone: (32-14) 571 211. http://ec.europa.eu/jrc
Telephone: direct line (32-14) 571 229. Fax: (32-14) 571 3. 1800
mail: jrc- eurl-PAH@ec.europa.eu

ANNEX 5. Questionnaire & Answers from participants

Questions, sent to the participants in the pre-trail

Lab details | Measured values | Questions and Answers

No.	Cue	Question	Answer
1	Lab experience MCPDEs	For how long (years) your lab has been analysing food for MCPDEs?	
2	Lab experience GEs	For how long (years) your lab has been analysing food for GEs?	
3	Samples per year	How many samples does your lab analyse for MCPD esters and GE in food per year?	
4	Accreditation	Is your laboratory accredited for the analysis of MCPD esters and/or GEs s in food?	☐ Yes, for MCPD esters in oil/fat ☐ Yes, for MCPD esters and GEs in oil/fat ☐ Yes, for MCPD esters in foodstuff ☐ Yes, for MCPD esters and GEs in foodstuff ☐ No
5	Analyst experience.	How many years of experience does the analyst have with analysis of MCPDEs and GEs in food?	
6	Instructions	Did you find the instructions distributed for this MVS adequate?	○ Yes ○ No
7	If NO, improvments	If NO, which parts do you think could be improved?	
8	ProLab/RingDat interface	What do you think about the reporting by ProLab/RingDat?	
9	Problems?	Did you have any problems in using this platform?	○ No ○ Yes
10	If YES, what kind of problems?	If YES, what kind of problems?	
11	Any other comments	Any other comments you wish to address?	
12	Method description	Did you find the Method description (SOP) adequate?	○ Yes ○ No
13	If NO, improvements	If NO, in which part(s) it could be improved?	
14	Able to follow the method	Were you able to follow the method in all details?	○ No ○ Yes
15	If NO, deviations	If NO, which parts required deviation from protocol? Please include paragraph number and describe the deviation applied.	
16	Problems during analysis	Did you encounter any problems during the analysis?	○ No ○ Yes
17	If YES, what/were	If YES, what were the specific problems and to which samples did they apply?	
18	Analytical process splitted?	Was the analytical process split over staff (e.g. Extraction was done by Person#1, instrumental analysis by Person#2)?	
19	Abnormalities noticed	Did you notice any abnormality, that however seem to have no effect on the result?	○ No ○ Yes
20	If YES, please describe	If YES, please describe and report for which samples (codes) they occured	
21	Familiar with steps	Were you familiar by practice with all the steps performed during the analysis?	○ No ○ Yes
22	If NO, please describe	If NO, please describe and report for which steps (Please refer to the respective paragraph number in the SOP)	
23	Overnight stops	Did you need to include any "overnight" stops in the analysis of the MVS samples without performing new calibration when resuming the sequence?	○ No ○ Yes
24	If YES, for which samples	If YES, please state for which samples and at what stage of the analysis?	
25	Signal integration mode	How did you intergate the signals?	○ Automatic ○ Manual ○ Automatic with verification
26	Re-integration	If you integrated automatically, for how many chromatograms was it necessary to re-intergare analyte peaks? (If none, put 0)	
27	Calibration	please specify the supplier of your neat calibration standards	
28	Date cal.stand.sol. prep.	Please specify the date when your calibration standards were prepared - intermediate and working standard solution	
29	Date oil	When did you analysed the oil sample (extraction)	
30	Date IF	When did you analysed the IF sample (extraction)	
31	Sequence	Did you analysed both samples in one sequence and with 1 calibration curve?	
32	Any other information	Any other information that you would like to add?	

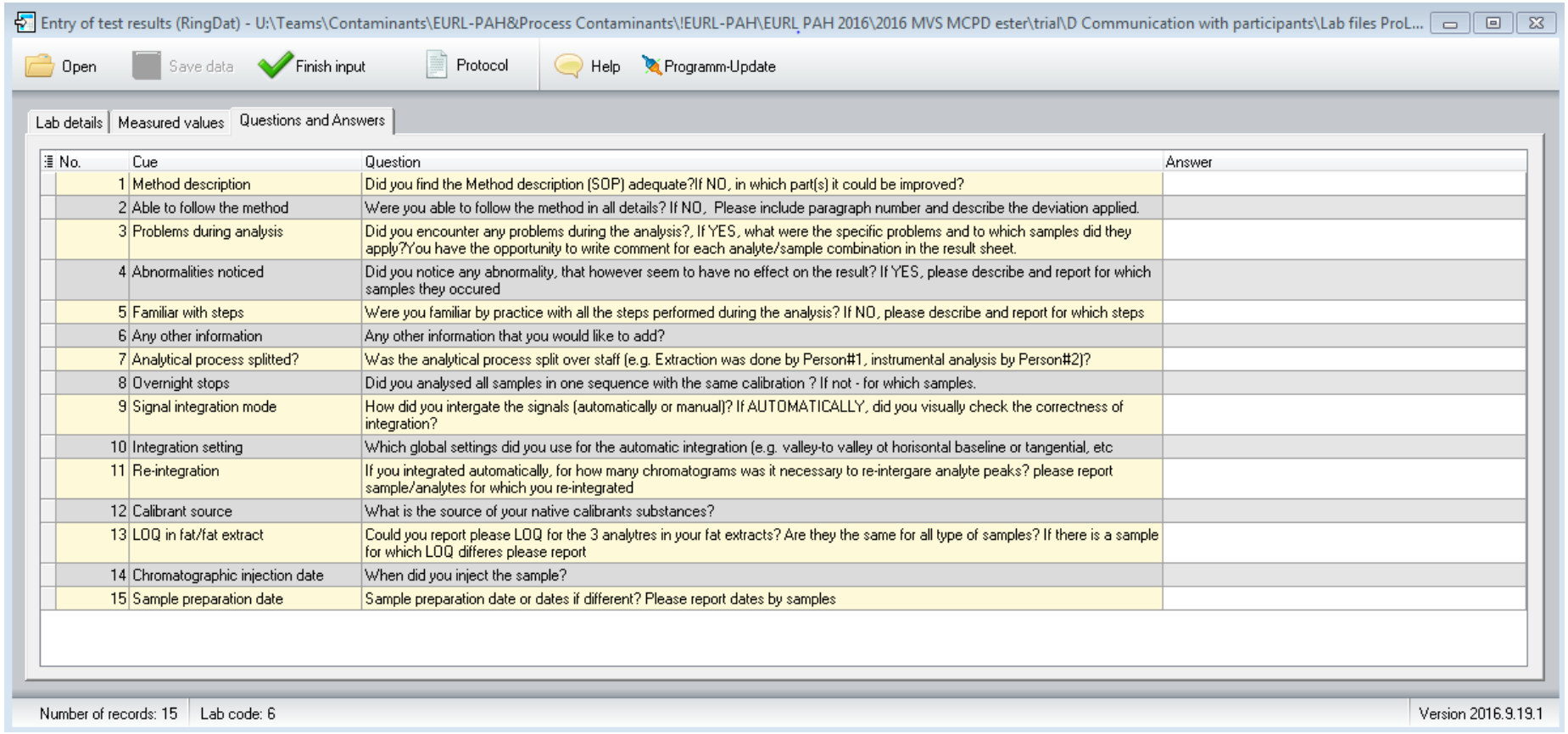
Answers from the participants in the pre-trial

Lab Code	1. Lab experience MCPDEs	2. Lab experience GEs	3. Samples per year	4. Accreditation	5. Analyst experience.	6. Instructions	7. If NO, improvements	8. ProLab/RingDat interface	9. Problems?	10. If YES, what kind of problems?
LC0003	5	3	20	Yes, for MCPD esters and GEs in oil/fat:	3	Yes		Good	No	
LC0005	2 years	2 years	250	No	2 years	Yes		effective	No	
LC0006	10	7	100	Yes, for MCPD esters in oil/fat: Yes, for MCPD esters and GEs in oil/fat: Yes, for MCPD esters in foodstuff: Yes, for MCPD esters and GEs in foodstuff:	5	Yes		uncomfortable usage	No	
LC0007	since 2009	since 2009	200	Yes, for MCPD esters in oil/fat: Yes, for MCPD esters and GEs in oil/fat: Yes, for MCPD esters in foodstuff: Yes, for MCPD esters and GEs in foodstuff:	5 years	Yes		practical	No	
LC0008	5	2	100	Yes, for MCPD esters in oil/fat: Yes, for MCPD esters and GEs in oil/fat: Yes, for MCPD esters in foodstuff: Yes, for MCPD esters and GEs in foodstuff:	5	Yes		LOQ and LOD of fat content make no sense	No	
LC0009	since 2007	since 2010	> 3000	Yes, for MCPD esters and GEs in foodstuff:	> 5	Yes			Yes	cannot open it, on network
LC0011	2005	2010	2600 in 2016	Yes, for MCPD esters in foodstuff:	12 years	Yes			Yes	Trying to save the data, everything disappeared; Consuming a lot of time; Error in the table F-MCPD on the basis
LC0013	2	1	10-20	Yes, for MCPD esters in oil/fat: Yes, for MCPD esters and GEs in oil/fat: Yes, for MCPD esters in foodstuff:	1-2	Yes		difficult to use because of problems with opening the program	Yes	McAfee Virenscanner did block opening the program
LC0014	10 years	10 years	30 samples	No:	1 years	Yes		no problem	No	
LC0016	14 years	9	<100	Yes, for MCPD esters in oil/fat: Yes, for MCPD esters in foodstuff:	10	Yes	N/A	Good	No	N/A
LC0017	6	6	300	No:	2.5	No	The instructions did not define "MU (abs)" (on the measured values tab).	OK	No	

Lab Code	11. Any other comments	12. Method description	13. If NO, improvements	14. Able to follow the method	15. If NO, deviations	16. Problems during analysis	17. If YES, what/were	18. Analytical process splitted?	19. Abnormalities noticed	20. If YES, please describe	21.Familiar with steps	22. If NO, please describe
LC0003	The proposed limits are expressed in mg/kg.	Yes		No	Insufficient IS solution to prepare the working standards as per SOP.	Yes	Qualifier ion for GEs, neither 146 nor 147 is suitable at low levels.	No	No		Yes	
LC0005		Yes		No	11.3 we did NOT perform the PLE (we do not have this equipment). We used the same solvents but we extracted with soxhlet. For that reason we only report the results in the extracted oil and not in the product.	Yes	The PBA did not dissolve for 100% in di-ethylether. Although the reaction seems to be good.	yes preparation and instrumental were split	No		No	11.3 we did NOT perform the PLE (we do not have this equipment). We used the same solvents but we extracted with soxhlet. For that reason we only report the results in the extracted oil and not in the product.
LC0006		Yes		Yes	GC-MS method better voluntary	Yes	s. our control material	no	No		Yes	
LC0007		Yes		Yes		No		No	No		Yes	
LC0008	11.4. 2-4 no SPE carried out //	Yes		Yes		No		no	No		Yes	
LC0009		Yes		No	ASE Extraction for IF: use Extrelut for Sand ; and n-Hexane for iso-Hexane	No		1	No		Yes	
LC0011		Yes		Yes		Yes	ASE causes discoloration and an aqueous phase; Method does not work for our in-house	1 technician analysing and calculation, 1 maintenance team	Yes	Different, unusual slope for the GE	Yes	
LC0013		Yes		Yes		Yes	Phase separation (ethyl acetate phase) at step 11.4 / 8. , addition of dye necessary (10µl cochenille red)	yes	No		No	11.4 step 2-7
LC0014		Yes		No	used polyacrylic acid (ſſ<1000um),Sand 20ſ-35 mesh	No		No	No		Yes	
LC0016		Yes	N/A	Yes	N/A	Yes	For both samples: SOP 11.4 point 8, The volume of EtOAc was increased to 3 x 1 ml; SOP 11.4 point 9, The solubility of PBA in Et2O was poor. We prepared and used (300 µl) a solution of PBA in EtOAc (0.2 g PBA in 10 ml EtOAc)	No	No	N/A	Yes	
LC0017		Yes		Yes		No		No	Yes	The 3-MBPD qualifier ion (m/z 174) was not present for any sample. However, we were able to quantify using m/z 240.	No	We typically run a direct method in our laboratory, so the indirect methods are somewhat unfamiliar in practice. However, we are fully knowledgeable of the techniques used.

Lab Code	23. Overnight stops	24. If YES, for which samples	25. Calibration	26. Date cal.stand.sol. preparation	27. Date oil	28. Date IF	29. Sequence	30. Signal integration mode	31. Intergaration	32. Re-integration	33. Any other information
LC0003	No		Supplier LGC, manufacturer TRC	Individual 23/01/2017, intermediate 14/02/2017 working stds 27/02/2017.	2/17/2028	2/17/2027	Yes	Automatic with verification	valley to valley (MassHunter agile 2)	Majority particularly qual ions for GEs.	
LC0005	No		TRC: Toronto Research Chemicals	2/06/2017	3/16/2017	3/16/2017	yes	Automatic	horizontal baseline	0	M(u) LOD and LOQ for this method unknown, method not validated
LC0006	Yes	1) after ASE evaporation 2) after SPE 3) Transesterification	TRC	16.02.2017	22.02.2017	22.02.2017	yes	Automatic with verification	valley-to-valley	0	Why using amber vials? Is rinsing of the vial at step 11.4.3/4 possible? Please specify the amount of Na2SO4. There is a wrong m/z for glycidyl in the method protocol (174 instead of 147). We used our own GC-MS method. We use Ottawa sand as fill material (20-30mesh), because the use of material with a higher mesh should be avoided according to our ASE manual.
LC0007	No		Toronto Research Chemicals	20.02.2017	22.02.2017	22.02.2017	Yes	Automatic with verification	horizontal baseline	50 % (including the chromatograms for the calibration standards)	The fat content in IF only determined by weighing the PLE-extract. The LODs and LOQs for Esters in IF are only calculated based on fat content.
LC0008	No		Chiron	01.03.2017	06.03.2017	06.03.2017	yes	Automatic with verification	horizontal baseline	0	The title is unclear, better "…..3-MCPD-, 2-MCPD- and ….." (with hyphen); it would be useful to receive native check standards
LC0009	Yes	Acidic transesterification for 16 h	TRC	28.02.17	7/3/2017	7/3/2017	yes	Automatic with verification	MassHunter	0 ; only for the prepared Blank	
LC0011	1		TRC	Stock solution Nov 2016, working sol. Feb 2017	05.04.2017	05.04.2017	yes	3	Baseline	CAL O all analytes + 3 additional chromatograms	FAPAS 2646 and FAPAS 2649 as reference material included; If higher fat content with in-
LC0013	Yes	all samples / 11.4 step 6	Biozol Diagnostics / EQ Laboratories GmbH	all prepared new in February 2017	15.03.2017	15.03.2017	yes	Automatic with verification	horizontal baseline (mainly)	about 2	
LC0014	Yes	Acidic transesterification	Wako, tronto research	13/03/2017	3/14/2017	3/14/2017	YES	Automatic	horizontal baseline	0	No
LC0016	No	N/A	TRC	06/01/2017	3/15/2017	3/15/2017	Yes	Manual	N/A	N/A	
LC0017	No		Toronto Research Chemicals	03/16/2017	3/16/2017	3/16/2017	Yes	Automatic with verification	Horizontal Baseline	12	As discussed via email the PLE procedure does not work for any U.S. formulas, but did work for the formula sample supplied in the pre-trial. In addition, the samples were initially prepared and analyzed using Gly-Ol (instead of Gly-P). The 3-MPBD results using Gly-Ol were inaccurate. The calibration curve samples were inconsistent and the concentrations for the oil and infant formula samples were very high, well outside the upper limit of the calibration curve. Using Gly-P produced an excellent calibration curve and 3-MBPD concentrations for the oil and infant formula samples that were very reasonable.

Questions, sent to the participants in the trail



No.	Cue	Question	Answer
1	Method description	Did you find the Method description (SOP) adequate?If NO, in which part(s) it could be improved?	
2	Able to follow the method	Were you able to follow the method in all details? If NO, Please include paragraph number and describe the deviation applied.	
3	Problems during analysis	Did you encounter any problems during the analysis?, If YES, what were the specific problems and to which samples did they apply?You have the opportunity to write comment for each analyte/sample combination in the result sheet.	
4	Abnormalities noticed	Did you notice any abnormality, that however seem to have no effect on the result? If YES, please describe and report for which samples they occured	
5	Familiar with steps	Were you familiar by practice with all the steps performed during the analysis? If NO, please describe and report for which steps	
6	Any other information	Any other information that you would like to add?	
7	Analytical process splitted?	Was the analytical process split over staff (e.g. Extraction was done by Person#1, instrumental analysis by Person#2)?	
8	Overnight stops	Did you analysed all samples in one sequence with the same calibration ? If not - for which samples.	
9	Signal integration mode	How did you intergate the signals (automatically or manual)? If AUTOMATICALLY, did you visually check the correctness of integration?	
10	Integration setting	Which global settings did you use for the automatic integration (e.g. valley-to valley ot horisontal baseline or tangential, etc	
11	Re-integration	If you integrated automatically, for how many chromatograms was it necessary to re-intergare analyte peaks? please report sample/analytes for which you re-integrated	
12	Calibrant source	What is the source of your native calibrants substances?	
13	LOQ in fat/fat extract	Could you report please LOQ for the 3 analytres in your fat extracts? Are they the same for all type of samples? If there is a sample for which LOQ differes please report	
14	Chromatographic injection date	When did you inject the sample?	
15	Sample preparation date	Sample preparation date or dates if different? Please report dates by samples	

Answers from the participants in the trial

Lab Code	1. Method description	2. Able to follow the method	3. Problems during analysis	4. Abnormalities noticed	5. Familiar with steps	6. Any other information	7. Analytical process splitted?	8. Overnight stops
LC0003	YES	YES	Standards prepared as directed in protocol, Samples D & J over ~20% calibration range for 3-MCPDE. Sample L ~5% over calibration range.	Chromatographic interference for sample M for both 2-MCPDE and d5-2-MCPDE.	YES	We found it necessary to filter the isooctane extract (we use Spin-X centrifuge filters) to prevent GC syringe needle blockage.	NO	YES
LC0006	YES	8.10 Glass fibre filters instead of Cellulose, 11.3 Evaporation of Solvent after PLE over night at 30°C	NO	NO	YES	6.1.16. mesh should not be higher than 20-30 according to the manufacturer of the PLE (ASE). 8.1. Why using amber glass?	NO	YES
LC0007	Yes	Yes	Not during this RV, but sometimes we can´t analyse the content of GE, because there is neither a signal for GE nor for the d-GE (ISTD). We do not know exactly the reason for this problem. Maybe there is a malfunction during the step of bromination.	No	Yes	All Samples were also measured in MRM-Mode (2-MCPDE 196.0->104.0; 3-MCPDE 196.0->147.0; GE 240.0 ->147.2). The results are comparable with the transmitted results (SIM-Mode).	No	No. Samples B, C, M, and O are analysed in a sequence w ith a separate calibration. Samples D, E, F, G, I, J, K, and L are analysed in a sequence w ith a separate calibration.
LC0008	Yes	Yes	No	No	Yes	Ion 240 for MPBD	No	Yes
LC0009	yes	yes	For MBPD m/z 240 is reported, because of blank problems on 242., But the results for 240 after blank compensation are also available	No	Yes.	The first removal of Ethylacetat Volume 600 µl was tricky	Only one person for the whole process	Two Sequences with two calibrations. One for oil samples and one for other food products
LC0011	Yes.	Yes.	No problems, some chromatograms not perfect.	No.	We normally do not use ASE. We perform AOCS 29a; fat extraction by Röse-Gottlieb (Mojonnier).	Detection works for GCMSMS also (in-house Method)	1 Person	Yes.
LC0013	yes	yes	yes, 11.4 point 8 Extractiion with ethyl acetate: The phase separation was difficult to be seen; that's why we added a small amount of a dye (10µl Cochenille red A - E124) to each sample/standard	No	Yes		Yes, Sample praparation Person1/ Instrumental Analysis Person2	No, 2 sequences with separate calibrations : B,C,M,O,D,E and F,G,I,J,K,L resp.
LC0014	yes.	no. In 11.2., I used Polyacrylic acid (<1000um) and Sand (48 - 150 mesh)	no.	no.	yes.	Ions (m/z 196 and 201) were used for 3-MCPD and 3-MCPD-d5 quantification, because m/z 147 baseline abundance was too high.	no.	yes.
LC0016	Would recommend supplying both ISTD and mixed calibration solutions to avoid uncertainties associated with standards prepared by different labs & chemicals from different sources.	Yes	No	No	Yes		Single analyst	All sample analysed on one sequence

Lab Code	9. Signal integration mode	10. Integration setting	11. Re-integration	12. Calibrant source	13. LOQ in fat/fat extract	14. Chromatographic injection date	15. Sample preparation date
LC0003	AUTOMATICALLY with manual corrections of intergration.	Agilent MassHunter Agile2	All	TRC (supplier LGC)	3-MCPDE 60µg/kg, 2-MCPDE 70µg/kg and GE 80µg/kg	06/07/2017	PLE extractions carried out 03/07/2017, bromination & hydrolysis carried out 05/07/2017, derivatisation 06/07/2017.
LC0006	Automatically with visually check	valley-to-valley		TRC Canada	2-MCPDE 5µg/kg, 3-MCPDE and GE 10µg/kg	06.07.2017	PLE: 03.07. SPE 04.07. Glycidyl conversion and transesterification 05.07. end of preparation 06.07.17
LC0007	Automatically w ith visual check	Horizontal baseline	more than 50 %	Toronto Research Chemicals	LOQ = 100 ìg/kg fat (for the 3 analytes in all samples)	07.07.2017 (B, C, M, and O); 18.07.2017 (D, E, F, G, I, J, K, and L)	04.07.-07.07.2017 (B, C, M, and O); 12.07.-17.07.2017 (D, E, F, G, I, J, K, and L)
LC0008	automatically with visual checking	horizontal baseline	all chromatogramms have been checked/corrected manually	Chiron Norway	30 µg/kg	7/5/2017	7/3 - 7/5/2017
LC0009	Integration is automatically and checked for correct integration	settings masshaunter default	blank samples need to be corrected native, but most are correct integrated	Toronto Research Chemicals TRC	LOQ-3MCPD [147]=100 µg/kg ; LOQ 2MCPD [196]= 100 µg/kg; LOQ MBPD [240]= 100 µg/kg	28.07.17 Food samples ; Oil samples 15.7.17	26.07.17 first Part; acidic transesterfication over night_27.07.17 second Part- for food samples / Oil samples 11 and 12.07.17 preparation
LC0011	Automatically + visually checked.	valley vallley horizontal	GCMS: 7 of 396; GCMSMS: 12 of 882	TRC	0.10 mg/kg result referring to fat/oil for all analytes	27th July	ASE: 24th July; Analysis: 25th+26th July; GCMSMS: 27th July; Calculation: 31st July
LC0013	Automatically with visually check of the correctness of intergration	integration of the Agilent MassHunter Quantitative Analysis Software		Biozol Diagnostics / EQ Laboratories GmbH	LOQ : 90µg/kg for MCPDE's and 60µg/kg for GE	same day after finishing sample preparation	
LC0014	Automatically. yes.	Horizontal baseline.	0	Wako Pure Chemical Industries, Ltd.	I didn't calculate LOQ.	26/07/2017	
LC0016	Manual	N/A	N/A	Labelled / unlabelled 3MCPDE from ICT Prague, Czech Republic; All other calibrants from TRC Canada.	LOQs for all samples: 3MCPDE, 22 µg/kg fat; 2MCPDE, 12 µg/kg fat; GES, 23 µg/kg fat	09-10/08/2017	N/A

ANNEX 6: Homogeneity

Mass fractions given here may be different from the consensus values of results of participants. These are rough estimates obtained with other calibration solutions. All data below is given in [µg/kg].

Sample B and O (oil)

Homogeneity according to IUPAC	Analyte		
	3-MCPD	2-MCPD	3-MBPD
Mean	0.47	0.26	1.02
$\hat{\sigma}$	0.082	0.050	0.163
σ^2_{all}	0.003	0.001	0.005
σ^2_{an}	0.025	0.015	0.049
critical value ($F_1\ \sigma^2_{all} + F_2\ \sigma^2_{an}$)	0.0042	0.0012	0.009
σ^2_{sam}	0.0008	0	0
σ^2_{sam} < critical	Passed	Passed	Passed

Sample D and J (potato chips)

Homogeneity according to IUPAC	Analyte		
	3-MCPD	2-MCPD	3-MBPD
Mean	4.24	1.88	0.32
$\hat{\sigma}$	0.547	0.278	0.061
σ^2_{all}	0.032	0.008	0.003
σ^2_{an}	0.164	0.083	0.018
critical value ($F_1\ \sigma^2_{all} + F_2\ \sigma^2_{an}$)	0.083	0.021	0.003
σ^2_{sam}	0.036	0.008	0.001
σ^2_{sam} < critical	Passed	Passed	Passed

Sample E and K (waffle 1)

Homogeneity according to IUPAC	Analyte		
	3-MCPD	2-MCPD	3-MBPD
Mean	0.72	0.45	0.11
$\hat{\sigma}$	0.116	0.081	0.023
σ^2_{all}	0.005	0.001	0.001
σ^2_{an}	0.035	0.024	0.007
critical value ($F_1\ \sigma^2_{all} + F_2\ \sigma^2_{an}$)	0.008	0.0017	0.001
σ^2_{sam}	0	0.0004	0
σ^2_{sam} < critical	Passed	Passed	Passed

Sample F and I (waffle 2)

Homogeneity according to IUPAC	Analyte		
	3-MCPD	2-MCPD	3-MBPD
Mean	0.95	0.54	0.07
$\hat{\sigma}$	0.150	0.095	0.016
σ^2_{all}	0.003	0.001	0.001
σ^2_{an}	0.045	0.029	0.005
critical value ($F_1\ \sigma^2_{all} + F_2\ \sigma^2_{an}$)	0.007	0.002	0.0004
σ^2_{sam}	0.001	0.0001	0.0001
σ^2_{sam} < critical	Passed	Passed	Passed

Sample G and l (cracker)

Homogeneity according to IUPAC	Analyte		
	3-MCPD	2-MCPD	3-MBPD
Mean	2.54	1.16	2.63
$\hat{\sigma}$	0.353	0.181	0.372
σ^2_{all}	0.190	0.012	0.056
σ^2_{an}	0.106	0.054	0.112
critical value ($F_1\ \sigma^2_{all} + F_2\ \sigma^2_{an}$)	0.213	0.018	0.080
σ^2_{sam}	0	0.003	0.006
σ^2_{sam} < critical	Passed	Passed	Passed

ANNEX 7: Reported results

	2-MCPDEs fat base						3-MCPDEs fat base						GEs fat base					
	S001	S002	S003	S004	S005	S006	S001	S002	S003	S004	S005	S006	S001	S002	S003	S004	S005	S006
Unit	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg
LC0003	274.0	223	1771.5	415	479	1181	638.5	773	4308.5	1019.5	1131.5	2669	1490	1430.5	299	71	95.5	2267
LC0006	288.0	295.4	1777.7	454.4	536.6	1243.3	505.8	619.9	3665.8	851.5	911.3	2210.1	1012	1499.4	270.4	90.6	158.3	2377.6
LC0007	274.5	337.5	1627	391	502	1113.5	584	743.5	4289	1010.5	1091	2498	968.5	1416.5	211	29.5	48.5	2385.5
LC0008	282.0	273	1714	431.5	541	1172.5	597	711.5	4283	1025.5	1167.5	2594	1016.5	1566.5	234.5	35	67.5	2430
LC0009	282.4	118.7	1626.2	476.4	590	1241.1	540.8	659.6	4589.3	1001.4	1124.1	2776.2	881.6	1237.8	278.8	77.7	110.1	2094.1
LC0011	259.0	268	1500.5	373.5	457.5	1004	605.5	749.5	4318.5	940.5	1096.5	2531.5	1129.5	1512.5	369	105.5	205.5	2384.5
LC0013	271.3	259.8	1535.8	435.4	483.4	1057.8	499.3	817.5	3911.9	865.7	971.8	1284.5	966.2	1548.7	261.4	66.6	87.5	2319.6
LC0014	295.5	294	1815	467.5	561.5	1290	585	688.5	4245	952	1100	2570	990	936	202	72.6	78.8	2165
LC0016	322.0	331.5	1732	501.5	619	1289	645.5	759.5	4073	1080	1264	2492	994.5	1430	558.5	141.5	141	2078
LC0017	not tested	not tested	not tested	not tested	not tested	not tested	not tested	not tested	not tested	not tested	not tested	not tested	not tested	not tested	not tested	not tested	not tested	not tested
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Method	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2

	2-MCPDEs product base				3-MCPDEs product base				GEs product base				fat content			
	S003	S004	S005	S006	S003	S004	S005	S006	S003	S004	S005	S006	S003	S004	S005	S006
Unit	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	g/100g	g/100g	g/100g	g/100g
LC0003	481.5	107	127	134.5	1171	263.5	300	303.5	81.5	18.5	25	257.5	27.2	25.8	26.5	11.4
LC0006	485.5	118.8	143.3	141.8	1001.1	222.6	243.4	252.2	73.8	23.7	42.4	271.3	27.3	26.2	26.7	11.4
LC0007	439.3	105.2	134.1	127.9	1158.1	271.6	291.3	287.1	57	7.9	12.9	273.9	27	26.9	26.7	11.5
LC0008	457.5	114	145.5	144	1143.5	270.5	315.5	318.5	62.5	9	18	298	26.7	26.4	27	12.3
LC0009	450.9	125.7	154.8	141.9	1273	264.3	294.9	317.5	77.3	20.5	28.9	239.6	27.8	26.4	26.3	11.4
LC0011	395.5	97.5	116.5	112	1139	246	280	282.5	97.5	27.5	52.5	266	26.4	26.2	25.6	11.2
LC0013	413.9	107.2	90.5	120.1	1053.7	213.6	183.7	268.5	70.6	16.5	17.3	263.1	26.9	24.7	17.9	11.3
LC0014	494.5	121	147	145.5	1160	246.5	288.5	291	55.1	18.8	20.7	245	27.3	25.9	26.2	11.4
LC0016	470.5	129	160.5	149	1106	278	328	288	151.5	36.5	36.5	240	27.1	25.7	25.9	11.6
LC0017	not tested	not tested	not tested	not tested	not tested	not tested	not tested	not tested	not tested	not tested	not tested	not tested	not	not tested	not tested	not tested
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Method	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-2	ISO 5725-	ISO 5725-2	ISO 5725-2	ISO 5725-2

ANNEX 8:

Standard Operating Procedure (as provided to participants)

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Directorate F – Health, Consumers & Reference Materials
Food & Feed Compliance (F.5)

Standard Operating Procedure for the Simultaneous Determination of

3-MCPD, 2-MCPD and Glycidyl Fatty Acid Esters in Various Food Matrices by Derivatisation in Organic Phase

In-house validated by the EC-JRC
2016

Contents

1. SCOPE AND APPLICATION

This standard operating procedure (SOP) specifies an indirect method for the simultaneous determination of fatty acid esters of 2-Chloro-1,3-propandiol (2-MCPD), 3-Chloro-1,2-propandiol (3-MCPD) and of glycidol in a wide variety of food products, with a fat content of more than 5 %, after extraction by pressurised liquid extraction (PLE), acid transesterification and derivatisation of the released free (non-esterified) form with phenylboronic acid (PBA). The PBA derivatives are consecutively measured by gas chromatography mass spectrometry (GC-MS) with electron ionisation (EI) in selected ion monitoring mode (SIM). Quantification of the analytes is carried out using 3-MCPD-ester-d5 (rac 1,2-bis-palmitoyl-3-chloropropanediol-d5), 2-MCPD-ester-d5 (1,3-Distearoyl-2-chloropropanediol-d5) and Gly-O-d5 (pentadeuterated glycidyl oleate) as internal standards. Results are expressed as free forms of 2-MCPD, 3-MCPD and as glycidol. The working range of the method is 25 µg kg^{-1} – 4 mg kg^{-1} for free forms of 2-MCPD and 3-MCPD and 12.5 µg kg^{-1} – 2 mg kg^{-1} for free form of glycidol. The method was applied for quantification of the three groups of analytes in eight different food categories including: a) bread and rolls, b) fine bakery wares, c) smoked fish products, d) fried and roasted meat; e) potato based snacks and fried potato products, f) cereal-based snacks, and g) margarines

2. PRINCIPLE

The test sample is immersed in liquid nitrogen and then grinded and homogenized, by means of a laboratory grinder or mortar and pestle, to a fine homogeneous powder. A test portion (5 g) is mixed with polyacrylate (5 g) and sand (15 g) and transferred into the extraction cell. The fat fraction is then extracted with tert-butyl methyl ether (tBME) by pressurised liquid extraction at a temperature of 40 °C. Then the organic extract is evaporated until constant weight is reached. The extracted amount of fat is determined gravimetrically. An aliquot of the fat extract (100 ± 5 mg) is dissolved in anhydrous tetrahydrofuran and mixed with internal standards (rac 1,2-bis-palmitoyl-3-chloropropanediol-d5, 1,3-distearoyl-2-chloropropanediol-d5 and pentadeuterated glycidyl oleate). Olive oil and margarines, consisting mostly of lipids, are directly spiked with stable isotope labelled internal standards and vortex-mixed with anhydrous tetrahydrofuran. A solid-phase extraction is performed afterwards in case of presence of partial acylglycerols, in particular MAGs.

Glycidyl esters are firstly converted into 3-monobromopropanediol (3-MBPD) monoesters in an acidic solution of sodium bromide. In the next step ester-bound analytes are transesterified with methanol in acidic medium. The transesterification is stopped by saturated sodium hydrogen carbonate solution. Methanol is then evaporated under a nitrogen stream at 40 °C and aqueous ammonium sulphate solution is added. Thereafter, the sample is defatted with n-hexane and the released free MCPD and glycidol are extracted with ethyl acetate and derivatised with phenylboronic acid. The samples is evaporated to dryness and re-dissolved in isooctane prior to GC-MS analysis.

Injection is performed in pulsed splitless mode. The chromatographic separation is obtained on a 5% diphenyl, 95% dimethyl polysiloxane column (30 m x 0.25 mm internal diameter and 0.25 µm film thickness capillary column). The analytes are ionised by electron ionization (EI) at 70 eV. The target analytes are recorded in Single Ion Monitoring (SIM) mode, and quantified by using the isotopically labelled internal standards.

The system is calibrated with MCPD esters and glycidyl esters, which are subjected to transesterification and derivatisation prior to measurement.

3. DEFINITIONS

Laboratory sample: sample as prepared for sending to the laboratory and intended for inspection or testing (i.e. the sample or subsample(s) received by the laboratory).

Test sample: sample prepared from the laboratory sample and from which test portions will be taken.

Test portion: the quantity of material drawn from the test sample and on which the test or observation is actually carried out (i.e. for this study the test portion is of 5 g).

Final extract: solution containing the analytes; obtained after the last evaporation step and reconstitution of the extract.

Labelled analogue: Stable isotope labelled analogues of MCPD and glycidyl esters. The labelled analogues are used to correct the losses of native compounds during analysis.

Quantifier ion (Q_1): ion monitored for quantifying the analytes.

Qualifier ion (Q_2): ion monitored in for confirmation of identity.

Procedural blank: a sample made up of all reagents foreseen for the preparation of a test portion and processed in all respects as a test portion. This kind of blank, tests the purity of the reagents but also other possible sources of contamination, like the glassware and the analytical instrument.

4. SAFETY

Protective equipment such as laboratory coat, and safety glasses have to be used. All handlings of reagents and organic solvents should be performed in a fume hood with adequate air flow.

3-MCPD is considered a potential carcinogen and just like its derivatives it is irritating to eyes, respiratory system and skin. Persons using these instructions should be familiar with normal laboratory practise. It is the responsibility of the user of these instructions to apply safety and health practices which are in agreement with the local requirements.

5. STANDARDS

The list of native substances and labelled analogues applied for the quantification of the target compounds included in the scope of this SOP are listed in Table 1.

5.1. Reference Substances

Table 1. Name, CAS number, molecular formula and molecular weight of native and labelled analytes

Name	Acronym	CAS #	Molecular formula	Molecular weight (g/mol)
rac 1,2-bis-palmitoyl-3-chloropropanediol	3-MCPD ester	51930-97-3	$C_{35}H_{67}ClO_4$	587.36
rac 1,2-bis-palmitoyl-3-chloropropanediol-d5	3-MCPD-d5 ester	1185057-55-9	$C_{35}H_{62}D_5ClO_4$	592.39
1,3-distearoyl-2-chloropropanediol	2-MCPD ester	26787-56-4	$C_{39}H_{75}ClO_4$	643.46
1,3-distearoyl-2-chloropropanediol-d5	2-MCPD-d5 ester	-	$C_{39}H_{70}D_5ClO_4$	648.49
glycidyl palmitate	Gly-P	7501-44-2	$C_{19}H_{36}O_3$	312.48
glycidyl oleate-d5	Gly-O-d5		$C_{21}H_{33}D_5O_3$	343.56

All 3-MCPD ester, 2-MCPD ester, Gly-P, 3-MCPD-d5 ester, 2-MCPD-d5 ester and Gly-O-d5 stock solutions are prepared in toluene and stored at 4 °C in the dark. The intermediate solutions of native substances and internal standards are prepared by diluting the stock solutions with toluene.

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

Other diesters of 2-MCPDand glycidol can be used as well.

6. CHEMICALS

6.1. General

Use only reagents of recognized analytical quality/standard, unless otherwise specified.

Note: Commercially available solutions with equivalent properties to the reagents listed may be used.

For storing of substances and commercially available solutions, supplier indications are followed. For opened commercial solutions or for in-house prepared solutions, the indications given in this procedure are intended to minimise the evaporation of the solvent and to protect the analytes from degradation.

For the preparation of solutions of native or labelled compounds, a microbalance is used. All quantities are expressed as mass concentration (weight/volume). Intermediate standard solutions are prepared volumetrically. All solutions and substances are being used at 20 °C.

6.1.1. Tetrahydrofuran, anhydrous

6.1.2. Methanol, analytical grade

6.1.3. *n*-Hexane, analytical grade

6.1.4. Ethyl acetate, analytical grade

6.1.5. 2,2,4-trimethylpentane (Isooctane), analytical grade

6.1.6. Diethyl ether, analytical grade

6.1.7. Toluene, analytical grade

6.1.8. *tert*-Butyl methyl ether (tBME), analytical grade

6.1.9. Water, grade I according to ISO 3696:1995 (Millipore Milli-Q)

6.1.10. Sulphuric acid (purity ≥ 95 %)

6.1.11. Sodium hydrogen carbonate (purity ≥ 99 %)

6.1.12. Sodium sulphate, anhydrous granular (purity ≥ 99 %)

6.1.13. Ammonium sulphate (purity ≥ 99 %)

6.1.14. Phenylboronic acid (PBA)(purity ≥ 97 %)

6.1.15. Sodium bromide, anhydrous (purity ≥ 99.5 %)

6.1.16. Sand, 50 – 70 mesh particle size

6.1.17. Sodium polyacrylate, Poly(acrylic acid), partial sodium salt-graft-poly(ethylene oxide) granular, 90-850 µm particle size

6.1.18. Reference material for quality control. A self-prepared test material may be applied for this purpose.

6.1.19 3 mL SPE cartridges with 500 mg aminopropyl (NH_2) e.g. Thermo Scientific ™ HyperSep ™.

6.1.20 Acetone, analytical grade

6.1.21 petroleum ether, boiling point 40- 60 °C, analytical grade

6.1.22 *iso*-Hexane, (2-methylpentane), analytical grade

7. GASES

7.1. Helium purified compressed gas (purity equivalent to 99.999 %)

7.2. Nitrogen purified compressed gas

8. APPARATUS

8.1. 10 ml amber glass vials with PTFE layered screw caps

8.2. Microbalance (if available), with a readability of 0.00001 g

8.3. Analytical balance, with a readability of 0.0001 g

8.4. Laboratory balance, with a readability of 0.01 g

8.5. Porcelain mortar and pestle, capacity of the mortar shall be at least 200 ml.

8.6. Ultrasonic bath

8.7. Vortex test tube shaker

8.8. Pressurised liquid extraction (PLE) apparatus comprising the following:

8.9. PLE cells, with 34 ml of volume

8.10. Cellulose Filters, 30 mm diameter

8.11. Sample carousel

8.12. Degasser

8.13. Extraction chamber

8.14. Solvent collection bottles, 250 ml of volume

8.15. Pressure control device, for the supply and release of the pressurizing gas in the extraction cell

8.16. Temperature control device

8.17. Instrument control and data processing system

8.18. Evaporation apparatus: Rotary evaporator capable of evaporation under controlled temperature and vacuum. The evaporation apparatus shall be equipped with either round bottom flasks or glass tubes of appropriate volumes: approximately 250 ml for the evaporation of PLE extracts (approximately 100 ml).

8.19. Glass Pasteur capillary pipettes, 230 mm length

8.20. Centrifuge

8.21. (Ceramic) knife or scalpel

8.22. Gas-chromatography – mass spectrometry (GC-MS) apparatus including computerised instrument control and data evaluation

8.23. Amber glass volumetric flasks, class A

9. STANDARD PREPARATION

The stock standard solutions are prepared gravimetrically. Intermediate solutions may be prepared volumetrically.

The presented standard concentrations are indicative only! Correct values have to be calculated based on the exact concentrations after weighing. The standard concentrations have also to be corrected for purity of the reference substances.

9.1. Single-substance stock solutions of native esters

Prepare for native 3-MCPD esters, 2-MCPD esters, and glycidyl esters (listed in Table 1) a solution in toluene (6.1.7) with a concentration of ~ 1 mg/ml. The single standard stock solutions are prepared by weighing of 10 (±0.1) mg of each neat substance using the microbalance (8.2). The substance is transferred into a flask 10 mL, toluene is added and weighed on an analytical balance. To dissolve the substances, each solution shall be sonicated for a couple of minutes. These solutions will be used for the preparation of

calibration standards. Table 2 provides an overview on the standard concentrations of the ester-bound analytes and on the concentration, which is equivalent to the free form.

The concentration of the stock solution is calculated using the mass fraction of the stock solution and the density of toluene (δ = 867 kg/m[3)].

Table 2: List and concentrations of single substance stock solutions of esters

Standard number	Substance	Concentration	Free form equivalent
9.1a	3-MCPD ester	1.0 mg/ml	188.2 µg/ml
9.1b	2-MCPD ester	1.0 mg/ml	171.8 µg/ml
9.1c	Gly-P	1.0 mg/ml	237.1 µg/ml

9.2. Single-substance stock solutions of stable isotope labelled MCPD and glycidyl esters

Prepare, from stable isotope labelled 3-MCPD ester, 2-MCPD ester, and glycidyl ester (listed in Table 1), a solution in toluene (6.1.7) with a concentration of 1 mg/ml.

Calibration and analysis of samples are designed in a way that the exact concentration of the stock standard solution of stable isotope labelled esters is of minor importance. It is however of paramount importance to add both to the calibration solution and to the sample the same amount of stable isotope labelled standard.

The single standard stock solutions are prepared by weighing of 10 (±0.1) mg of each neat substance using the microbalance (8.2). The substance is transferred into a flask 10 mL toluene is added and weighed on an analytical balance. To dissolve the substances, each solution shall be sonicated for a couple of minutes. These solutions will be used for the preparation of the internal standard solution and for matrix matched calibration standards. Table 3 provides an overview on the standard concentrations of the ester-bound analytes and on the concentration which is equivalent to the free form.

The concentration of the stock solution is calculated using the mass fraction of the stock solution and the density of toluene (δ = 867 kg/m^3).

Table 3: List and concentrations of single substance stock solutions of stable isotope labelled esters, including concentration expressed as free 3-MCPD, 2-MCPD, glycidol equivalents

Standard number	Substance	Concentration	Free form equivalent
9.2a	3-MCPD-d5 ester	1.0 mg/ml	195.1 µg/ml
9.2b	2-MCPD-d5 ester	1.0 mg/ml	178.2 µg/ml
9.2c	Gly-O-d5	1.0 mg/ml	230.3 µg/ml

9.3. Mixed intermediate labelled MCPD and glycidyl ester solution

Prepare, with the individual stock solutions of labelled esters (9.2a, 9.2b, 9.2c) listed in Table 3 a mixed intermediate solution in toluene with a concentration of approximately 25 µg/ml glycidyl esters and 50 µg/ml for MCPD esters by pipetting 1250 µl of standard stock solutions (9.2c) and 2500 µl each of the of standard stock solutions (9.2a, 9.2b) into a 50 ml volumetric flask (8.23) and fill up to mark with toluene.

9.4. Mixed intermediate solution of native MCPD and glycidyl esters

Prepare, from the single-substance stock solutions of native esters (9.1a, 9.1b, 9.1c), a mixed intermediate solution in toluene with a concentration of approximately 25 µg/ml glycidyl esters and 50 µg/ml for MCPD esters by pipetting 1250 µl of standard stock solutions (9.1c) and 2500 µl each of the of standard stock solutions (9.1a, 9.1b) into a 50 ml volumetric flask (8.23) and fill up to mark with toluene.

9.5. Preparation of calibration standards

Table 4 provides a scheme for the preparation of calibration standards for the analyte content range of about 100 µg kg^{-1} – 4 mg kg^{-1} for free forms of 2-MCPD and 3-MCPD and 50 µg kg^{-1} – 2 mg kg^{-1} for free form of glycidol in extracted fat. The amount of intermediate solution is pippeted into a 10 mL flask and the flask is filled up to the mark with toluene. The concentration values given in Table 4 are target values. The actual concentrations have to be calculated based on the actual concentration of the stock solutions.

Table 4: Preparation scheme for calibration standards

	Volume of mixed intermediate solution of native MCPDEs and GEs (9.4)	Nominal concentration of MCPDs (free form)	Nominal concentration of Glycidol (free form)
	mL	µg/mL	µg/mL
Cal 1	0.1	0.1	0.05
Cal 2	1	1	0.5
Cal 3	2	2	1
Cal 4	3	3	1.5
Cal 5	4	4	2

Table 5: MW values for the used MCPD and glycidyl esters and their corresponding free forms

	MW ester	**MW free form**
3-MCPD ester	587.36	110.54
3-MCPD-d5 ester	592.39	115.57
2-MCPD ester	643.46	110.54
2-MCPD-d5 ester	648.49	115.57
Gly-P	312.48	74.08
Gly-O-d5	343.56	79.11

9.6. Preparation of labelled MCPD and glycidyl ester solution

2 mL of mixed intermediate labelled solution (9.3) is pippeted into a 10 mL flaked and the flask is filled up to the mark with toluene.

100 µL of this solution will be used for the spiking into the fat extract and for calibration (for internal standardisation). Store this solution in the dark and at a temperature below 10 ºC. A solution stored in this way is stable for at least two months. If longer stability is proven, the solution can still be applied.

9.7. Preparation of matrix matched calibration

A matrix matched calibration is performed. Therefore 100 µl of each calibration standard and 100 µl of mixed labelled ester solution (9.6) is added to 100 mg of blank oil. Additionally one 'zero' level sample is prepared by adding 100 µl of toluene and 100 µl of mixed labelled ester solution (9.6) is added to 100 mg of blank oil. These samples undergo the same treatment as normal samples starting from point 11.4.

The addition of 100 mg of blank oil to the calibration samples improves the robustness of the method, since the oil matrix helps retain the analytes during the evaporation step.

10. SOLUTIONS

10.1. Glycidyl conversion

1. ***Acid aqueous solution of sodium bromide (3 mg/ml).*** Prepare a concentrated aqueous solution of sodium bromide by dissolving 1 g of sodium bromide (6.1.15) in 10 ml of ultra-pure water (6.1.9). Transfer 180 µl of the concentrated solution into a 10 ml volumetric flask (8.23). Add 5.5 ml of ultra-pure water (6.1.9) and afterwards 0.3 ml of sulphuric acid (6.1.10). Shake vigorously.

It is advisable to freshly prepare the solution on daily basis

2. ***Sodium hydrogen carbonate solution (0.6 %, w/v).*** Weigh 0.6 g of sodium hydrogen carbonate (6.1.11)in a 100 ml volumetric flask and fill up to the mark with ultra-pure water (6.1.9). Use ultrasonic bath to ensure the complete dissolution of the reagent.

10.2. Acid transesterification

1. ***Transesterification reagent*****:** Sulphuric acid/methanol solution (*1.8 % v/v*). Pipette 50 ml of methanol (6.1.2) into an empty 100 ml volumetric flask, add afterwards 1.8 ml of sulphuric acid (6.1.10) and fill then up to mark with methanol. (Note 1)

It is advisable to freshly prepare the solution on daily basis

2. ***Stop reagent***: Sodium hydrogen carbonate solution (saturated). Weigh 4.8 g of sodium hydrogen carbonate (6.1.11) in a 50 ml volumetric flask and fill up to mark with ultra-pure water (6.1.9). Use an ultrasonic bath to ensure the dissolution of the reagent.

10.3. Ammonium sulphate solution

Weigh 20 g of ammonium sulphate (6.1.13) in a 50 ml volumetric flask and fill up to the mark with ultra-pure water (6.1.9). Use ultrasonic bath to ensure dissolution of the reagent.

10.4. Derivatisation reagent

Dissolve 0.4 g of phenylboronic acid (PBA) (6.1.14) in 10 ml of diethyl ether (6.1.6). Shake vigorously.

Some batches of PBA do not dissolve in this solvent. Another batch has to be used in such case.

11. PROCEDURE

11.1. Sample treatment

As a general precaution, all of the sample material received by the laboratory shall be used for obtaining a representative and homogeneous laboratory sample without introducing contamination.

11.2. Test sample preparation

a) To obtain the test portion weigh, 5 g ± 0.1 g of the homogenised test sample into an aluminium weighing boat. Add 5 g of polyacrylic acid (6.1.17) and 15 g of sand (6.1.16). Mix thoroughly until the sample is finely ground and visually homogeneous.

The sand and the polyacrylic acid are weighed with a laboratory balance.

b) Oils and margarines, consisting mostly of lipids, skip point 11.3 and follow directly from point 11.4.

11.3. Sample extraction by PLE

Transfer the test portion into the extraction cell of the PLE apparatus after having checked that all the seals and O-rings are in good status and having placed the filter.

The extraction takes place under the following conditions:

Table 6. Pressurized liquid extraction conditions

Pressure	1500 psi
Temperature	40 ºC
Pre-heat time	5 minutes
Heat time	5 min
Static time	5 min
Flush volume	60 %
Purge time	180 seconds
Static cycles	2
Solvent:	*tert*-Butyl methyl ether 100 %

After the extraction, the extractant (< 60 ml) is decanted into the evaporation vessel with known tare weight and evaporated at 40 ºC until dryness. The weight of the evaporation vessel containing the extract is recorded after reaching constant weight. The difference between tare weight of the evaporation vessel and weight after evaporation of the extractant is attributed to the extracted oil/fat.

11.4. SPE, conversion and transesterification

1. A portion of 100 mg of oil/fat (±5 mg) is transferred with a Pasteur pipette or a spatula into a 1.5 ml vial and 100 µl of mixed labelled ester solution (9.6) is added. Add 500 µl of n-hexane : ethylacetate (85+15, v/v) and shake vigorously on a vortex mixer for 15 seconds.
2. The SPE cartridge is conditioned with 2 mL n-hexane : ethylacetate (85+15, v/v).
3. The sample is loaded onto the cartridge.
4. The target compounds are eluted with 10 mL n-hexane : ethylacetate (85+15, v/v). The eluate is evaporated under a gentle stream of nitrogen at 40 °C. The residue is thereafter reconstituted in 2 mL tetrahydrofuran.

IMPORTANT NOTE: *SPE extraction could be omitted in case justified evidence exists that the sample is free from partial acylglycerols, in particular MAGs.*

5. **Conversion:** Add 30 µL of acid aqueous solution of sodium bromide (10.1.1) to the sample, shake vigorously (vortex) and incubate the mixture at 50 °C for 15 min. Stop the reaction by the addition of 3 ml of 0.6% aqueous solution of sodium hydrogen carbonate (10.1.2). In order to separate the oil/fat from the water phase, add 2 ml of *n*-hexane (6.1.3) and shake vigorously. After separation of the two phases, transfer the upper layer to an empty test tube (8.1) and evaporate to dryness under a nitrogen stream (at 40 °C). Dissolve the residue (oil) in 1 ml of anhydrous tetrahydrofuran

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

The clear separation of the organic phase from the water phase can be difficult. Therefore, it might be necessary to centrifuge the sample.

The evaporation of the organic phase under nitrogen stream must be carefully monitored and stopped immediately after the evaporation of the solvent.

6. **Acid transesterification:** Add 1.8 ml of sulphuric acid/methanol solution to the sample and shake vigorously (vortex) for 10 s. Close the cap of the test tube (8.1) tightly and incubate the mixture at 40 °C for 16 h. After the incubation period, the ester cleavage is stopped by the addition of 0.5 ml sodium hydrogen carbonate saturated solution to the sample. Shake (vortex) for 10 s. Evaporate the organic solvent (methanol) of the mixture under a nitrogen stream at 40°C.

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

7. **Salting-out:** Add 1.3 ml of ammonium sulphate solution. Add 1ml *n*-hexane and shake for 10 s with a vortex. Discard the upper phase that contains fatty acid methyl esters dissolved in *n*-hexane by using Pasteur pipettes. Repeat this step with another 1ml of *n*-hexane.

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

8. **Extraction:** Extract the free form of 2- and 3-MCPD as well as 3-MBPD from the aqueous phase with 3 x 0.6 ml of ethyl acetate, shake each time for 10 s (vortex) and transfer the upper phases to an empty glass test tube containing a small amount of anhydrous sodium sulphate.
9. **Derivatisation:** Add 150µl of the derivatisation reagent to the organic solvent (1.8 ml of ethyl acetate), shake for 15s and incubate in an ultrasonic bath for 5 minutes. To complete the derivatisation reaction, evaporate the extracts to dryness at 40 °C under a low stream of nitrogen.
10. Dissolve the residue in 300 µl of isooctane by shaking the mixture for 10 s (vortex), centrifuge the final solution at 3500 rpm and transfer the supernatant to an empty GC vial (a glass insert of about 150 µl of volume is typically used).

The evaporation of the extracts under nitrogen stream can be carried out at maximum of 40 °C to facilitate the evaporation of ethyl acetate, but it must be stopped as soon as the solvent is evaporated in order to avoid loss of the highly volatile phenylboronic derivatives.

If the evaporation of extracts is carried out at elevated temperatures the test tube must be allowed to cool down to room temperature before dissolving the residue.

12. INSTRUMENTAL CONDITIONS

12.1. GC conditions

(a) Injection volume: 1.0 µL

(b) Injection mode: pulsed splitless

Pulse pressure: 200 kPa
Pulse time: 0.30 min
Purge flow: 30.0 ml/min
Purge time: 2.0 min

(c) Injection temperature: 250 °C

(d) Carrier gas: helium

(e) Flow rate: 1.2 ml/min

(f) Temperature program: 60 °C (1 min), from 60 °C to 150°C at 6 °C/min, 2 min at 150 °C, from 150 °C to 300 °C at 10 °C/min.

12.2. MS conditions

(a) Transfer line temperature: 300 °C

(b) Ion source temperature: 250 °C

(c) Quadrupole temperature: 150 °C

(d) Ionization mode: EI, SIM mode

(e) Parameters for SIM mode:

(i) phenylboronic derivative of 3-MCPD (*m/z*): 147 (Q_1); 196 (Q_2);
(ii) phenylboronic derivative of 3-MCPD-d5 (*m/z*): 150 (Q_1); 201 (Q_2)
(iii) phenylboronic derivative of 2-MCPD (m/z): 196 (Q_1); 198 (Q_2);
(iv) phenylboronic derivative of 2-MCPD-d5 (*m/z*): 201 (Q_1); 203 (Q_2)
(v) phenylboronic derivative of 3-MBPD (*m/z*): 242 (Q_1); 147 (Q_2);
(vi) phenylboronic derivative of 3-MBPD-d5 (*m/z*): 245 (Q_1); 150 (Q_2).

Acquisition time window: 5-20 min.

Table 8. Retention times and m/z-ratios of native and stable isotope labelled MCPDs and MBPD.

Compounds	Retention time, (min)	Q_1 , (*m/z*)	Q_2 , (*m/z*)
3-MCPD-d5	17.25	150	201
3-MCPD	17.33	147	196
2-MCPD-d5	18.02	201	203
2-MCPD	18.13	196	198
3-MBPD-d5	19.49	245	150
3-MBPD	19.57	242	147

Ions (m/z 196 and 201 for 3-MCPD and 3-MCPD-d5, respectively and m/z 150 and 147 for 3-MBPD and 3-MBPD-d5, respectively) can also be used for quantification purposes.

12.3. Sample Analysis

Each sequence encompasses a procedural blank to assess interferences/contamination deriving from the applied reagents and apparatus. A reference material (quality control sample) shall be also included in the batch, for checking the method performances along time. Calibration standards are also injected at the end of the sequence, or after at least 10 sample injections.

13. DATA ANALYSIS & REPORTING

13.1. Calibration curve

The calibration curve is obtained by plotting the signal ratios of the PBA derivatives of the native analytes and the PBA derivatives of the corresponding labelled standards on the abscissa, against the amounts of native analytes (expressed **in ng** of free 3-MCPD, 2-MCPD, glycidol equivalent) added into the test tube prior to derivatisation.

Ions at m/z 147 (3-MCPD), 150 (3-MCPD-d5), 196 (2-MCPD), 201 (2-MCPD-d5), 240 (3-MBPD) and 245 (3-MBPD-d5) are used for quantification. The calibration function is defined for each analyte by linear regression, and can be described by **Equation 2**.

$$\frac{A_{native}}{A_{labelled}} = a * C_{native} + b \qquad \textbf{Equation 2}$$

Where:

A_{native}	is the area of the quantifier ion of the native analyte peaks
$A_{labelled}$	is the area of the corresponding stable isotope labelled analogue peaks
a	is the slope of the calibration function
C_{native}	is the amount of native analytes added into the test tube prior to derivatisation (in ng)
b	is the intercept of the calibration function

The injections of calibration standards shall be performed in random order.

13.2. Calculations

The concentration of the free form of analyte in the sample is reported in µg/kg according to Eq. 3.

$$X_{native} = \frac{\left(\frac{A_{native}}{A_{labelled}} - b\right)}{\frac{a}{W_{sample/\ fat}}} \qquad \textbf{Equation 3}$$

X_{native}	is the concentration of native analytes (in µg/kg) in the analysed fat /test sample.
A_{native}	is the area of the native analyte peak of the test sample
$A_{labelled}$	is the area of the corresponding stable isotope labelled analyte peak

$W_{sample/fat}$: a) weight of the extracted fat used for further analysis, or b) weight of test portion, if mixed labelled ester process solution (9.6) was added to the test portion prior to extraction (both values in g)

The conversion of the analyte content expressed on fat basis into analyte content expressed on product basis (µg/kg) is described by Equation 4:

$$C_P = X_{native} * \frac{F_{extracted}}{W_{sample}}$$ **Equation 4**

C_p: Concentration of the native compound in the sample (**in µg/kg**)

$F_{extracted}$: Amount of fat extracted from the test portion (**in g**)

W_{sample}: Weight of the test portion (**in g**)

14. REFERENCES

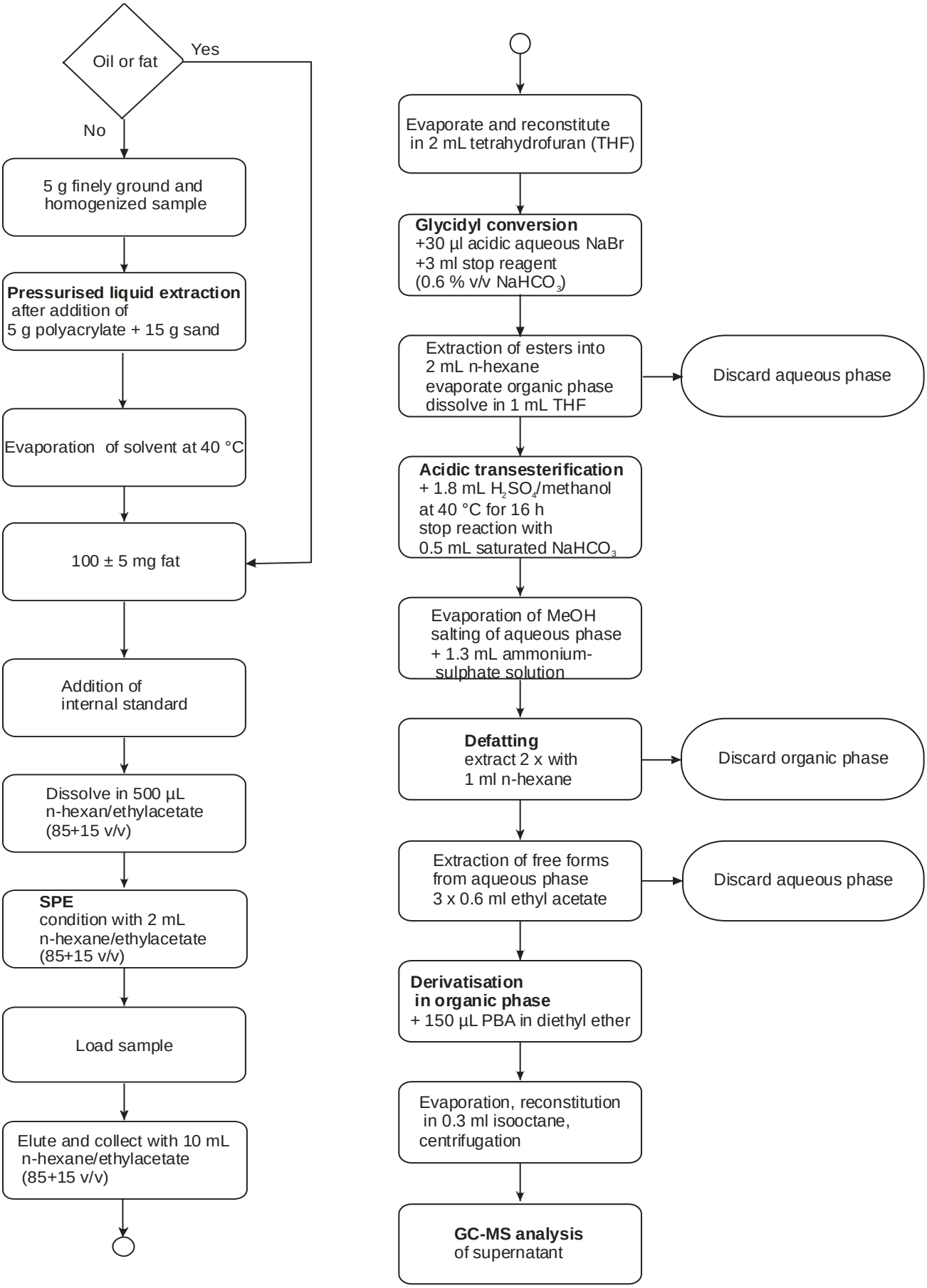
AOCS Official Method Cd 29a-13

Ermacora, A., Hrnčiřík, K. (2012). J Am Oil Chem Soc. 89:211-217.

Ermacora, A., Hrnčiřík, K. (2013). J Am Oil Chem Soc. 90:1-8.

Kraft, R., Brachwitz, H., Etzold, G., Langen, P., Zöpel, H-J (1979). J Prakt Chem 321: 756-768.

ANNEX 1: WORK FLOW FOR THE ANALYSIS OF ESTER-BOUND 3-MCDP, 2-MCDP AND GLYCIDOL



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