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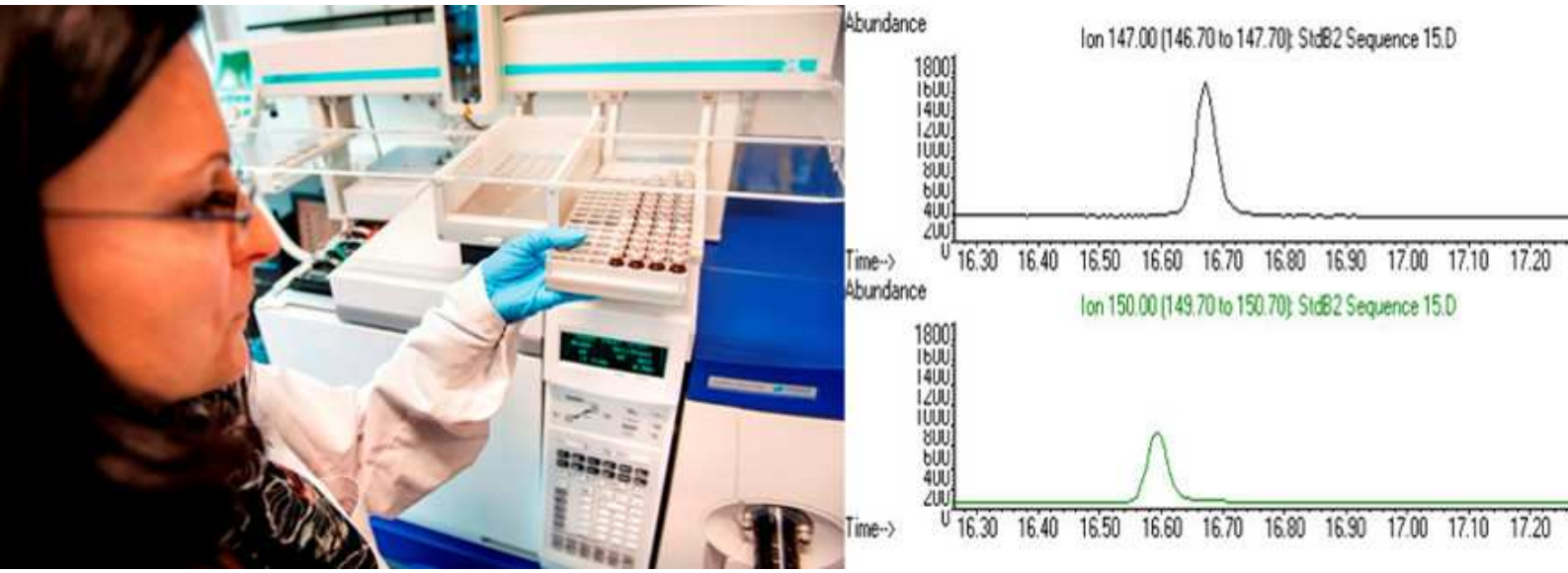

Development and validation of analytical methods for the analysis of 3-MCPD (both in free and ester form) and glycidyl esters in various food matrices and performance of an ad-hoc survey on specific food groups in support to a scientific opinion on comprehensive risk assessment on the presence of 3-MCPD and glycidyl esters in food

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Abstract

3-Monochloropropane-1,2-diol (3-MCPD), and 2-monochloropropane-1,3-diol (2-MCPD), are substances that might be generated in the processing of food. EU legislation specifies maximum levels for 3-MCPD in hydrolysed vegetable proteins and soya sauce. However, besides the free forms of 2- and 3-MCPD high levels of esterified MCPD forms were found in fats and oils. Another group of substances identified in fats and oils are glycidyl esters (GE). In order to provide reliable occurrence data on the levels of both bound and free forms of those substances, the European Food Safety Authority (EFSA) requested the Joint Research Centre (JRC) to develop suitable analysis methods and test the analysis methods on different kinds of food. Consequently two analytical methods were developed. One of the developed methods allows the determination of ester-bound analytes, whereas the other analysis method is suitable to determine free 2-MCPD and free 3-MCPD. Reliability of analysis results and robustness of the analysis methods were the main focus during method development and optimisation. The analytes were extracted with organic solvents under mild conditions. GEs are converted to monobromopropanediol esters (MBPD esters) prior to transesterification. MCPD esters and MBPD esters were transesterified followed by derivatisation of the analytes with phenyl boronic acid (PBA) in organic solvent. The PBA derivatives were measured by gas chromatography mass spectrometry (GC-MS) applying stable isotope labelled analogues of the analytes for quantification. The performance of both analysis methods was compliant with criteria specified by EFSA. The analytical methods were applied for the analysis 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. Analysis results were compiled and reported to EFSA in standard sample description (SSD) format.

This report describes the work commissioned by the European Food Safety Authority (EFSA) to the Joint Research Centre under the Service Level Agreement (SLA/EFSA-JRC/DCM/2013/01). It is presented in the format requested by EFSA and is published as well as EFSA supporting publication 2015: EN-779.

EFSA supporting publication 2015: EN-779

EXTERNAL SCIENTIFIC REPORT

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[1]

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ABSTRACT

3-Monochloropropane-1,2-diol (3-MCPD), and 2-monochloropropane-1,3-diol (2-MCPD), are substances that might be generated in the processing of food. EU legislation specifies maximum levels for 3-MCPD in hydrolysed vegetable proteins and soya sauce. However, besides the free forms of 2- and 3-MCPD high levels of esterified MCPD forms were found in fats and oils. Another group of substances identified in fats and oils are glycidyl esters (GE). In order to provide reliable occurrence data on the levels of both bound and free forms of those substances, the European Food Safety Authority (EFSA) requested the Joint Research Centre (JRC) to develop suitable analysis methods and test the analysis methods on different kinds of food. Consequently two analytical methods were developed. One of the developed methods allows the determination of ester-bound analytes, whereas the other analysis method is suitable to determine free 2-MCPD and free 3-MCPD. Reliability of analysis results and robustness of the analysis methods were the main focus during method development and optimisation. The analytes were extracted with organic solvents under mild conditions. GEs are converted to monobromopropanediol esters (MBPD esters) prior to transesterification. MCPD esters and MBPD esters were transesterified followed by derivatisation of the analytes with phenyl boronic acid (PBA) in organic solvent. The PBA derivatives were measured by gas chromatography mass spectrometry (GC-MS) applying stable isotope labelled analogues of the analytes for quantification. The performance of both analysis methods was compliant with criteria specified by EFSA. The analytical methods were applied for the analysis 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. Analysis results were compiled and reported to EFSA in standard sample description (SSD) format.

KEY WORDS

MCPD esters, glycidyl esters, free MCPD, food

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SUMMARY

The Service Level Agreement (SLA/EFSA-JRC/DCM/2013/01) between the European Food Safety Authority (EFSA) and the Joint Research Centre (JRC) had the aim to develop and in-house validate analytical methods for determining 3-monochloropropane-1,2-diol (3-MCPD), 2-monochloropropane-1,3-diol (2-MCPD), their fatty acid esters and glycidyl esters in food. Methods recently specified by the American Oil Chemists' Society (AOCS) had to be used as basis for method development and optimisation. The developed methods had to be tested on food samples, which were specified by EFSA both in terms of number and matrix. Additionally EFSA required that the methods shall be able to quantify 2-MCPD- and 3-MCPD esters as well as glycidyl esters at a level as low as 100 µg/kg fat. The requested limits of quantification of (LOQ) free 3-MCPD and 3-MCPD were 25 µg/kg food (expressed on whole weight basis).

Two analysis methods were developed, one for the determination of 2-MCPD, and 3-MCPD esters and glycidyl esters in food, the other targeting the determination of the free forms of 2-MCPD and 3-MCPD. Both analysis methods are based on the extraction of the analytes from the food sample with organic solvents, and include derivatisation of the analytes, which is performed in analogy to AOCS Official Method CD 29a-13. Gas chromatography mass spectrometry (GC-MS) with electron ionisation in selected ion monitoring mode was used as a measurement technique for all targeted analytes. Both analysis methods were in-house validated. The obtained data confirmed the desired sensitivity of the methods; LOQs were well below the specified threshold values. Precision of the analytical methods was better than EU legislation specifies for the determination of 3-MCPD in hydrolysed vegetable protein and soy sauce.

The analysis methods were 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 in Standard Sample Description (SSD) format, whereas the analysed food items were coded by FoodEx2 codes.

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BACKGROUND AS PROVIDED BY EFSA

3-Monochloropropane-1,2-diol (3-MCPD) is a food processing contaminant. It is classified as a possible human carcinogen for which the Scientific Committee on Food established in 2001 a tolerable daily intake (TDI) of 2 μg/kg b.w. In food, 3-MCPD can be present in the free from or in form of esters with different fatty acids (3-MCPD esters). Following recent data collection on the results from monitoring activities in the EU Member States, EFSA published on 26 September 2013 a Scientific Report on "Analysis of occurrence of 3-monochloropropane-1,2-diol (3-MCPD) in food in Europe in the years 2009-2011 and preliminary exposure assessment". The preliminary exposure assessment revealed that most of the 64 population groups included in the analysis were exposed at levels below the TDI. However for 8 population groups, the 95th percentile of exposure ('high consumers') resulted above the TDI. The report also 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 particular the LC limits, in order to reduce the uncertainty in occurrence and exposure estimates.

In order to gather reliable occurrence data to support the exposure assessment for the opinion, improved and validated analytical methods for the analysis of 3-MCPD and glycidyl esters in the relevant food matrices are required. Recently (between 2012 and 2013) three improved analytical methods for the analysis of 3-MCPD esters and glycidyl esters in fats and oils have been developed and in August 2013, after a collaborative study with 20 participants from eight countries, the American Oil Chemists' Society (AOCS) has validated and adopted them as official AOCS methods (AOCS Cd 29a-13, Cd 29b-13, Cd 29c-13). 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. The development of these methods is an urgent task since it is the pre-condition for the generation of reliable data on the occurrence of 3-MCPD and glycidyl esters in the relevant food categories (at least those highlighted in the report).

Once the methods will be available, their implementation in the EU Member States will allow establishing a suitable occurrence monitoring. Such exercise requires time and is expected to produce data far after the anticipated deadline for the EFSA opinion. An effective approach to develop the necessary analytical methods and guarantee quick availability of reliable occurrence data in time for the EFSA opinion would be setting up and supporting an ad-hoc project with an organisation with proven competence in chemical analysis and in the particular domain of process contaminants, thus capable of developing robust and validated methods and generating reliable data in the given timeframe.

The present Service Level Agreement is conceived to this purpose and foresees the development of analytical methods and the creation of a base of data sufficient to have a more reliable assessment of the human exposure to 3-MCPD and glycidyl esters in Europe. The tasks should be finished by the end of August 2014. The focus will be on the food categories affected by these food contaminants and for which recent reliable data are not present in the EFSA chemical occurrence database and are not foreseen to be submitted by the Member States by the above mentioned date. The list of the food categories is in the terms of reference.

TERMS OF REFERENCE AS PROVIDED BY EFSA

The contractor shall develop and apply in-house validated state-of-the-art analytical methods for the analysis of free and esterified forms of 3-MCPD and glycidyl esters in processed foods sampled from the European market in order to:

- make available robust and validated analytical methods to be used by other European laboratories involved in generating occurrence data of 3-MCPD and glycidyl esters.
- generate a set of analytical data which can be used by EFSA for assessing dietary exposure to the above mentioned substances in Europe in the following food categories, (at least 70 data points per food group; for the last 2 food groups depending on the available data, a different number of samples may be agreed during the execution of the contract):
    - Bread and bread rolls
    - Fine bakery wares
    - Smoked fish products
    - Smoked meat products
    - Fried and roasted meat
    - Potato-based snacks and fried potato-based products
    - Cereal-based snacks
    - Infant and follow-on formulas (in case the data available in the Member States are not sufficient)
    - Margarines and fats/oils (in case the data available in the Member States are not sufficient)

Sampling shall be random but is not expected to be performed in all geographical areas in Europe for all food groups, based on the assumption that the levels of process contaminants should in most food groups be similar across Europe. However, for smoked meat products, smoked fish products and fine bakery wares sampling shall be performed in different areas (Mediterranean, Central Europe, Northern Europe and Eastern Europe).

Details of the assignment

The analytical methods shall be based on the present best available science. In particular, the limit of quantification should be at or below 100 µg/kg (referred to fat) for the analysis of the esters. For the free form of 3-MCPD, the limit of quantification should be at or below 25 µg/kg. For each sample, both the free and the ester-bound form of 3-MCPD shall be analysed. For each sample, at least 4 entries shall be generated in the analytical results database:

3-MCPD (free);

3-MCPD (esters) [expressed as 3-MCPD moiety];

3-MCPD (total) [this is the sum of the previous two results expressed as 3-MCPD moiety];

Glycidyl esters [expressed as glycidol moiety].

All the results are expected to be expressed in µg/kg product, whole weight.

If the analytical method provides in one run also values for other related substances (e.g. 2-MCPD), these should also be separately reported.

Each entry related to the same sample should have the same sample code and other descriptors, apart from the code of the parameter analysed.

The sampling shall be planned in order to cover the different most common foods in every food group and avoid repeating foods from the same production lot. For heterogeneous food groups, like fine bakery wares, the major focus should be on the sub-groups with higher fat content (in the dough) and stronger heat treatment (e.g. A009V Biscuits (sweet and semi-sweet), A00CC Puff pastry, A00BV Shortcuts (pies -tarts)) but should also include some tests on other groups (e.g. A00AG Choux pastry, A00AN Cakes [particularly the bakery bases] and other to be selected from the FoodEx2 structure)

This contract/grant was awarded by EFSA to: The Institute for Reference Materials and Measurements of the Joint Research Centre, hereinafter referred to as "JRC"

Contractor

Contract title: 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

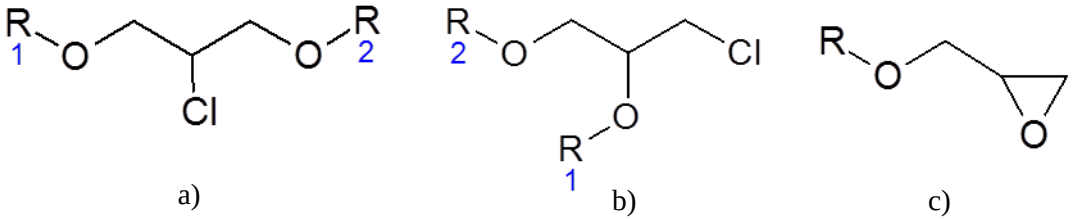
Contract number: SLA/EFSA-JRC/ DCM/ 2013/01

INTRODUCTION AND OBJECTIVES

3-monochloropropane-1,2-diol (3-MCPD) is a substance, which is generated during food processing. It was found in food items such as acid hydrolysed vegetable protein (HVP), soy sauce, different food ingredients and bakery products (Velisek et al., 2008; Baer et al., 2010). Maximum levels of 20 µg/kg were established in the European Union (EU) for the content of 3-MCPD in HVP and soya sauce by Commission Regulation (EC) No 1881/2006[2]. Provisions for methods of sampling and analysis for the official control of 3-MCPD are laid down in Commission Regulation (EC) No 836/2011[3].

First reports on the presence of 3-MCPD esterified with fatty acid esters (3-MCPD esters) were published in the 1980s (Davidek et al., 1980; Cerbulis et al., 1984). However, 3-MCPD esters got only a few years ago full attention of risk managers due to the finding of high levels of them especially in refined vegetable oils and products made of refined vegetable oils. High levels (above 4 mg/kg) were found in hydrogenated fats, palm oil and solid frying fats (BfR, 2007). Besides 3-MCPD esters, fatty acid esters of 2-monochloropropane-1,3-diol (2-MCPD) and of glycidol (3-hydroxy-1,2-epoxypropane) were found in refined fats and oils (Weisshaar and Perez, 2010).

The initial conservative assumption of risk assessors of a quantitative release of 3-MCPD from 3-MCPD esters in the human intestine was confirmed by recent studies (EFSA, 2008; Buhrkem et al., 2011; Barocelli et al., 2011). The toxicities of both 3-MCPD and of glycidol were assessed by the International Agency for Research on Cancer (IARC) (IARC, 2000; IARC 2012). Glycidol has been classified as probably carcinogenic to humans (Group 2A), whereas 3-MCPD is considered as potentially carcinogenic to humans (Group 2B). The chemical structures of 2-MCPD esters, 3-MCPD esters and glycidyl esters (GEs) are depicted in Figure 1:



a) b) c)

Figure 1: Structure of 2-MCPD esters (a), 3-MCPD esters (b) and glycidyl esters (c). R, R1 and R2 represent acyl groups of fatty acid.

Recent years were characterised by the development of methods for the determination of both 2- and 3- MCPD esters, and GEs in edible fats and oils (Crews et al., 2013). Initial difficulties caused by the mutual transformation of the three groups of compounds during analysis were tackled and several methods were standardised by international organisations for the determination of MCPD esters (both 2-MCPD esters and 3-MCPD esters) as well as GEs in fats and oils (AOCS 2012; AOCS 2013a; AOCS 2013b; AOCS 2013c).

However, only little information is available on the determination of MCPD esters and GEs in processed food. Küster et al. (2010) published an analytical method for the simultaneous determination of MCPD esters, GEs as well as free forms of MCPD in different food. This method was later on adapted for the simultaneous determination of GEs and MCPD esters in different

[2] Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs, OJ L 364, 20.12.2006, p. 5-31.

[3] Commission Regulation (EC) No 836/2011 of 19 August 2011 amending Regulation (EC) No 333/2007 laying down the methods of sampling and analysis for the official control of the levels of lead, cadmium, mercury, inorganic tin, 3-MCPD and benzo(a)pyrene in foodstuffs, OJ L 215, 20.8.2011, p. 9–15.

foodstuffs (Küsters et al., 2011). Zelinkova et al. (2009) determined 3-MCPD esters in infant and baby foods. Information of the performance of analytical methods for the determination of MCPD esters and GEs is also available from the German Federal Institute for Risk Assessment (BfR), which organised a comparative study on the determination of MCPD esters in different foods such as infant formula, mayonnaise, and vegetable oil based creams and spreads (Fry et al., 2013). However, only the papers presented by Küsters et al. (2010, 2011) contain data on method performance for food with large differences in composition.

The aim of this project, as specified in the Service Level Agreement (SLA), was to develop robust and reliable analytical methods for the determination of 3-MCPD esters and glycidyl esters as well as the free form of 3-MCPD in food. The methods had to be tested on more than 500 samples belonging to different food categories, which were identified by the European Food Safety Authority (EFSA). However, the scope of the developed analytical methods was extended to 2-MCPD and 2-MCPD esters and the number of analysed samples exceeded 600 at the end of the project.

MATERIALS AND METHODS

1. Sampling plan and sampling

EFSA and JRC agreed mid of March 2014 upon the number and distribution of samples to be analysed in the frame of this project. They are laid down in the sampling plan, which is presented in Appendix A. The sampling plan did not cover all food items available to consumers due to time and resource limitations. However, the project has to be considered as support to an EU wide monitoring of food, it can certainly not replace it.

Irrespective of all limitations, the obtained data shall provide sufficient information to identify priorities for future monitoring activities.

In view of the time constraints and considering the structure of EU food businesses, it was agreed to restrict sampling of food items provided by multinational companies to Belgium and neighbouring countries, assuming that goods supplied by multinational companies to these markets may be considered reasonably similar across Europe with respect to the presence of these process contaminants. Sampling was extended over a larger number of countries for products produced mostly by small and medium sized enterprises where the production practices may be assumed to be more variable. It was also agreed to apply convenience sampling, i.e. without applying a statistically designed programme taking food consumption and census data into consideration. Nonetheless, a broad variability of products within the individual categories was collected. This was accomplished by sampling different products/brands in different countries and different shops. Both premium labels and private labels were considered in the sampling plan. The agreed number of samples was met or exceeded in all categories except barbecued marinated sausage, and smoked herring. Marinated sausages for barbecuing are in contrast to marinated meat commercially not available, whereas the offer of smoked herring is in most food stores poor. Smoked salmon, smoked mackerel, and smoked trout dominated the refrigerated display cases of the visited supermarkets, whereas herring was usually sold either raw or canned in oil.

In summary, more than 600 food items were sampled in 17 EU Member States. About 95 % of these samples were acquired in 10 countries, applying a convenience sampling regime, as mentioned before. The samples were produced in 22 countries.

2. Processing of food prior to analysis

Most food items were analysed as sold without any processing. Only food belonging to the categories marinated- and non-marinated barbecued meat and sausages, and oven baked potato products were processed by IRMM staff prior to analysis.

Barbecued meat samples were prepared on a charcoal grill, at a distance of 10 cm above the charcoal. All samples were cooked "well done", overcooking was avoided.

Oven baked potato products were prepared in a household oven at 220 °C for 12 to 20 minutes, depending on the times recommended by the producers. If time ranges were specified a value in the middle of the range was chosen for preparation of the respective product.

A special case presents the category home cooked pan-fried meat. Colleagues working at IRMM donated domestically cooked samples belonging to this category.

3. Considerations for the design of an analytical method for the determination of MCPD esters and glycidyl esters

The design of the analytical methods was based on the available experiences within the analytical community and was identified as the best solution allowing generating the data requested by EFSA in the defined timeframe. As mentioned before, very little information was available on the performance of analytical methods for a broad range of foodstuff. This lack of knowledge was accompanied by doubts expressed already beforehand on the reliability of currently available methods. The latter may be reasoned by the experiences made with artefact formation in the analysis of MCPD esters and GEs in edible oils. Artefacts formed by the analysis procedure led to the withdrawal of previously proposed analytical methods (Crews et al., 2013). Overall, the process of developing reliable methods for determining simultaneously MCPD esters and glycidyl esters in edible oils took several years. Hence, it is understandable that experts in the field expressed concerns about the feasibility of achieving the goals of this project within a short time period. Consequently, it was decided to design the required method based on principles which have been successfully applied before for one or the other food matrix. Two distinct analysis routes were in use within the analytical community – the determination of intact esters on the one hand, and the determination of MCPD- and glycidyl moieties released from the esters on the other hand. The former approach was successfully applied for the determination of glycidyl esters in edible oil, but finds its limitations in the determination of MCPD esters due to the large number of possible MCPD mono- and di-esters (AOCS, 2012). The commercial availability of reference materials, matrix interferences, differences in sensitivity of measurement instruments for MCPD ester isomers, and the levels of achievable limit of detection (LOD) and limit of quantification (LOQ) impair the suitability of direct analytical methods additionally (Crews et al., 2013).

Toxicologically relevant parts of MCPD esters and GEs are measured by indirect analytical methods, which usually consist of transesterification of the esters to release the MCPD/glycidyl moiety followed by derivatisation and gas chromatographic - mass spectrometric (GC-MS) determination (Crews et al., 2013). A method comparison study on the determination of MCPD esters and GEs in edible oils, which was conducted by the Joint Research Centre (JRC) in 2012, identified three analytical procedures with comparable performance (Karasek et al., 2013). These methods became in 2013 standards of the American Oil Chemists' Society (AOCS) (AOCS, 2013a-c). The method including acidic transesterification of the ester bound forms of the analytes was used with slight modifications as a building block for the design of the analytical method applied in this project. A portion of the extracted fat was processed after addition of stable isotope labelled internal standards. In the next step glycidyl esters were converted into monobromopropanediol esters (MBPD esters), in analogy to the procedure described in the AOCS Official Method CD 29a- 13 (AOCS, 2013a). They were consequently transesterified in acidic medium together with MCPD esters, and derivatised with phenyl

boronic acid to the respective dioxaborolane derivative prior to GC-MS measurement. The modifications comprised the extraction of the free forms of the analytes after transesterification into ethyl acetate, and consequent derivatisation of them in an organic solvent instead of in an aqueous solution as described in the AOCS method (AOCS, 2013a). The benefit of this additional extraction step was lower consumption of derivatisation reagent, which provided in the GC-MS measurement lower background levels and consequently LODs far below the minimum required level.

This second part of the sample preparation was preceded by an extraction step, which aimed to separate the fat fraction containing the analytes from the solid food matrix. Despite the comparative study organised by German Federal Institute for Risk Assessment (BfR) (Fry et al., 2013) did neither indicate significant effects of extraction on artefact generation nor on potential analyte decomposition, it was decided to apply the mildest conditions possible during sample preparation. The conditions proposed by Küsters et al. (2011) (room temperature) would fulfil this demand. However, the method applied by Küsters was not chosen for two reasons. The major reasons were that swelling of samples during extraction and problems related to phase separation could hamper the analysis. Both were expected to occur for at least part of the matrices studied in this project. For example it can be expected that many bakery wares contain emulsifiers which could complicate phase separation, as the bakery industry accounts for about 50 % of the emulsifier market (Brandt, 1996). Another reason was that to our knowledge experiences with the implementation of the method in a laboratory outside the German Federal Armed Forces were not available.

The BfR study demonstrated that extraction methods comprising several extraction cycles provide high levels of recovery. The variability of these results was much lower than of results obtained with methods based on a single extraction step (e.g. sonication). However, other effects, such as the type of internal standard used, could have contributed to the level of variability observed in the BfR study.

Pressurised liquid extraction was selected for the current project for reasons of user-friendliness, control of extraction conditions and level of automation. After the extraction fat was recovered for all products from the extraction solvent, weighed, and a portion of about 100 mg was spiked with stable isotope labelled internal standards for further analysis, except breads and rolls, porridge and other food matrices with labelled total fat contents below 5 %, which were spiked with the internal standard solution directly prior to the extraction.

It is true that the addition of internal standards prior to extraction would allow expressing results directly on product basis. However, the varying fat content of food would either require adaptation of the consecutive sample preparation to take account of the variable amount of fat extracted, or subsampling of a defined portion of extracted fat, thereby sacrificing a large portion of internal standards. The latter would be costly and change method performance parameters. A third option was to adapt the amounts of internal standards added to the samples depending on the expected fat contents. This might be the most attractive in theory, but had limitations in practice for several reasons. Processed food from restaurants and retail shops does not contain nutrient labels. Hence, the fat content can only be guessed, which might lead to the addition of wrong amounts of internal standards.

Therefore, it was preferred to spike a defined portion of the extracted fat fraction with a defined volume of a solution containing deuterated MCPD and glycidyl esters. Potential bias in the preparation of solutions of labelled esters was cancelled out by fortifying the fat portions and calibration solutions with the same volumes of internal standard solution.

The result on fat basis was then converted into the analyte content value expressed on product basis using the determined fat content. This approach has the benefit of providing simultaneously results on fat basis and on product basis, and allows to better monitor the performance of the method, as each sample contains the same amount of labelled standard. Especially the latter aspect proved to be beneficial due to the limited experience with this type of analysis at the start of the project. However, the chosen approach could introduce bias in case of incomplete fat extraction. Hence, special attention was paid to the extraction of fat from the investigated food.

The fat content determined for a particular product is influenced by the applied method. Different types of lipids contribute to the fat content depending of the fat determination method applied. Besides acyl glycerols, phospholipids, waxes, sterols, and free fatty acids contribute to varying degrees to the fat content of foods. The determination of total fat according to e.g. Weibull-Stoldt method or Röse-Gottlieb method requires hydrochloric acid respectively ammonia to digest matrix proteins. Fat is consecutively extracted into an organic solvent or solvent mixtures. Such harsh conditions were avoided in the current project as hydrochloric acid would have led to artefact formation.

The amount and composition of extracted fat depends of the ability of the extractant to access and dissolve the respective lipid fractions present in the food matrix. Bound lipids (lipoproteins and glycolipids) and encapsulated lipids are usually not extracted by conventional fat extraction procedures due to a lack of accessibility/solubility. However, it is questionable whether lipids other than acylglycerols contribute to the formation of MCPD esters and GEs. To our best knowledge, literature does not contain any respective information. Hence, it might be sufficient to extract only acylglycerols for the determination of MCPD esters and GEs, which simplifies extraction procedures. Manirakiza et al. (2011) studied the influence of the extraction method on the determination total lipids from different food samples. They identified hot solvent extraction as most suitable for the determination of total lipids from solid food. A mixture of n-hexane and acetone (4:1) provided the best results. However, this mix has the disadvantage of co-extracting water from fresh samples. Additionally free MCPD is co-extracted by the *n*-hexane-acetone mix.

In the current project all extractions for the determination of MCPD esters and GEs were performed with 100 % *tert*-butyl methyl ether (tBME). The choice of the solvent was based on experiences made in the extraction of fat from food samples with different types of solvents. The comparability of the extraction yield with the yield provided by alternative extraction protocols was evaluated and respective data are given in the "Results" section.

The free form of MCPD was determined separately, despite it might be possible to combine the determination of MCPD esters and free MCPD in one method. However, this was not explored in the frame of this project due to time constraints.

Figure 2: provides an outline of the analytical method for the determination of MCPD esters and GEs in food. The detailed description of the analysis method is provided in Appendix B.

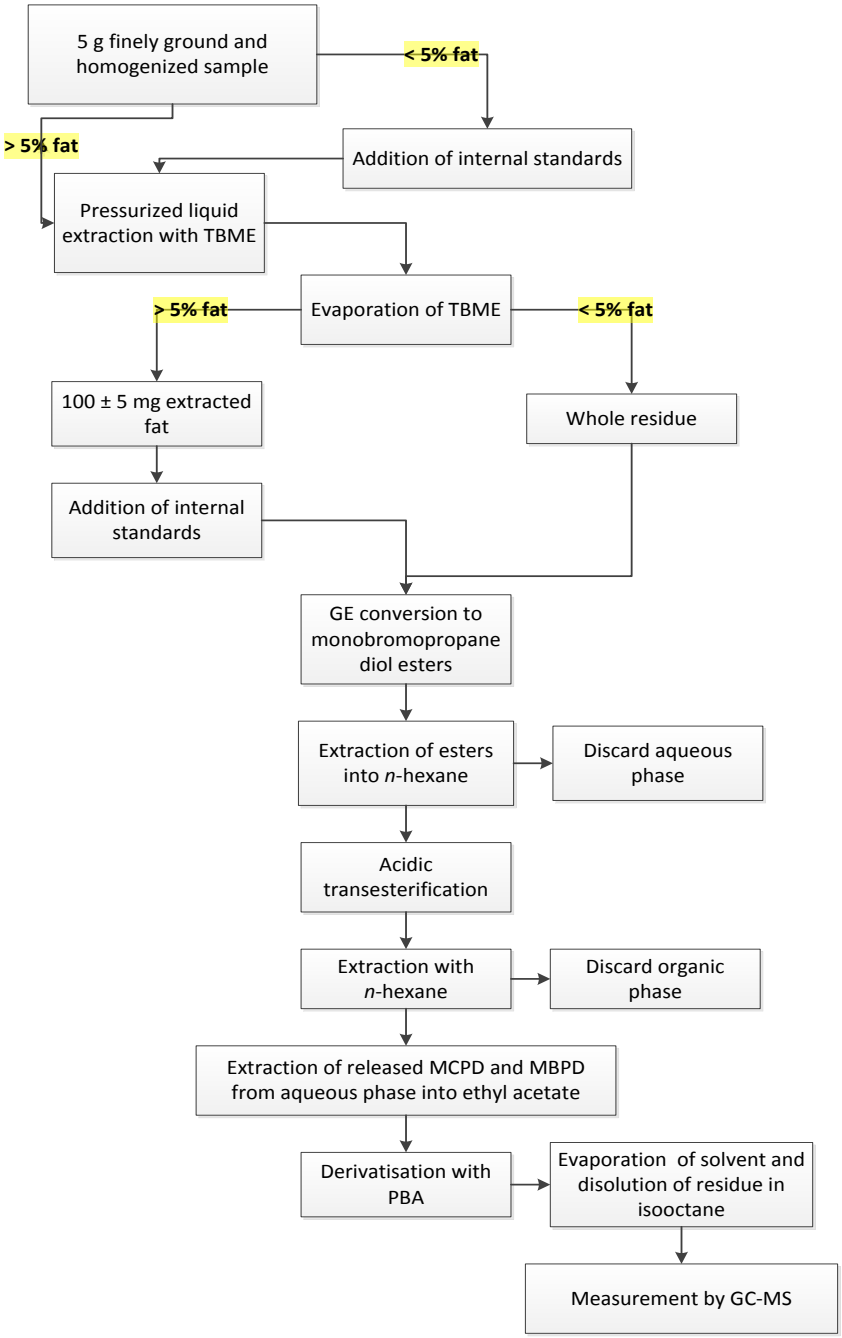


Figure 2: Flow chart of the analytical method applied for the determination of MCPD esters and GEs in food

GC-MS: gas chromatography mass spectrometry, GE: glycidyl ester MCPD: monochloropropanediol, MBPD: monobromopropanediol, PBA: phenylboronic acid, TBME: *tert*-butyl methyl ether

4. Considerations for the design of an analytical method for the determination of free 3-MCPD and free 2-MCPD in food

A lot of experience and literature is available for the determination of free 3-MCPD and 2-MCPD in food. First reports on chloropropanols in food date back to 1978 (Velisek et al., 1978). Since then different approaches were developed. Due to the physical characteristics of free MCPDs, which are absence of a chromophore, high polarity and low molecular weight, many analytical methods are based on aqueous extraction of the analytes, if necessary clean up, and derivatisation. Different derivatisation reactions were proposed and reviewed in literature (Hamlet et al., 2002; Wenzl et al., 2007; Hamlet and Sadd, 2009). The most frequently applied derivatisation reagents are heptafluorobutyrylimidazole (HFBI) and phenylboronic acid (PBA). The former is used in the European Standard EN 14573:2004, whereas the latter is preferred for the analysis of MCPD esters (CEN, 2004; AOCS, 2013a-c). As the scope of the project was to determine both bound and free forms of MCPD in food, it was decided to use PBA for the derivatisation of the analytes. The European Standard EN 14573:2004 was considered for application for the determination of free MCPDs. However, sample preparation specified in EN 14573:2004 is laborious and not environmental friendly due to high solvent consumption. Hence, it was decided to develop an analytical method which allows high sample throughput and which is more favourable regarding use of resources.

The critical steps in the determination of free MCPD are extraction and subsequent derivatisation. Different extraction procedures were tested. However, sample-handling problems were experienced with aqueous extractants partially during extraction (swelling of matrix) and partially after extraction by emulsion formation. The best compromise consisted of the extraction of finely ground food samples with a mix of *n*-hexane and acetone (1:1 v/v). Water is added to the mix after separation of the extractant from the solid residue to form two phases. The upper, organic phase is discarded and the aqueous phase is collected and completely evaporated. The evaporation of the aqueous extract was accomplished with a vacuum centrifuge and a stream of nitrogen to remove the last small drop in the test tube. The extracted matrix (residue) served as a catcher for the analytes. The dry residue was dissolved in ethyl acetate and the analytes derivatised with PBA. Finally, the ethyl acetate diethyl ether mix was evaporated and the analytes were taken up in isooctane prior to the GC-MS measurement.

The limiting factor for the sample throughput in this procedure was the evaporation of the aqueous solution, which was necessary to remove acetone. Residues of acetone caused difficulties in liquid-liquid extraction of the analytes into ethyl acetate, which was performed prior to derivatisation, analogous to the analysis of MCPD esters and GEs. Therefore, complete evaporation of the aqueous phase was preferred. The time-consuming evaporation step was scheduled during night. A 48 positions vacuum centrifuge was used for that purpose. Consequently, it was possible to analyse up to 48 samples per day.

Figure 3: provides an outline of the analytical method for the determination of free 3-MCPD and free 2-MCPD in food. The detailed description of the analysis method is provided in Appendix C.

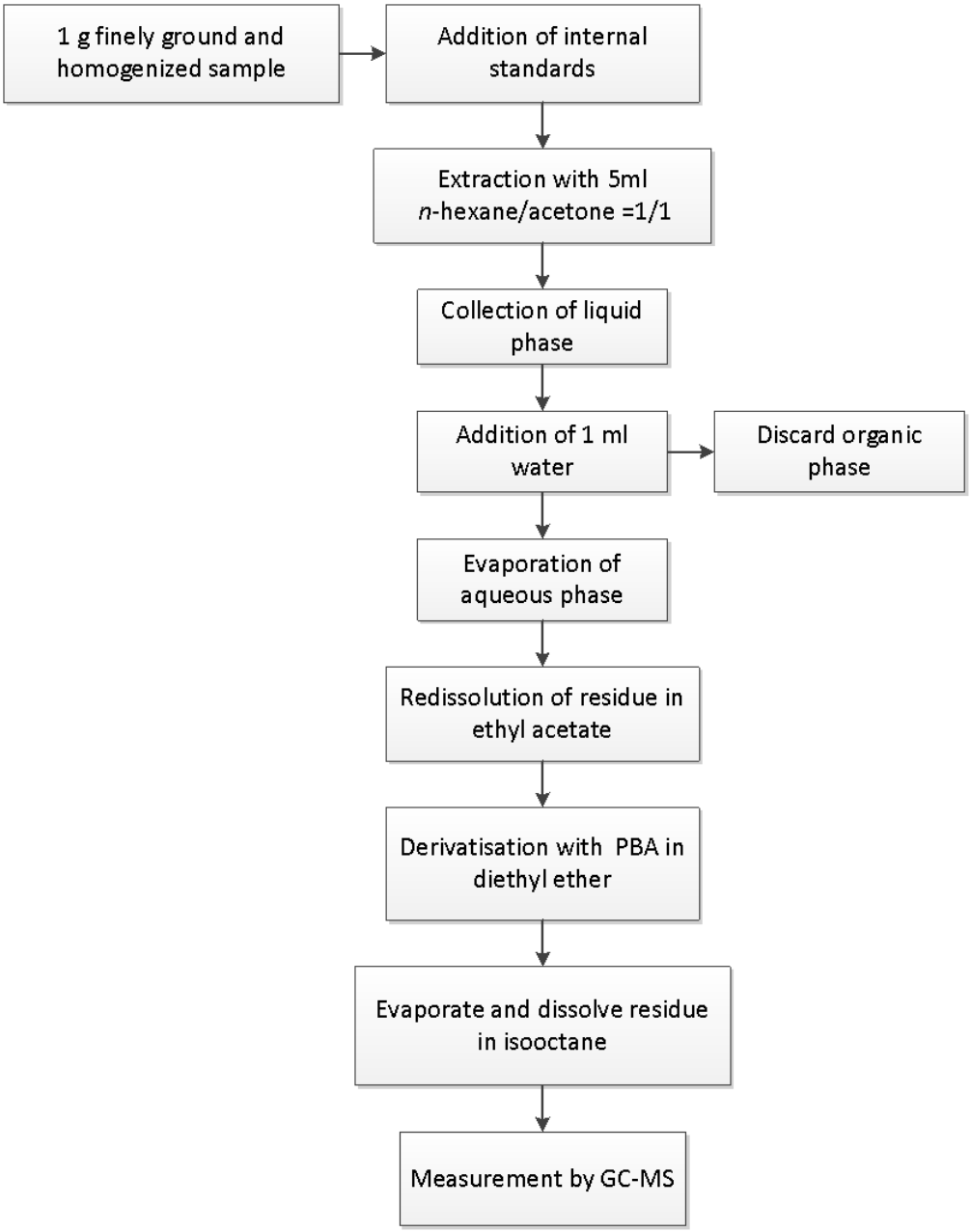


Figure 3: Outline of the analytical method for the determination of free-3-MCPD and free 2-MCPD in food
GC-MS: gas chromatography mass spectrometry, PBA: phenylboronic acid

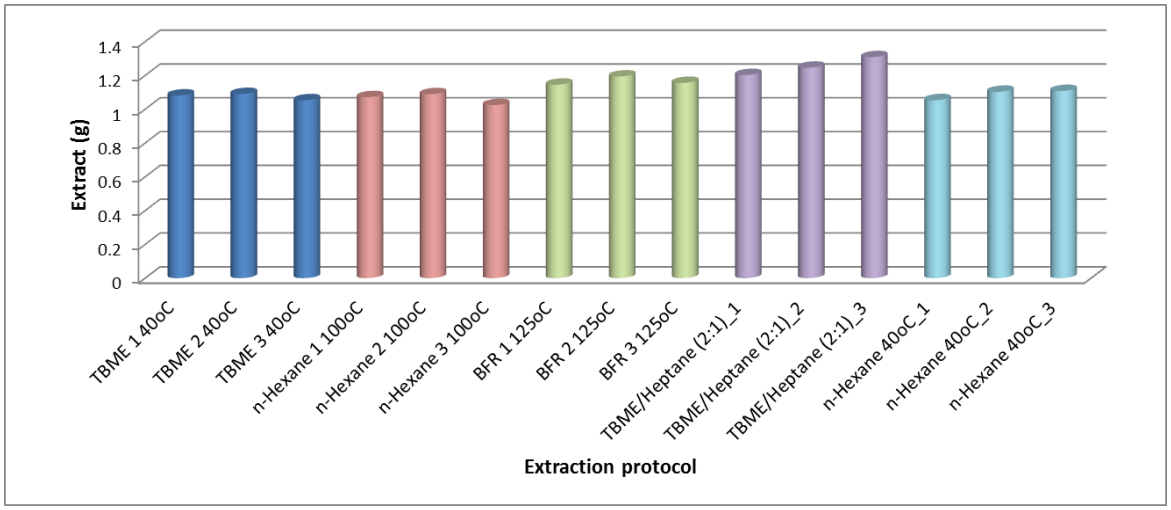
RESULTS

1. Performance of an analytical method for the determination of MCPD esters and GEs in food

1.1. Extraction efficiency

The efficiency of fat extraction of the applied procedure was compared to the extraction efficiency provided by other procedures. Two of them were used by other researchers for the determination of MCPD esters and GEs in infant formula (petroleum ether/isohexane/acetone) and in margarine (TBME/*n*-heptane) (BfR, 2013; Ermacora and Hrncirik, 2014).

Figure 4: presents the comparison of different extraction procedures for the extraction of a Belgian waffles sample. This sample falls into the category fine bakery ware and is characterised by a labelled fat content of 22.2 g fat per 100 g product. It contains among other ingredients lecithin, palm oil, coconut oil, rapeseed oil and butter. Each experiment was performed in triplicate. Dark blue columns represent results obtained with the extraction method applied in this project for the analysis of food samples.

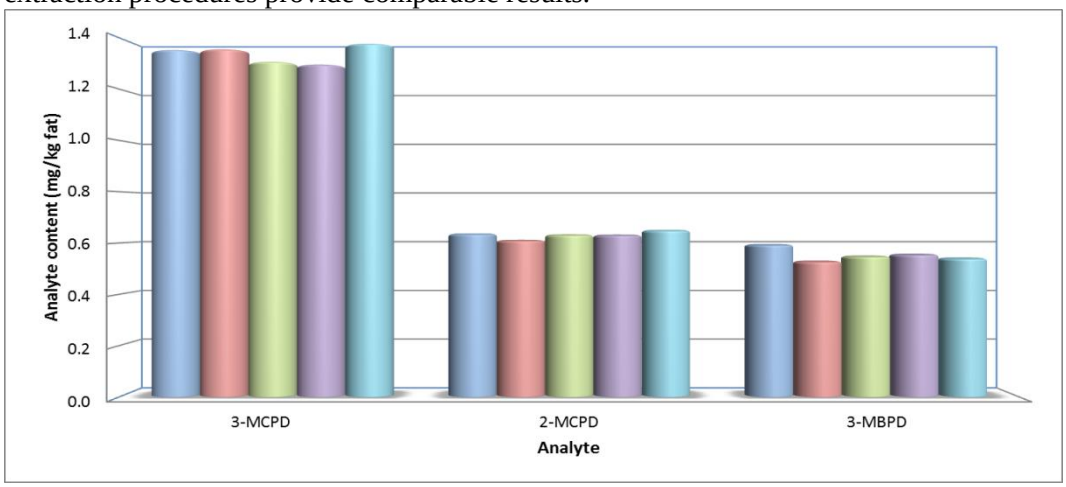


Legend: blue columns: TBME, 40°C; red columns *n*-hexane at 100 °C; green columns: method proposed by BfR (petroleum ether/isohexane/acetone=2/2/1), 125 °C; purple columns: TBME/n-heptane=2/1 at 60 °C as proposed by Ermacora and Hrncirik (2014); light blue columns: *n*-hexane at 40 °C.

Figure 4: Comparison of extraction procedures for waffles: Dark blue columns represent results obtained with the extraction method applied in this project for the analysis of food samples.

The results obtained by the different extraction methods agreed within 10 % with each other, and with the labelled total fat content within 5 %. The extractions with TBME/*n*-heptane gave results exceeding the labelled total fat content by between 8.3 % and 17.9 %. However, it shall be noted that the labelled fat content cannot be used as a reference for the particular sample, as it might represent only the average fat content of the particular product, potentially covering besides the extracted fat also other lipid fractions.

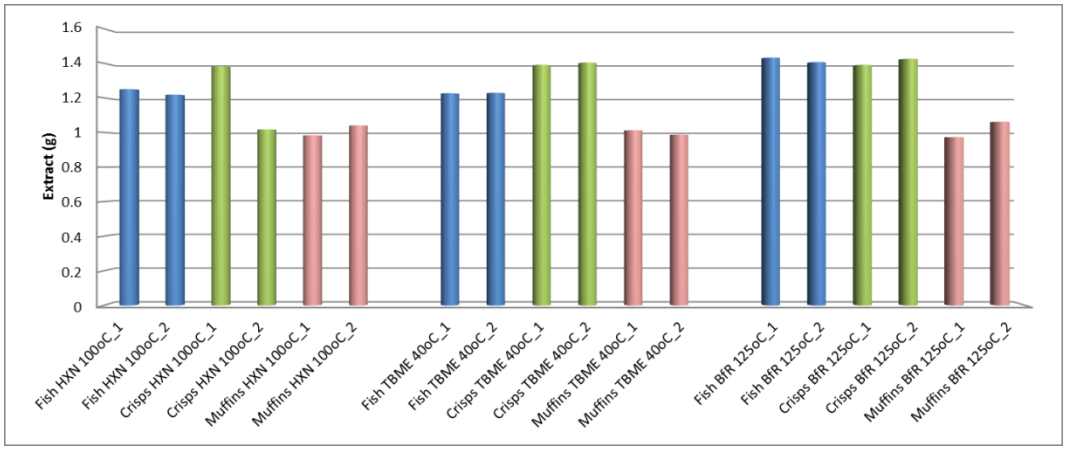
The extracted fat fractions were consecutively analysed for their MCPD ester and GE content. They agreed very well as demonstrated in Figure 5: . Hence, it may be concluded that the applied extraction procedures provide comparable results.



Legend: blue columns: TBME, 40 °C; red columns *n*-hexane at 100 °C; green columns: method proposed by BfR (petroleum ether/isohexane/acetone=2/2/1), 125 °C; purple columns: TBME/n-heptane=2/1 at 60 °C as proposed by Ermacora and Hrncirik (2014); light blue columns: *n*-hexane at 40 °C.

Figure 5: Analyte content measured in extracts obtained from waffles with different extraction protocols: Dark blue columns represent results obtained with the extraction method applied in this project for the analysis of food samples.

Similar experiments were performed for other matrices. Figure 6: shows extraction yields obtained with three different extraction protocols for fish, potato crisps, and muffins. The labelled fat content of muffins was 20 g per 100 g product, which translates into 1 g fat per 5 g extracted sample. The respective figures for potato crisps are 28.7 % fat /100 g product which is equal to 1.43 g per 5 g.



Legend: blue columns: smoked fish; green columns: potato crisps, red columns muffins

Figure 6: Comparison of extraction procedures for smoked fish, potato chips, and muffins: The block in the middle represents data obtained with the extraction procedure applied in this project for the analysis of food samples.

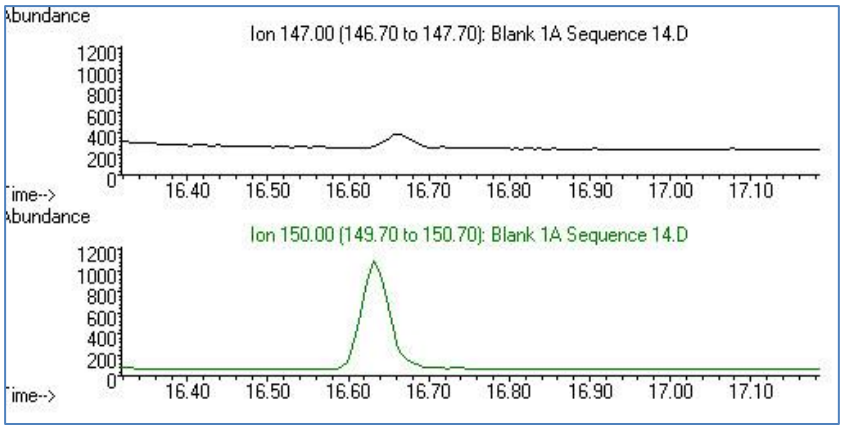
The fat extractions gave comparable results for muffins and potato crisps. A slight difference was experienced for the smoked fish sample, for which the extraction procedure proposed by BfR gave about 10 % higher extraction yields compared to the extraction with TBME at low temperature and with *n*-hexane at 100 °C.
Extraction efficiency was monitored for the waffle sample, which was used for quality control, over the whole period of the study. The average extraction yield of more than 40 extractions conducted over a period of about 6 months was 1.092 ± 0.051 g fat / 5 g sample, which corresponds to 98.4 ± 4.6 % of the labelled fat content.
The average extraction yield, of samples analysed for monitoring purposes was 97 ± 10 % of the labelled total fat content, These samples comprised potato based products, smoked meat and smoked fish products, fine bakery wares, and cereal based snacks.

1.2. Linearity

Linearity of the instrument response was tested for the measurement of 3-MCPD, 2-MCPD and 3-MBPD in the content range between 20 µg/kg and about 1850 µg/kg. Instrument calibration was performed with standards of the respective esters 3-MCPD 2-MCPD, 3-MBPD, as well as their stable isotope labelled analogues. They were mixed and subjected to the whole analysis procedure, starting with the bromination of glycidyl esters. The derivatives were at the end extracted into isooctane and analysed by GC-MS.
However, to avoid heteroscedasticity, the calibration range was split into two parts. The lower part, which was applicable to most of the analysed samples, covered the range 20 µg/kg to about 750 µg/kg, whereas a second calibration was curve was made for the range from about 600 µg/kg to about 1800 µg/kg.
Different calibration models were tested on the data sets. Linear calibration models were found for all analytes most appropriate. Lack-of-fit tests were passed in all cases.

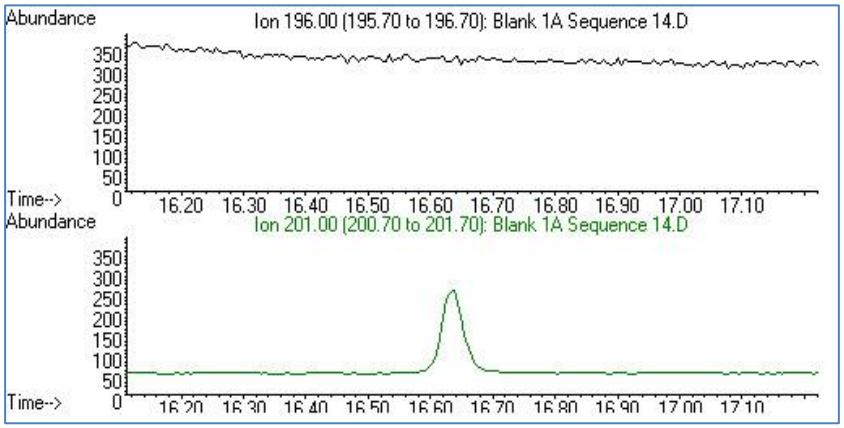
1.3. Selectivity

Selectivity was evaluated based on blank samples, which were spiked with internal standards and subjected to the whole analysis procedure. The stability of background levels was controlled for each analysis sequence by means of at least one blank extra virgin olive oil sample which was analysed with each batch of samples.
Recorded single ion chromatograms were evaluated for interfering peaks. Significant interferences were recorded neither at the retention time of the peak corresponding to 3-MCPD (Figure 7:), nor for 2-MCPD (Figure 8:).



Legend: upper chromatogram: trace corresponding to 3-MCPD, lower chromatogram: trace corresponding to 3-MCPD-d5

Figure 7: Single ion chromatograms for derivatives of 3-MCPD and 3-MCPD-d5

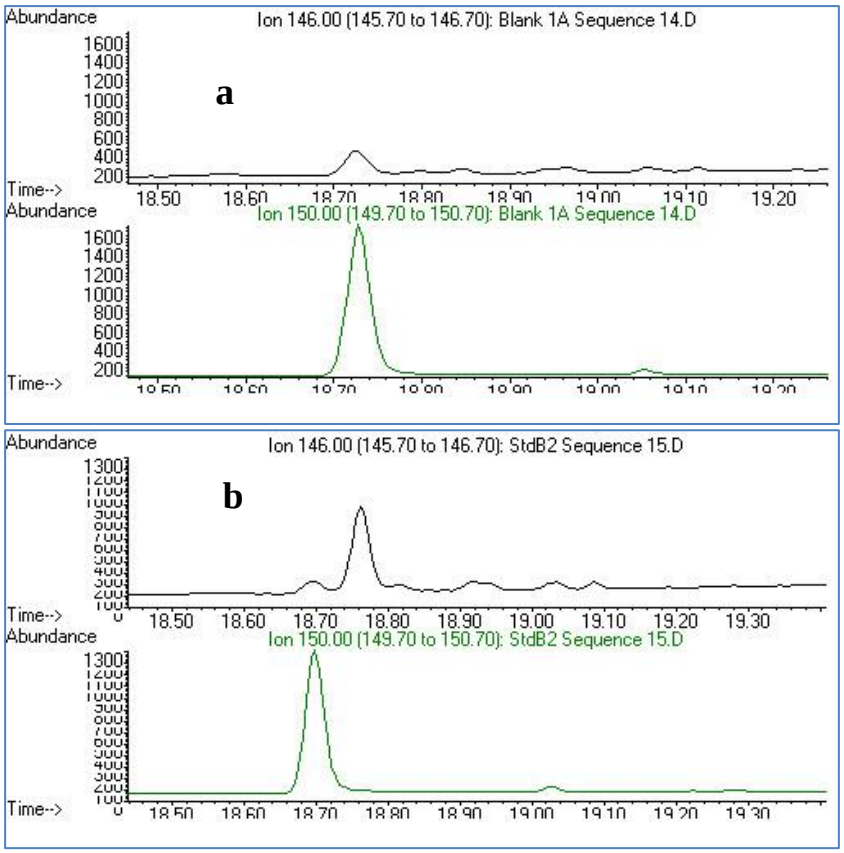


Legend: upper chromatogram: trace corresponding to 2-MCPD, lower chromatogram: trace corresponding to 2-MCPD-d5

Figure 8: Single ion chromatograms for derivatives of 2-MCPD and 2-MCPD-d5

The measurement of 3-MBPD derivatives was in terms of selectivity more difficult than the other two analytes. Initial experiments revealed that the signal at m/z-ratio 146 was less affected by interferences than the more abundant m/z=147.

Figure 9a and b show single ion chromatograms of the derivatives of 3-MBPD (m/z=146, Rt=18.76 min) and 3-MBPD-d5 (m/z=150, Rt=18.70 min) for a blank sample (extra virgin olive oil) (a) and a calibration standard (b). As can be seen the interference in the chromatogram of the blank sample is separated from the analyte (black trace in b). This is important, as both this interference and the interference at 18.80 min could be much more pronounced than the displayed chromatograms would suggest. However, sufficient separation was achieved with the applied conditions.



Legend: upper chromatograms: traces corresponding to 3-MBPD, lower chromatograms: traces corresponding to 3-MBPD-d5

Figure 9: Single ion chromatograms for derivatives of 3-MBPD and 3-MBPD-d5 for a blank sample (a) and a calibration standard (b)

1.4. Limit of detection and limit of quantification

LOD and LOQ are important parameters for the handling of left censored data in exposure assessment. For this reason, EFSA set stringent requirements on LOQ. LOQ for MCPD esters and GEs had to be at 100 µg/kg or lower, expressed on fat basis, where MCPD esters had to be expressed as free MCPD and GEs as glycidol. The DIN standard 32645:2008-11 was used as basis for the estimation of LOD and LOQ (DIN, 2008). LOD and LOQ were estimated via the standard deviation of blank samples spiked to a suitably low level with the targeted analytes). Spiking was necessary in order to get integrateable signals.

Ten blank fat samples were spiked with a mixture of 1,2-bis-palmitoly-3-chloropropanediol, 1,3-distearoyl-2-chloropropanediol, and glycidyl oleate to a level of about 50 µg/kg each of the free forms of 3-MCPD, 2-MCPD and glycidol. Each sample was analysed once, applying the whole transesterification and derivatisation procedure. Extraction was omitted as the LOQ had to be estimated directly in fat. Homoscedasticity was assumed for the content range between LOD and the spiking level, and the probabilities of type I and type II errors (α and ß errors) were set to 0.05. Equation 1 was used for the estimation of LOD, whereas Equation 2 was applied for LOQ.

$$x_{LOD} = 3.86 \times \frac{s_{y,B}}{b}$$ Equation 1

x_{LOD}: content level of LOD; $s_{y,B}$: standard deviation of the peak area of pseudo-blanks; b: slope of calibration curve

$$x_{LOQ} = 7.2 \times \frac{s_{y,B}}{b}$$ Equation 2

x_{LOQ}: content level of LOQ; $s_{y,B}$: standard deviation of the peak area of pseudo-blanks; b: slope of calibration curve

The factors 3.86 and 7.2 take into account the number of experiments and the chosen error probabilities.

Table 1: Estimates of LOD and LOQ for the determination of MCPD esters and glycidyl esters in fat extracted from food

	LOD[a] µg/kg fat	**LOQ[a] µg/kg fat**
3-MCPD from esters	7	13
2-MCPD from esters	8	15
Glycidol from GEs	17	31

(a) LOD and LOQ estimates rounded up to next integer

1.5. Precision

Method precision was evaluated by different means. Initial estimates were derived from the replicate analysis of spiked edible oil samples.

More important than repeatability was intermediate precision, which was derived from the analysis of quality control (QC) samples that were included in each batch of analysis. The QC samples comprised spiked edible oil and a waffle sample (labelled fat content 8.3 g/ 25 g ~ 33.2 %). Intermediate precision was also evaluated for a potato crisps sample, which was analysed 13 times over a period of six weeks. Intermediate precision values represent the relative standard deviations of 48 measurements of waffles, and of the olive oil spiked to about 730 µg/kg per analyte. The olive oil spiked to about 50 µg/kg was analysed 64 times over the period of the project.

Table 2 contains the intermediate precision estimates for each of the four different QC samples. From the data it can be concluded that the precision is compliant with applicable threshold values set in EU legislation for precision of analysis of free 3-MCPD in soy sauce and hydrolysed vegetable proteins (see Table 3). It becomes also obvious that precision decreases with decreasing content levels. The achieved level of precision is also compliant with precision data derived from the collaborative trial (AOCS, 2013a).

For information, AOCS official method Cd 29a-13, which was used as a basis for the method applied in this project, was validated by collaborative trial for the determination of MCPD esters and glycidyl esters in edible oils. The concentration range of the different analytes (expressed on basis of their free forms) was between about 200 µg/kg to 47000 µg/kg. Repeatability relative standard deviations at the lower end of the tested concentration range were between about 12 % and 16 %. Reproducibility relative standard deviations were between about 15 % and 44 %.

Table 2: Intermediate precision estimates derived from the analysis of QC samples

Matrix **Number of samples analysed**	**Spiked oil low level** **64**	**Spiked oil high level** **48**	**Waffles** **48**	**Potato crisps** **13**
	% (µg/kg)[a]	**% (µg/kg)[a]**	**% (µg/kg)[a]**	**% (µg/kg)[a]**
3-MCPD from esters	10.6 (55)	3.7 (737)	4.6 (250)	9.1 (78)
2-MCPD from esters	5.6 (53)	4.0 (756)	4.0 (132)	8.6 (47)
3-MBPD from glycidyl esters	15.3 (55)	4.7 (720)	instable[b]	11.0 (74)

(a) Values within brackets represent the analyte content expressed as free 3-MCPD, free 2-MCPD and glycidol. They are expressed on whole weight basis.

(b) QC data indicated a decrease of the glycidyl ester content over time.

Compared to 2-MCPD esters, the higher standard deviations for the measurements of 3-MCPD esters and glycidyl esters at low content levels might be caused by higher variability of background levels at the mass trace used for quantification of these substances, and linked to that higher variability in peak integration.

1.6. Trueness

The evaluation of trueness was challenging, which is linked to the complexity and variability of the investigated matrices. Even if there were a certified reference material for the determination of MCPD esters and GEs in food available, commutability of the information for other food matrices would be questionable. There might be always the possibility of missing part of the analytes in the analysis of a particular food sample with very special properties due to incomplete extraction. This question was addressed by comparison of results obtained by different extraction techniques.

Needless to say that none of them represents the golden standard that would assure true values. However, the outcome of the experiments allows assuming that the risk not to detect large bias is low. Performing in depth studies on each single matrix analysed was not possible within the given time frame of the project.
Instead of spending the available resources on extensive extraction experiments, it was preferred to monitor the trueness of the sample preparation and measurement part of the method. This part of the method starts from the extracted fat. For this purpose a blank extra virgin olive oil sample was spiked with the esters of 2-MCPD and 3-MCPD, and glycidyl oleate to both a low content level (about 50 µg/kg each) and to a high level (about 750 µg/kg each). These samples were analysed together with the tested food samples. The performance for these QC samples and the agreement with the spiked content allowed concluding that the analysis system was stable over time, and produced results close to the true value (see Figure 14: to Figure 16:).

1.7. Performance over time - Quality control

The performance of the analytical method was monitored throughout the whole project, applying different means. Blank levels, stability of calibration, extraction efficiency, and stability of analysis results for a QC sample were monitored. A commercial Belgian waffle was chosen as QC sample because it contained esters of 3-MCPD, 2-MCPD and glycidol, and next to palm oil, coconut oil and rapeseed oil, whole eggs, butter, flower, sugar, yeast, salt, water and soy lecithin. It may be regarded from the analytical point of view as complex matrix. In addition to artefact formation or analyte degradation, analysis could be negatively affected by the presence of emulsifiers, and bias might be encountered by incomplete extraction of fat, which might be encased in the matrix. All these possibilities were discussed in different expert meetings prior to the start of the project. However, hardly any experience was available at that time that would have allowed excluding any of these potential threats to the credibility of the produced analysis results. However, high variability of analysis results and trends over time would be expected if any of the mentioned scenarios would occur during analysis at significant level.
A blank extra virgin olive oil was measured with each batch of samples. The order of blank samples, QC samples, and test samples was randomised for each batch of samples. The analyte content of the blank extra virgin olive oil was below the level of LOD. Quantifiable amounts of the analytes were not detected in any of the analysed replicates. Hence, it can be postulated that cross-contamination does not occur.
The blank olive oil was spiked to two different levels as explained before, and measured with the food test samples.
The QC programme applied throughout the study is summarised in Figure 10.

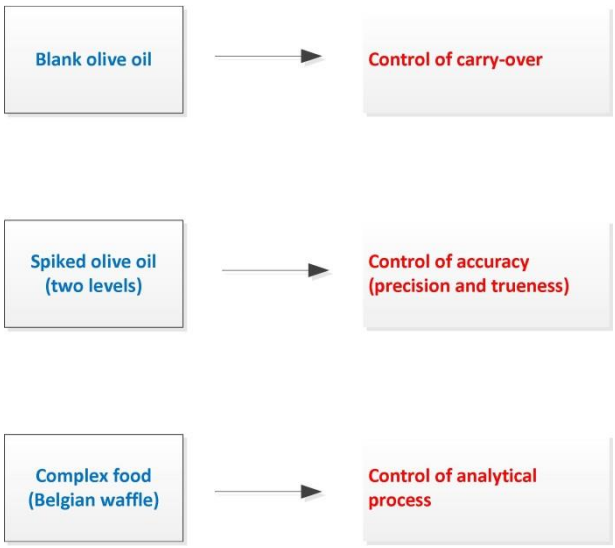


Figure 10: Overview of quality control measures

Data recorded for QC samples were plotted as control charts (Shewhart charts), which display the analysis results, the reference values (indicated as green lines) as well as upper and lower warning limits (yellow lines), and upper and lower action limits (red lines). The warning limits equal the reference value plus/minus two times the target standard deviations, whereas the action limits are formed with three times the target standard deviations. Content values are given in all charts based on the weight of the whole food.

The mean values of fifteen replicate analyses of the waffle sample formed the reference values for this QC sample, whereas the spiking levels were used as reference values for the olive oil samples. The estimation of realistic control levels requires information on the within laboratory reproducibility (intermediate precision) of the applied analytical method. This information can only be gained by replicate analysis over time. The timing of the project did not allow to study the performance of the analytical method over longer periods before starting the actual occurrence data collection exercise. Hence preliminary target values for tolerable precision were derived from specifications set in Commission Regulation (EC) No 836/2011[4] for the determination of free 3-MCPD (see Table 3). Based on initial experiments, which provided low repeatability standard deviations, it was decided to use half of the precision values provided by the modified Horwitz equation as preliminary target values in the calculation of control limits (Table 3). It should be noted that the determination of free 3-MCPD entails neither bromination nor transesterification steps, which should be beneficial for precision of this type of analysis. Therefore, it was not certain whether these control limits would be sustainable. In the course of the measurements conducted in this project, it turned out that this level of precision could be met for all analyte/QC sample combination except for glycidyl esters at low content levels (about 55 µg/kg in olive oil). At his content level, the precision threshold for the determination of glycidyl esters would have to be increased by about 4 %, from 11 % to 15 %.

Simply increasing the threshold levels could entail the risk of downgrading out-of-control situations,

[4] Commission Regulation (EC) No 836/2011 of 19 August 2011 amending Regulation (EC) No 333/2007 laying down the methods of sampling and analysis for the official control of the levels of lead, cadmium, mercury, inorganic tin, 3-MCPD and benzo(a)pyrene in foodstuffs, OJ L 215, 20.8.2011, p. 9–15.

while keeping them at the initial level would lead to certain values to be indicated as outliers. Hence it was tested whether outliers were among the data, and whether the distribution of data complied with the expected normal distribution. Outlier tests and Shapiro-Wilk normality tests were executed for this purpose. Neither outliers, nor significant deviations of the data distributions from normal distributions were detected. Hence, it is justified to assume that for each analyte/QC sample combination the obtained results belong to a single normally distributed population.
For consistency reasons the initially set control limits were kept for preparation of the QC graph for the determination of glycidyl esters in olive oil at low content levels. The respective graph is shown in Figure 16.

Table 3: Precision levels specified in Commission Regulation (EC) No 836/2011 for the determination of free 3-MCPD and precision levels applied for calculating threshold levels for QC

Content level	Precision derived from modified Horwitz equation	Relative precision applied for QC
<120 µg/kg	22 %	11.0 %
130 µg/kg	21.6 %	10.8 %
250 µg/kg	19.6 %	9.8%
750 µg/kg	16.6 %	8.3%

The established quality control charts for the determination of the analytes in the waffles QC sample demonstrate that the determination of 3-MCPD esters and 2-MCPD esters was under control over the period of the project. The achieved relative standard deviations were for derivatives of 3-MCPD about 5 % and for 2-MCPD about 4 %, which is significantly below the 7.5 % relative standard deviation which was used for setting control limits. Figures 11 and 12 show the respective QC charts.
However, the situation was different for the measurement of glycidyl esters. Figure 13 presents the QC chart for 3-MBPD derivatives, which are comparable with regard to variability of analysis with 2-MCPD and 3-MCPD data, but which shows a negative trend, indicating that this analyte was not stable over time. The glycidyl ester content (expressed as glycidol) decreased from initially about 135 µg/kg, to about 105 µg/kg.

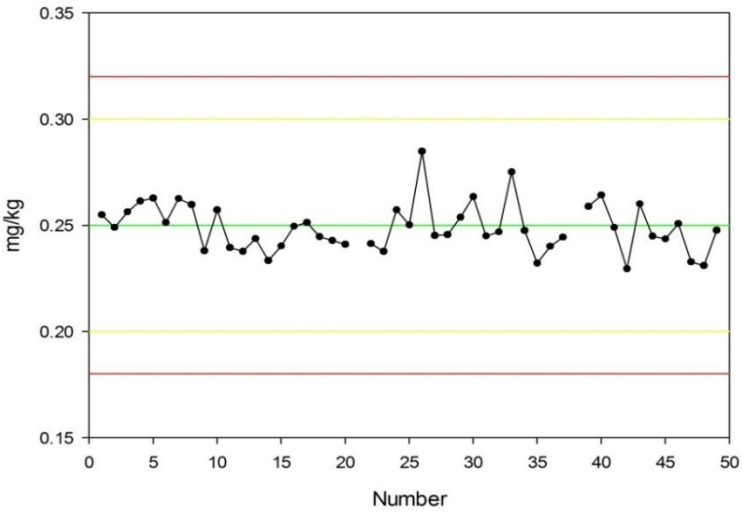


Figure 11: Shewhart chart for the determination of 3-MCPD esters in waffles

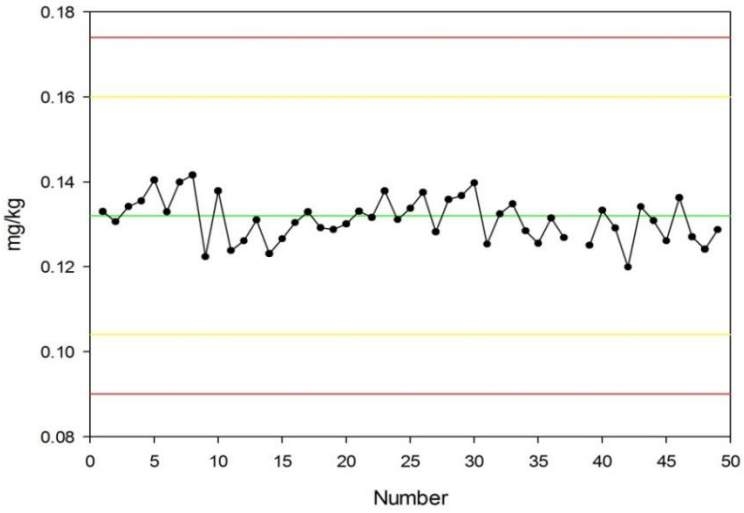


Figure 12: Shewhart chart for the determination of 2-MCPD esters in waffles

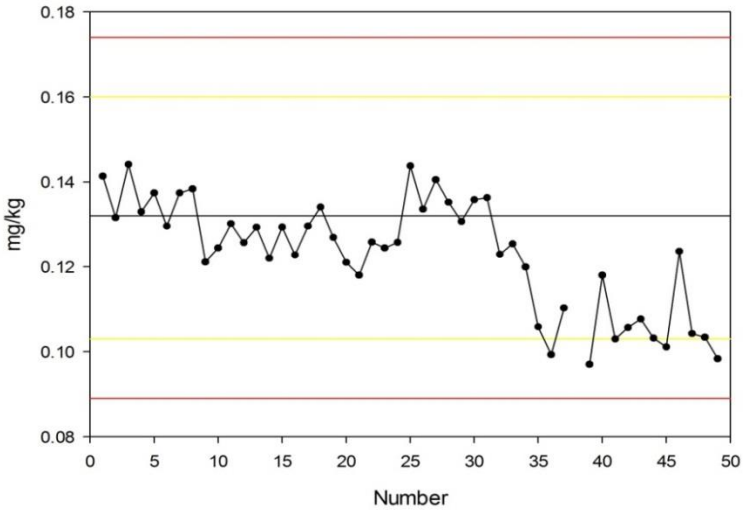
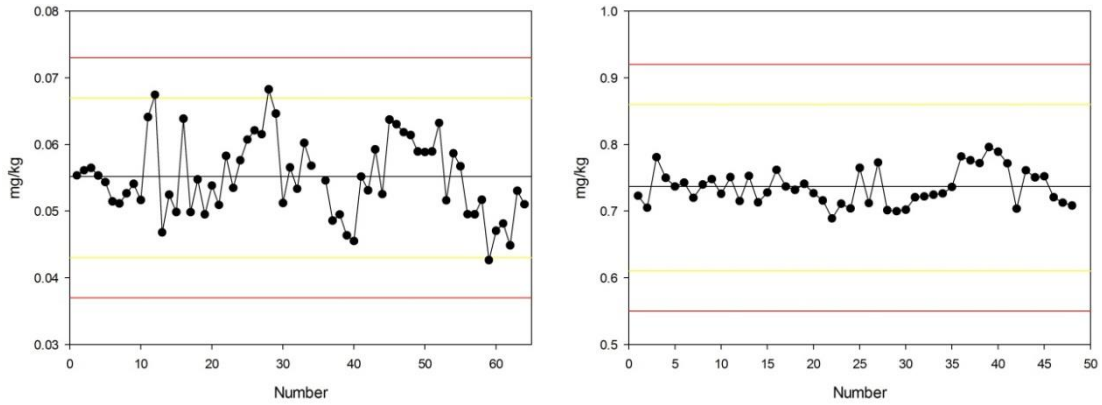


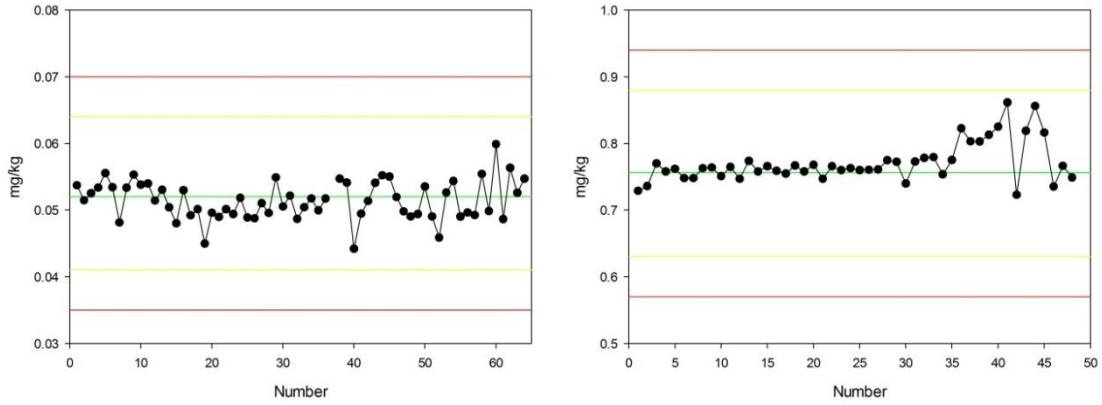
Figure 13: Shewhart chart for the determination of glycidyl esters in waffles

Shewhart charts for spiked olive oils are presented in Figures 14 to 16.



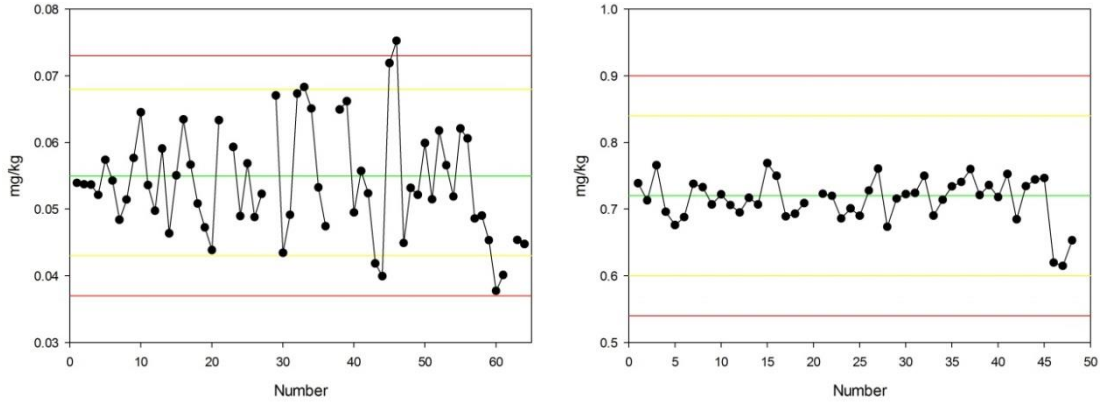
Reference values for the left panel: 55 µg/kg, for the right panel: 737 µg/kg

Figure 14: Shewhart charts for the determination of 3-MCPD esters in spiked olive oils



Reference values for the left panel : 53 µg/kg, for the right panel: 756 µg/kg

Figure 15: Shewhart charts for the determination of 2-MCPD esters in spiked olive oils



Reference values for the left panel: 55 µg/kg, for the right panel: 720 µg/kg

Figure 16: Shewhart charts for the determination of glycidyl esters in spiked olive oils

2. Performance of an analytical method for the determination of free 3-MCPD and free 2-MCPD in food

2.1. Linearity

Linearity of the instrument response was tested for the measurement of both free 3-MCPD and free 2-MCPD in the content range between 2.5 µg/kg and about 370 µg/kg. Instrument calibration was performed with standards in methanol, which were subjected to derivatisation. The derivatives were extracted into isooctane and analysed by GC-MS.
However, to avoid heteroscedasticity, the calibration range was split into two parts. The lower part, which was applicable to most of the analysed samples, covered the range 2.5 µg/kg to 120 µg/kg.
Different calibration models were tested on the data sets. Linear calibration models were found for both 3-MCPD and 2-MCPD most appropriate. Lack-of-fit tests were passed in both cases.

2.2. Selectivity

Selectivity was evaluated based on blank samples, which were spiked with internal standards and subjected to the whole analysis procedure. The stability of background levels was controlled for each analysis sequence by means of at least one blank extra virgin olive oil sample which was analysed with each batch of samples.
Recorded single ion chromatograms were evaluated for interfering peaks. Significant interferences were recorded neither at the retention time of the peak corresponding to 3-MCPD nor for 2-MCPD.

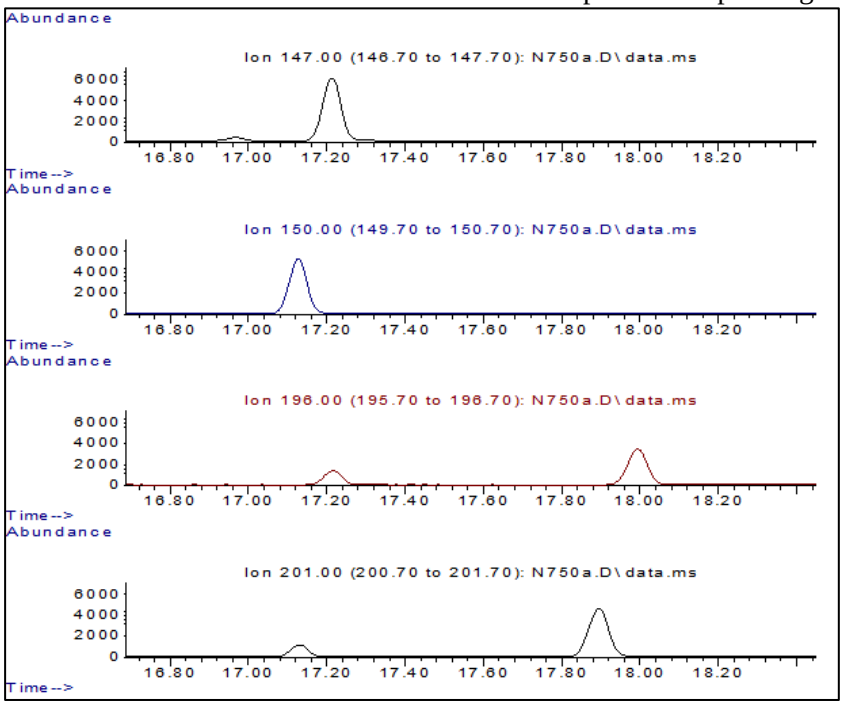


Figure 17: Single ion chromatograms for derivatives of 3-MCPD, 3-MCPD-d5, 2-MCPD, and 2-MCPD-d5 (from top to bottom)

2.3. Repeatability and intermediate precision

Repeatability was estimated from replicate analysis of three different matrices for native (unspiked) samples and samples spiked with 2- and 3-MCPD to about 30 µg/kg and 60 µg/kg. The analyses were carried out on a single day by one operator.
The achieved repeatability relative standard deviations were for all samples and analytes below 4 %. It should be noted that these values are achievable only under optimal conditions (clean GC inlet, clean column, and clean ion source). Analysis under routine conditions, which included large sequences of 40 to 50 injections revealed precision levels that were about 3 times poorer.
Intermediate precision levels were deduced from the analysis of quality control (QC) samples, which were included in each analysis sequences.
The relative standard deviations of results of in total 53 analyses, obtained under intermediate precision conditions, were for free 3-MCPD about 7 % and for free 2-MCPD about 9 %.
Both values are compliant with provisions set in EU legislation[5].

2.4. Limit of detection and limit of quantification

Ten blank or low contaminated food samples were spiked with a mixture of free 3-MCPD, and free 2-MCPD, to a level of about 10 µg/kg each. The samples comprised cookies, cereals, fish and meat samples, potato crisps, and bread. Each sample was analysed both native (unspiked) and spiked, applying the whole analysis procedure. The signal differences between the spiked and the native samples were attributed to the spiked amounts of analytes. Homoscedasticity was assumed for the content range between LOD and the spiking level, and the probabilities of type I and type II errors (α and ß errors) were set to 0.05. LOD and LOQ were estimated via the standard deviation of the signal differences between spiked and native samples. Equation 3 was used for the estimation of LOD, whereas Equation 2 was applied for LOQ.

$$x_{LOD} = 4.9 \times \frac{s_{y,net}}{b} \qquad \text{Equation 3}$$

x_{LOD}: content level at LOD; $s_{y,net}$: standard deviation of net signal (difference of peak area between spiked and native sample); b: slope of calibration curve

$$x_{LOQ} = 9.6 \times \frac{s_{y,net}}{b} \qquad \text{Equation 4}$$

x_{LOQ}: content level at LOQ; $s_{y,net}$: standard deviation of net signal (difference of peak area between spiked and native sample); b: slope of calibration curve
The factors 4.9 and 9.6 take account of the number of experiments and the chosen error probabilities.

Table 4: Estimates of LOD and LOQ for the determination of MCPD esters and glycidyl esters in fat extracted from food

	LOD[a] µg/kg (whole weight)	LOQ[a] µg/kg (whole weight)
3-MCPD free form	7	14
2-MCPD free form	5	9

(a) estimates are rounded up to next integer

[5] Commission Regulation (EC) No 836/2011 of 19 August 2011 amending Regulation (EC) No 333/2007 laying down the methods of sampling and analysis for the official control of the levels of lead, cadmium, mercury, inorganic tin, 3-MCPD and benzo(a)pyrene in foodstuffs, OJ L 215, 20.8.2011, p. 9–15.

2.5. Recovery

Recovery was evaluated from procedural blank samples and from low contaminated samples which both were spiked with different amounts of analytes. Recovery values were calculated from the net signals between spiked and native samples. The test sample matrices comprised smoked sausage samples, potato crisps and pan-fried meat samples. Each experiment was performed in duplicate.
Results of the different samples were pooled and the average recovered amount of analyte was plotted against the spiked amount. Figure 18 presents the plot of recovered average amounts depending on the spiking level.
As can be seen, recovery values were for both analytes close to 100 %.

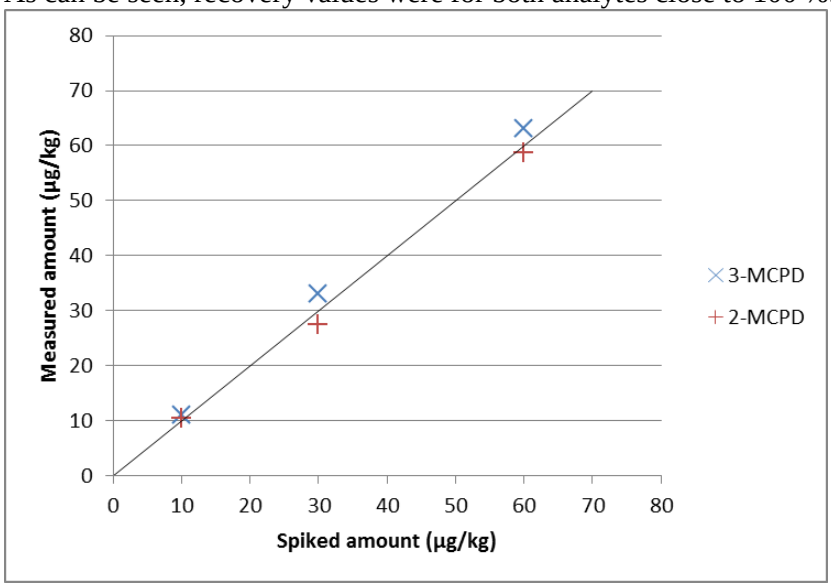


Figure 18: Average measured analyte contents depending on the spiked amounts.

2.6. Comparability of results with results obtained by EN 14573:2004

The comparability of results obtained by the developed method with results produced by the European Standard EN 14573:2004 was studied on a naturally contaminated smoked sausage sample. It shall be stressed that extreme smoking conditions were applied on purpose for the production of this sample, in order to generate elevated contamination levels. Data of this sample are not included in the data set reported to EFSA.
The sample was analysed by both methods in duplicate applying the whole analysis procedure. Results are shown in Table 5.

Table 5: Comparison of results obtained by developed method and EN 14573:2004 (standard method)

		3-MCPD	2-MCPD
Developed method	µg/kg	215.6	8.9
		207.6	8.7
Standard method	µg/kg	211.4	8.6
		205.0	8.0

2.7. Quality control

A smoked meat material was used for QC. It was spiked with the native analytes to about 47 µg/kg free 3-MCPD and about 20 µg/kg 2-MCPD. Both content values are expressed on whole weight basis. The lower of the two values is close the concentration of the maximum acceptable LOQ specified by EFSA (25 µg/kg), whereas the higher is about twice of this level. The QC sample was included in each analysis sequence. It was subjected to the whole analysis procedure. Test samples and QC samples were analysed in random order.
Figures 19 and 20 show quality control charts for the determination of free 3-MCPD and free 2-MCPD in the QC sample. Action levels are indicated by red lines, whereas yellow lines indicate warning levels. The reference value is indicated by the black line. They were formed provisionally by assuming half of the tolerable reproducibility as specified in Commission Regulation (EU) No 836/2011[6] as target standard deviation. The relative standard deviations of more than 50 determinations covering the period of the project were about 7 % in case of 3-MCPD and about 9 % in case of 2-MCPD.

[6] Commission Regulation (EC) No 836/2011 of 19 August 2011 amending Regulation (EC) No 333/2007 laying down the methods of sampling and analysis for the official control of the levels of lead, cadmium, mercury, inorganic tin, 3-MCPD and benzo(a)pyrene in foodstuffs, OJ L 215, 20.8.2011, p. 9–15.

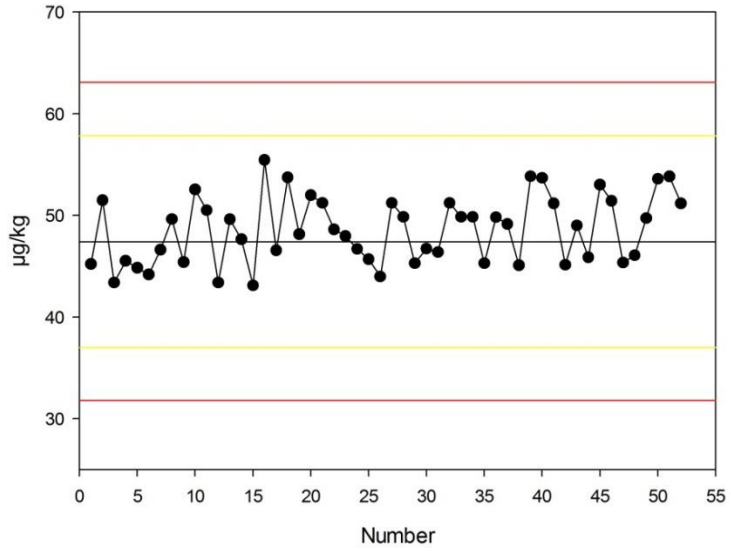


Figure 19: Quality control chart for the determination of free 3-MCPD in the QC sample.

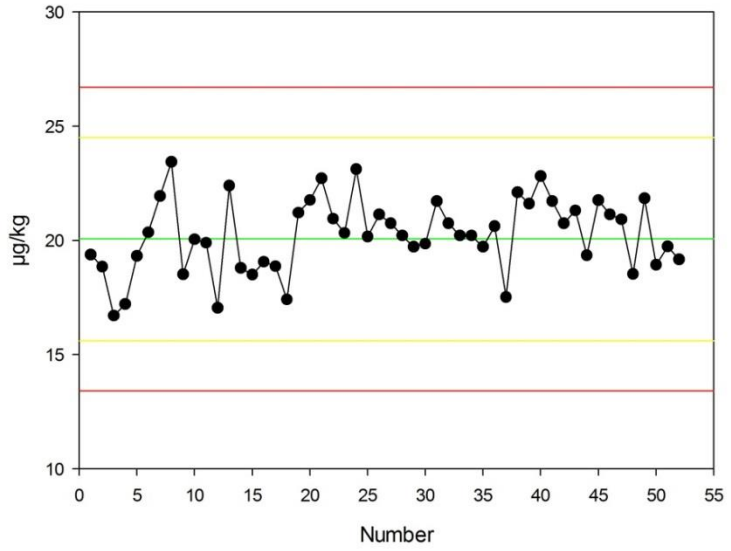


Figure 20: Quality control chart for the determination of free 2-MCPD in the QC sample.

3. Experiences made in the application of the methods over time

3.1. General

Both methods include extraction of the analytes from a small sample portion into organic solvents. For achieving the presented method performance levels, it is of paramount importance to grind the samples to fine homogeneous powders prior to analysis. All samples were ground by means of a laboratory grinder with rotating blades. Most samples were frozen with small amounts of liquid nitrogen prior to grinding. Care has to be taken that the samples do not take up large amounts of humidity after freezing and grinding, as powders might be hygroscopic.

3.2. Determination of MCPD esters and GEs in food

The analytical method is based on the extraction of fat from the food matrix, the determination of the amount of extracted fat by gravimetry, followed by subsampling of the fat fraction for further analysis. Internal standards are added to the sample in case of most food products only after extraction. This avoids sacrificing large amounts of internal standards in case of high fat products, as the consecutive sample preparation does not tolerate large amounts of fat. However, this procedure requires attention for the correct determination of the extracted amount of fat. Therefore, the extractant has to be evaporated (at moderate conditions) until constant weight is obtained.

Extraction of fat from infant formula was not successful under the specified extraction conditions. The achieved extraction yields were for this matrix in the range of only 20 % of the labelled total fat content. However, pressurized liquid extraction (PLE) with solvent mixtures containing acetone, as proposed by BFR method 22 and BFR method 23 (Fry et al, 2013), yields quantitative fat extraction.

3.3. Determination of free 2-MCPD and free 3-MCPD

Different experiments conducted during the method development phase identified the varying uptake of water by different food matrices as an issue hampering the analysis if aqueous/organic solvent mixtures were used for extraction. Recovery of the extractant from the solid food matrix provided problems in case of strongly swelling matrices. It was decided to extract the analytes into an organic phase, and to separate co-extracted fat, which might contain also esters of MCPD, after separation from the food matrix. However, some food samples showed also with purely organic solvents strong swelling and caused difficulties in separating the extractant from the matrix, which in turn affected recovery of the internal standards. A second extraction step improved their recovery.

After extraction and addition of water to the extractant, the separation of the organic and the aqueous phase was sometimes difficult. The organic phase got for some food matrices gel-type consistency, which was difficult to pipette. In addition, formation of emulsions at the interphase between organic and aqueous phases occurred for many samples. In both cases, it was decided to sacrifice a small part of the aqueous phase containing the analyte, which turned out beneficial regarding instrument stability.

Free MCPD might be lost during evaporation of the aqueous extract to dryness (prior to redissolution of the residue in ethyl acetate). Therefore, harsh evaporation conditions have to be avoided.

Stability of instruments

Different analysts, who are active in this field of analysis, reported issues related to stability of the instruments, which are explained by the injection of residual derivatisation reagent into the GC inlet. The application of the proposed methods did not cause any problems in that respect. Esters and free forms of the analytes were measured on two different GC-MS instruments. Both instruments were equipped with new columns at the beginning of the project. These columns had not to be replaced, even after analysing more than 600 samples, QC, blank samples and calibration standards not

included. Slight peak tailing was observed in chromatograms of the measurements of free forms of analytes after running long sequences of 100 samples. Cutting the column at the inlet side by about 50 cm remediated this problem. Inlet liners and septa were replaced after 50 to 70 injections. The performances of the ion sources of both instruments were stable. They were cleaned according to the normal maintenance schedule.

3.4. Quality/stability of derivatisation reagent

In the course of the project it came to the attention of the authors that different qualities of derivatisation reagent seem to be on the market, provided even by the same supplier under identical product numbers. Colleagues from an official food control laboratory informed the authors that they experienced a decrease in performance of their analytical method by employing a new batch of PBA. Experiments were performed in reaction to these findings to clarify the issue. A 5-year-old batch of PBA available to the JRC behaved differently from the batch of PBA used over the period of the project. PBA from the actual batch dissolved completely in diethyl ether, whereas the same amount of PBA was only partially soluble in an even increased volume of diethyl ether. For the time being, it cannot be explained whether this was the consequence of degradation of the older batch of PBA. However, it is worth to keep this possibility in mind.

3.5. Reporting of results

The results of the analysed samples were reported to EFSA in Standard Sample Description (SSD) format. Instructions for reporting were taken from the technical report on specific requirements for chemical contaminants' data submission (EFSA, 2014). Additionally all samples were coded by means of the FoodEx2 classification system.

CONCLUSIONS

Two analytical methods were developed and in-house validated for (a) the determination of 2-MCPD esters, 3-MCPD esters, and glycidyl esters, and (b) for the determination of free 2-MCPD and free 3-MCPD in a broad variety of food samples, such as bread and rolls, fine bakery wares, smoked fish products, fried and roasted meat products, potato based snacks and fried potato products, cereal-based snacks, and margarines. Performance characteristics of the methods met the specifications provided by EFSA. They met also the requirements on analytical precision defined in EU legislation for the official control of the levels of 3-MCPD in foodstuffs.
A future goal might be the integration of the two analyses into one analysis method, allowing the simultaneous determination of both free and ester bound MCPD as well as GEs.

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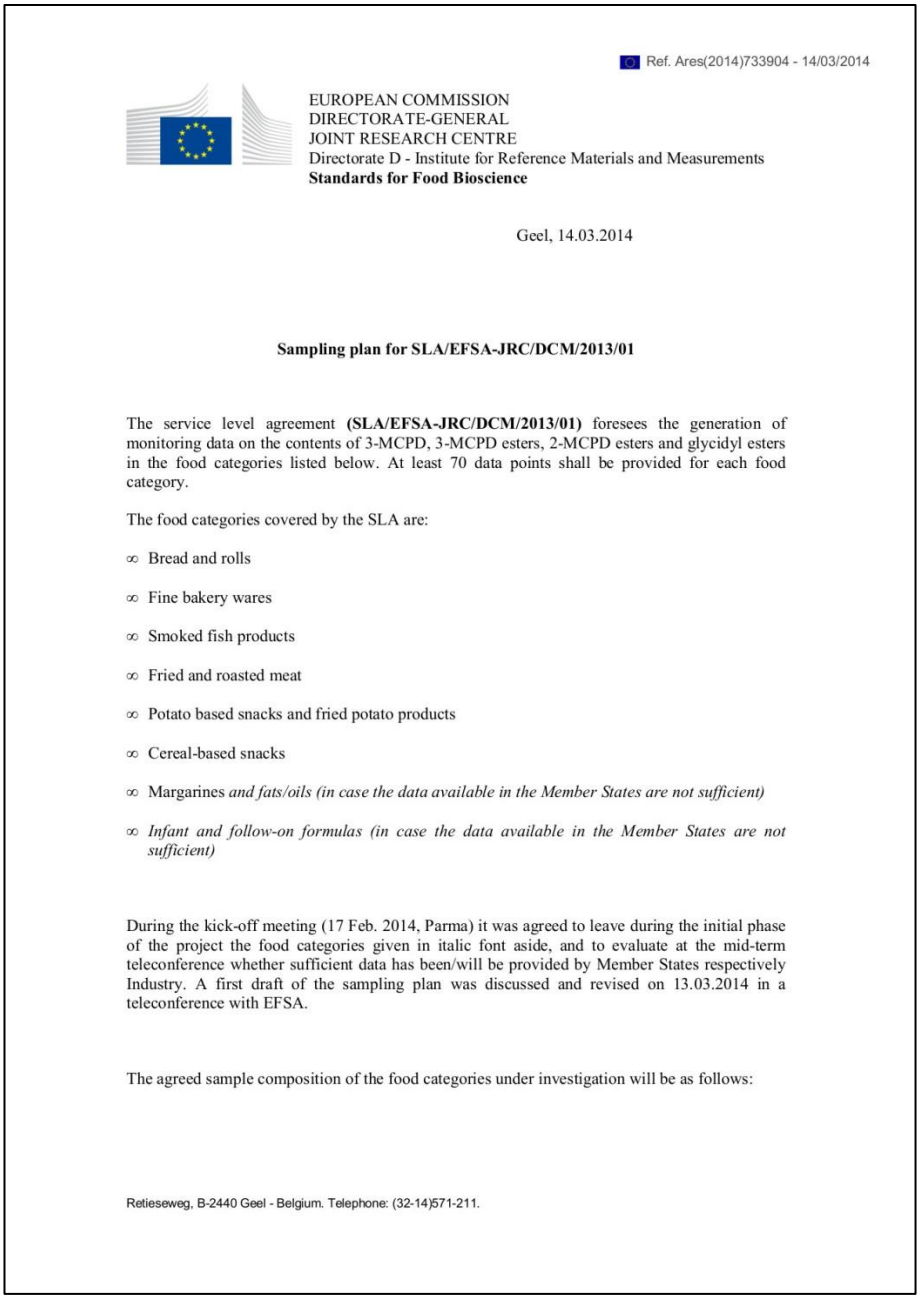
APPENDICES

Appendix A. Sampling plan



Ref. Ares(2014)733904 - 14/03/2014

EUROPEAN COMMISSION
DIRECTORATE-GENERAL
JOINT RESEARCH CENTRE
Directorate D - Institute for Reference Materials and Measurements
Standards for Food Bioscience

Geel, 14.03.2014

Sampling plan for SLA/EFSA-JRC/DCM/2013/01

The service level agreement **(SLA/EFSA-JRC/DCM/2013/01)** foresees the generation of monitoring data on the contents of 3-MCPD, 3-MCPD esters, 2-MCPD esters and glycidyl esters in the food categories listed below. At least 70 data points shall be provided for each food category.

The food categories covered by the SLA are:

- Bread and rolls
- Fine bakery wares
- Smoked fish products
- Fried and roasted meat
- Potato based snacks and fried potato products
- Cereal-based snacks
- Margarines *and fats/oils (in case the data available in the Member States are not sufficient)*
- *Infant and follow-on formulas (in case the data available in the Member States are not sufficient)*

During the kick-off meeting (17 Feb. 2014, Parma) it was agreed to leave during the initial phase of the project the food categories given in italic font aside, and to evaluate at the mid-term teleconference whether sufficient data has been/will be provided by Member States respectively Industry. A first draft of the sampling plan was discussed and revised on 13.03.2014 in a teleconference with EFSA.

The agreed sample composition of the food categories under investigation will be as follows:

Retieseweg, B-2440 Geel - Belgium. Telephone: (32-14)571-211.

1) Bread and bread rolls

It was agreed during the kick-off meeting to base the sample composition on consumption figures, which were provided by EFSA. However, a minimum number of ten samples per category was requested by EFSA.

Consequently the different classes of bread and rolls will be represented in the monitoring data with the number of samples indicated in the last column of Table 1.

Table 1: Consumption figures received from EFSA and number of samples for category bread and rolls

Classifier	Consumption figures (Number of entries)	Relative proportion (%)	Number of samples
Wheat bread and rolls	363752	56.2	30
Rye bread and rolls	97963	15.1	11
Mixed wheat and rye bread and rolls	144405	22.3	16
Multigrain bread	14385	2.2	10
Unleavened bread, crispbread, rusk	26917	4.3	10
Total	647422	100.0	77

Sampling will be performed in retail stores of southern and central Europe covering countries such as Italy, Austria, Germany, The Netherlands, Belgium, France, Czech Republic, Greece

2) Fine bakery wares

It was agreed during the kick-off meeting to base the sample composition on consumption figures, which were provided by EFSA.

Information was provided on two main classes of bakery ware, comprising many subclasses. The corresponding sample composition including the distribution of samples for subclasses of pastries and cakes (given in italic font) was agreed with EFSA. Details are presented in Table 2.

Table 2: Consumption figures received from EFSA and number of samples for category fine bakery ware

Classifier	Consumption figures (Number of entries)	Relative proportion (%)	Number of samples
Pastries and cakes	78639	63.4	50
Puff pastry (e.g. croissants, apple strudel …)			*10*
Hot surface cooked pastries (e.g. waffels, pancakes…)			*10*
Shortcrusts (e.g. flan tart, cheese pies, fruit pie-tarts…)			*10*
Fatty cake products (e.g. muffin, …)			*10*
Yeast leavened pastries (e.g. brioche, Berliner, doughnuts…)			*10*
Cookies	45398	36.6	20
Total	647422	100.0	70

Sampling will be performed in retail stores of southern and central Europe covering countries such as Italy, Spain, Greece, Austria, Germany, The Netherlands, Belgium, France, and Czech Republic

3) Smoked fish

Smoked fish is produced primarily from salmon, herring, mackerel, sprats and trout. Sampling will take account of these fishes with following sample distribution:

Table 3: Sample distribution for category smoked fish

Classifier	Relative proportion (%)	Number of samples
Herring	29	20
Salmon	29	20
Mackerel	14	10
Sprats	14	10
Trout	14	10
Total	100.0	70

Trade of smoked fish takes place on the European scale. The products are available in all countries. Hence, samples representing different production countries will be taken in Belgium, The Netherlands, and Germany.

4) **Smoked meat products**

In the kick-off meeting, it was agreed to cover in sampling both different regions of Europe as well as industrial products and products from small companies/farmers, sold locally. The sample distribution between samples from industrial production and samples from small companies was set to 55 to 15.

With the condition of minimum ten samples per category, following sample distribution was derived from the consumption data provided by EFSA.

Table 4: Sample distribution for category smoked meat products

Classifier	Consumption figures (Number of entries)	Relative proportion (%)	Number of samples
Preserved ham, pork	58862	56.5	25
Preserved bacon	11939	11.5	12
Uncooked smoked sausage	4655	4.5	10
Cooked smoked sausage	19617	18.8	13
Semi dry sausage	8952	8.6	10
Total	104095	99.9	70

Smoked meat products will be sampled on a broad European scale. The aim will be to cover products of about 15 EU MS.

5

5) Fried and roasted meat

In the kick-off meeting it was agreed to sample one third of the samples (15 samples) from commercial sources such as KFC, Burger King, Mc Donalds etc. However, this figure was reduced (to 15 samples) after the first review of the sampling plan by EFSA. Twenty samples shall be domestically prepared (pan-) fried meat samples. The last third of samples (24 samples) will constitute barbecued meat and sausage samples. The last category shall comprise both meat and sausage samples, which shall equally represent marinated and non-marinated food items).

Table 5: Sample distribution for category fried and roasted meat

Classifier	Relative proportion (%)	Number of samples
Commercial meat samples (retail, restaurants…)	20	15
Home cooked pan fried meat samples	27	20
Marinated barbecued meat samples	13	10
Non-marinated barbecued meat samples	13	10
Marinated barbecued sausage samples	13	10
Non-marinated (fresh) barbecued sausage sample	13	10
Total	99	75

The commercial meat samples will be sampled in the BENELUX area, as the production practices of these fast-food restaurants are to a large extend standardised throughout the EU. Home-cooked meat samples will be collected in the BENELUX from different households. Attention will be given to cover different country/culture dependent cooking practices. Barbecued meat and sausage samples will be prepared at IRMM from raw products from different sources in the BENELUX.

6) Potato products

During the kick-off meeting several sub-groups of potato products were identified.

There is the group of industrially prepared (finished) potato products such as potato crisps and potato sticks. They are prepared from sliced potatoes or from potato dough. The might flavoured or unflavoured.

The second group of important potato products comprises potato chips (French fries). These products are processed either in restaurants or at home. Croquettes and French fries made from potato dough will be covered in this group as well.

A third group of products comprise oven baked potato products (oven baked French fries, oven baked croquettes).

Table 6: Sample distribution for category potato products

Classifier	Relative proportion (%)	Number of samples
Potato crisps from sliced potatoes, flavoured and non-flavoured and fried small potato sticks	20	15
Potato crisps from potato dough, flavoured and non-flavoured	20	15
Potato chips from sliced potatoes (French fries)	20	15
Potato chips from potato dough and oil fried croquettes	20	15
Oven baked potato products (include also home made products like pan fried potato pieces or Roesti..)	20	15
Total	100	75

Potato crisps (both from sliced potatoes and from dough) and fried potato sticks will be sampled primarily in Belgian retail stores, as it can be assumed that the products of the big brands are same all over Europe. However the set of samples will also comprise some locally produced products or private labels.

Potato chips from sliced potatoes as well as oil fried potato dough products will be sampled in different restaurants, as it is assumed that processing conditions and the quality and type of the used oil have bigger influence on the final product than the raw materials. However, the

recommended end-points of processing should be rather similar throughout EU, which is the consequence of the acrylamide issue. Hence sampling will primarily take place in central Europe.

Oven baked products will be prepared at IRMM from different commercial raw materials, applying the recommended processing conditions.

7) Cereal based products (snacks)

Several classes of cereal products were identified during the kick-off meeting and consumption data were provided by EFSA. The sample composition will reflect the consumption and the following numbers of samples

Table 7: Sample distribution for category cereal based products

Classifier	Consumption figures (Number of entries)	Relative proportion (%)	Number of samples
Cereal flakes	27629	60.2	30
Muesli	7763	16.9	10
Cereal bars	1720	3.7	10
Popped cereals	2340	5.1	10
Porridge	5276	11.5	10
Total	45918	100.0	70.0

The products falling in these categories are usually produced on the industrial scale. It is justified to assume that production happens in a limited number of factories. Hence, the variability a particular product, from a particular manufacturer, sold in different countries might be limited. However, in sampling we will try to cover potential brand dependent variability of the analyte contents. Hence, sampling will target both popular brands as well as private labels from different countries. Sampling will take place in retail stores of the BENELUX, Germany, France, Austria, Czech Republic, Spain, and Italy.

8) Infant formula and margarine:

If data that will be provided by official food control and by industry for food falling in these two categories are considered by EFSA insufficient for exposure assessment, a sampling plan for filling identified gaps will be discussed and agreed during the mid-term teleconference. However thirty margarine samples shall be tested anyway. As margarines are produced in EU by a limited number of companies these samples will be purchased primarily in the BENELUX.

Table 8: Number of margarine samples

Classifier	Number of samples
Margarine	30

9

Appendix B. Standard operating procedure for the determination of 2-MCPD esters, 3-MCPD esters and glycidyl esters in food

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Reference Materials and Measurements

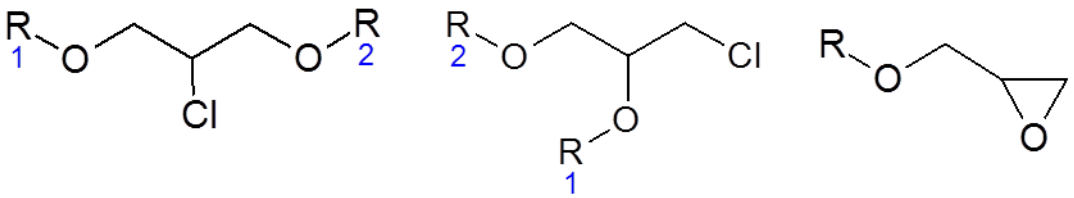
Standard Operating Procedure for the Simultaneous Determination of 3-MCPD, 2-MCPD and Glycidyl Fatty Acid Esters in Various Food Matrices

In-house validated by the EC-JRC-IRMM
2014

1. Scope and application

This standard operating procedure (SOP) specifies an indirect method for the simultaneous determination of fatty acid esters of 2-MCPD, 3-MCPD and of glycidol in a wide variety of food products after extraction by pressurised liquid extraction (PLE), acid transesterification and derivatisation of the released free (non-esterified) form in ethyl acetate 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 (EI). 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 0.020 mg kg-1 – 1.850 mg kg-1. The method was applied for quantification of the three groups of analytes in seven 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.
The method is not suitable for the determination of MCPD esters and glycidyl esters in infant formula.

Figure 1: Structure of 2-MCPD esters (a), 3-MCPD esters (b) and glycidyl esters (c)
R, R1 and R2 represent acyl groups of fatty acid.



a) **b)** **c)**

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. Depending on the labelled (expected) total fat content of the test sample one of two different sample preparation routes will be followed.

A): If the labelled (expected) total fat content of the test sample is above 5 % (weight/weight), the test portion is immediately extracted.

B) If the labelled (expected) total fat content of the test sample is below or equal to 5 % (weight/weight), stable isotope labelled internal standards (rac 1,2-bis-palmitoyl-3-chloropropanediol-d5, 1,3-distearoyl-2-chloropropanediol-d5 and pentadeuterated glycidyl oleate) are added into the extraction cell prior to sample extraction.

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, and the sample preparation continues depending on the previously chosen route.

In case of A): 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).

In case of B): The whole amount of extracted fat is used for further sample preparation. Isotope labelled substances are not added at this stage, as they are already contained in the sample.

Consequently, glycidyl esters are 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 stream of nitrogen at 40 °C and aqueous ammonium sulphate solution is added. Thereafter, the sample is defatted with n-hexane and the released free MCPD and free MBPD are extracted with ethyl acetate and derivatised with phenylboronic acid. The sample 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 3-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 procedure the test portion is of 5.0 ± 0.1 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	-	$C_{39}H_{75}ClO_4$	648.49
glycidyl palmitate	Gly-P	7501-44-2	$C_{19}H_{36}O_3$	312.48
glycidyl oleate-d5	Gly-O-d5	5431-33-4-unlabelled	$C_{21}H_{33}D_5O_3$	343.56

All 3-MCPD, 2-MCPD, 3-MBPD 3-MCPD-d5, 2-MCPD-d5 and 3-MBPD-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.

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. Isooctane (2,2,4-trimethylpentane), 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 (purity ≥ 97%)
6.1.15 Sodium bromide, anhydrous (purity ≥ 99.5%)
6.1.17. Sand, 50 – 70 mesh particle size
6.1.18. Sodium polyacrylate, Poly(acrylic acid), partial sodium salt-graft-poly(ethylene oxide) granular, 90-850 µm particle size
6.1.19. Reference material for quality control
A self-prepared test material may be applied for this purpose.

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 33 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 into a 10 ml amber volumetric flask (8.23) using the microbalance (8.2). Toluene is added up to the mark. 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.

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.

Note: Isotope labelled MCPD and glycidyl esters are very expensive and supplied in small quantities. Therefore, it is advisable to transfer the whole amount of substance with some toluene (including rinsing steps) from the original container (ampoule) into the volumetric flask and determine the transferred amount by differential weighing of the original container preferably on a microbalance.
Note: 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 into a 10 ml amber volumetric flask (8.23) using the microbalance (8.2). Toluene is added up to the mark. 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.

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	215.6 µg/ml

9.3. Mixed labelled MCPD and glycidyl ester solution

Prepare, with the individual stock solutions of labelled esters (9.7a, 9.7b, 9.7c) listed in **Table 3** a solution in toluene with a concentration of approximately 5 µg/ml by pipetting 500 µl of each individual stock solution of labelled ester (9.7a, 9.7b, 9.7c) into a 20 ml volumetric flask (8.23) and fill to mark with toluene.
50 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.

9.4. Mixed intermediate solution I 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 5 µg/ml by pipetting 250 µl of each of the standard stock solutions (9.1a, 9.1b, 9.1c) into a 50 ml volumetric flask (8.23) and fill up to mark with toluene.

9.5. Mixed intermediate solution II of native MCPD and glycidyl esters

Prepare, with the mixed intermediate solution I of native esters (9.4) a solution in toluene with a concentration of approximately 0.5 µg/ml by pipetting 1000 µl into a 10 ml volumetric flask (8.23) and fill up to mark.

9.6. Preparation of calibration standards

Table 4 provides a scheme for the preparation of calibration standards for the analyte content range of about 20 µg/kg to 1850 µg/kg extracted fat. Bold figures indicate volumes of standard solutions that have to be pipetted per calibration level into an empty screw cap test tube (8.1) for bromination. Two millilitres of tetrahydrofuran (6.1.1) are added and bromination, transesterification and derivatisation are performed as described in 11.3. The concentration values given in Table 4 are target values. The actual concentrations have to be calculated for each standard/analyte combination applying Equation 1.

Table 4: Preparation scheme for calibration standards

	Volume of mixed labelled MCPD and glycidyl ester process solution (9.3)	Nominal concentration of standard solution (9.3)	**Volume of mixed intermediate solution I of native MCPD and glycidyl esters (9.4)**	**Volume of mixed intermediate solution II of native MCPD and glycidyl esters (9.5)**	Nominal concentration of mixed intermediate solutions	Native 3-MCPD in test tube prior to transesterification	Labelled 3-MCPD in test tube prior to transesterification	Ratio native analytes / labelled analytes
	µL	µg/mL	µL	µL	µg/mL	ng	ng	
Cal 1	**50**	5.0	**20**		0.5	1.9	48.8	0.04
Cal 2	**50**	5.0	**60**		0.5	5.6	48.8	0.12
Cal 3	**50**	5.0		**20**	5.0	18.8	48.8	0.39
Cal 4	**50**	5.0		**40**	5.0	37.6	48.8	0.77
Cal 5	**50**	5.0		**60**	5.0	56.5	48.8	1.16
Cal 6	**50**	5.0		**80**	5.0	75.3	48.8	1.54
Cal 7	**50**	5.0		**100**	5.0	94.1	48.8	1.93
Cal 8	**50**	5.0		**120**	5.0	112.9	48.8	2.32
Cal 9	**50**	5.0		**150**	5.0	141.2	48.8	2.89
Cal 10	**50**	5.0		**200**	5.0	188.2	48.8	3.86

$$C_{Cal} = C_I * V_I * R_M \quad \textbf{Equation 1}$$

C_{Cal}: Equivalent of free form of native (labelled) substance in test tube (in ng)
C_I: Concentration of intermediate standard solution used to prepare calibration standard (in µg/ml)
V_I: Volume of intermediate standard solution pipetted into test tube (in µl)
R_M: Ratio of mol masses of native (labelled) free forms of 3-MCPD, 2-MCPD respectively glycidol and the corresponding esters used to prepare calibration standards.

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

	MW ester	MW free form	R_M
	g /mol	g/mol	
3-MCPD ester	587.36	110.54	0.1882
3-MCPD-d5 ester	592.39	115.57	0.1951
2-MCPD ester	643.46	110.54	0.1718
2-MCPD-d5 ester	648.49	115.57	0.1782
Gly-P	312.48	74.08	0.2371
Gly-O-d5	343.56	74.08	0.2156

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.
2. *Sodium hydrogen carbonate solution (0.6 %, w/v)*. Weigh 0.6 g of sodium hydrogen carbonate in a 100 ml volumetric flask and fill up to the mark with ultra-pure water. 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.
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 (6.1.7). Shake vigorously.

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) F*ood matrices with labelled (expected) total fat contents above 5 %:* 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 (6.1.18) and 15 g of sand (6.1.17). Mix thoroughly until the sample is finely ground and visually homogeneous. 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.

B) F*ood matrices with labelled (expected) total fat contents equal or below 5 %:* 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 (6.1.18) and 15 g of sand (6.1.17). Mix thoroughly until the sample is finely ground and visually homogeneous. 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. Add 50 µl of mixed stable isotope labelled ester solution (9.3) prior to the pressurized liquid extraction directly into the PLE extraction cell containing the test portion.

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

NOTE: Correct determination of the extracted amount of fat is difficult in case of low fat products. Therefore, for breads and rolls, porridge and other food matrices with labelled (expected) total fat contents below 5 %, 50 µl of mixed stable isotope labelled ester solution (9.3) are added prior to the pressurized liquid extraction directly into the PLE extraction cell containing the test portion.

11.3. Sample extraction by PLE

The extraction takes place under the following conditions:

Pressure	ambient
Temperature	40 ºC
Pre-heat time	0 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 (~100 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. Depending on the route chosen in 11.2 sample preparation is continued.

1. In case of 11.2 option A): A portion of 100 ± 5 mg of oil/fat) is transferred with a Pasteur pipette or a spatula into a 10 ml screw cap glass tube and 50 µl of mixed labelled ester solution (9.3) is added. Add 2 ml of anhydrous tetrahydrofuran (6.1.1) and shake vigorously on a vortex mixer for 15 seconds. Proceed with the conversion of glycidyl esters, as described below.
   In case of 11.2 option B): The whole amount of extracted fat is dissolved in 2 mL tetrahydrofuran (6.1.1) and transferred entirely into a 10 ml screw cap glass tube. Proceed with the conversion of glycidyl esters, as described in the following.
2. Glycidyl ester 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.
3. 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.
4. 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.
5. 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.
6. 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. 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).

12. Instrumental conditions

12.1. GC conditions

(a) Injection volume: 1.0 µL
(b) Injection mode: pulsed splitless
(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*): 146 (Q_1); 240 (Q_2);
(vi) phenylboronic derivative of 3-MBPD-d5 (*m/z*): 150 (Q_1); 245 (Q_2).

Acquisition time window: 5-20 min.

Table 6: 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	16.87	150	201
3-MCPD	16.95	147	196
2-MCPD-d5	17.61	201	203
2-MCPD	17.70	196	198
3-MBPD-d5	18.90	150	245
3-MBPD	18.96	146	240

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), (2-MCPD-d5), 146 (3-MBPD) and 150 (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 Equation 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

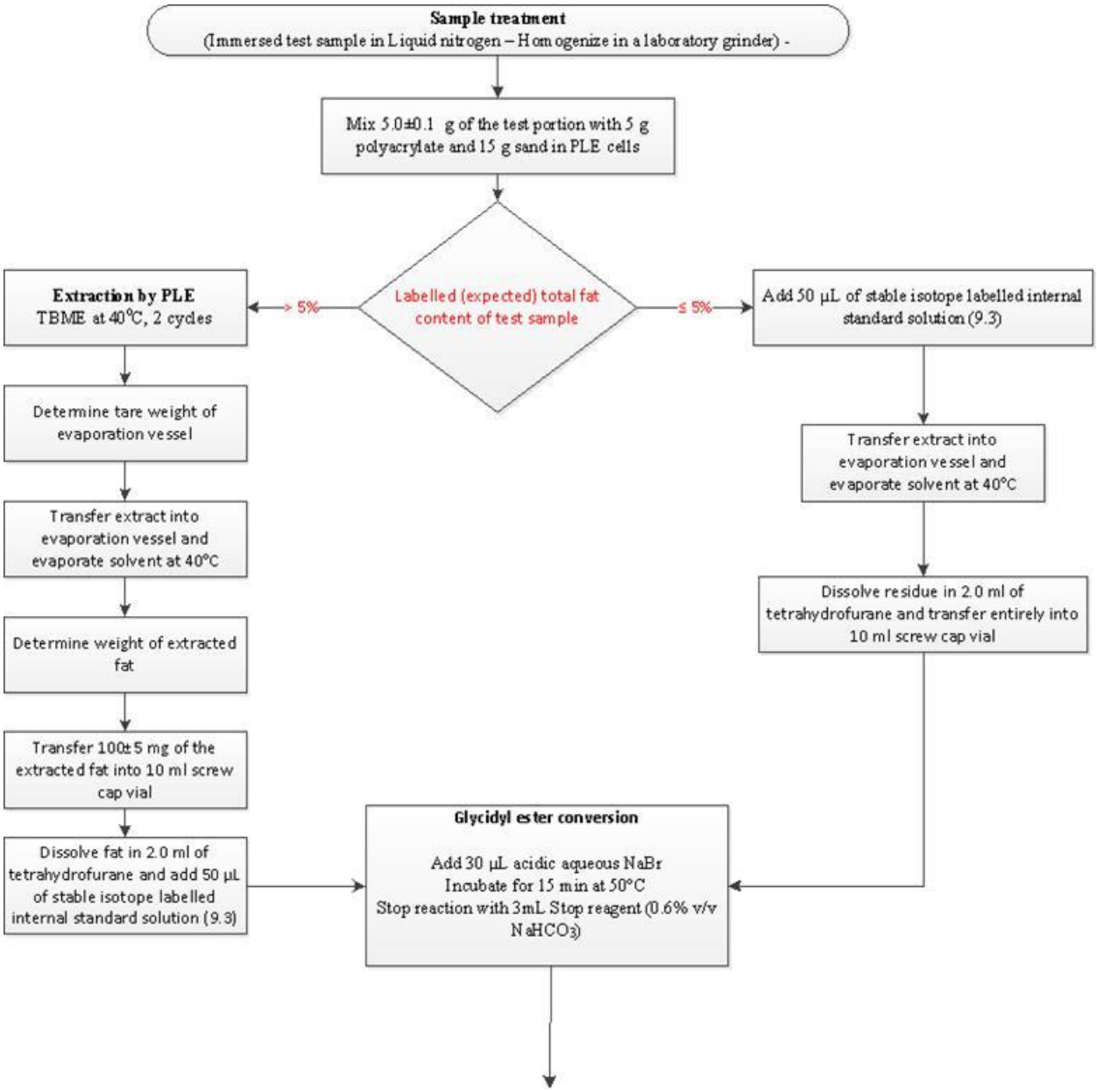
$W_{sample/fat}$: a) weight of the extracted fat used for further analysis, or b) weight of test portion, if mixed labelled ester process solution (9.8) was added to the test portion prior to extraction (**both values in g**)

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

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

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**)

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



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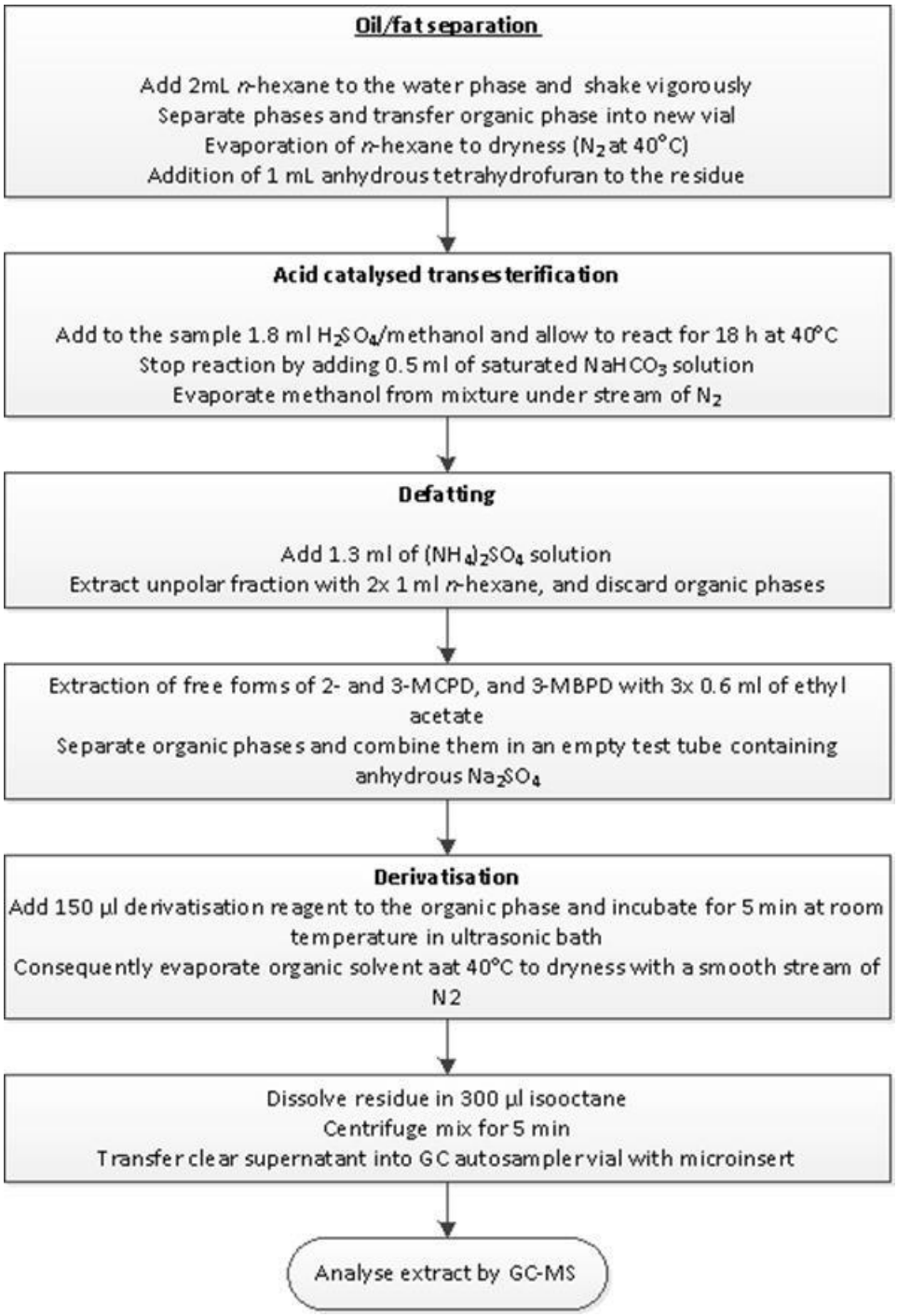


Appendix C. Standard operating procedure for the determination of free 3-MCPD and free 2-MCPD in food

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Reference Materials and Measurements

Standard Operating Procedurefor theDetermination of Free 3-MCPD and Free 2-MCPD in Food

In-house validated by the EC-JRC-IRMM
2014

1. Scope and application

The method is suitable to determine free forms of **3-monochloropropane-1,2-diol (3-MCPD) and 2-monochloropropane-1,3-diol (2-MCPD) in several food matrices** at concentrations between 9 µg/kg (3-MCPD) respectively 14 µg/kg (2-MCPD) and 720 µg/kg.

2. Safety

MCPDs are harmful to humans
A provisionally maximum tolerable daily intake (PMTDI) of 2.0 µg/kg body weight has been established for 3-MCPD.
Consequently, personal protective equipment such as laboratory coat, gloves and safety glasses has to be used for all sample preparation steps. All handlings of MCPDs and organic solvents should be performed in a fume hood with adequate air flow.

3. Principle

A test portion is weighted into 10 ml screw cap vials and stable isotope labelled free forms of 2-MCPD and 3-MCPD are added. The sample is then extracted with a n-hexane-acetone mixture by vigorously shaking. The extract is phase separated by adding water. The aqueous phase containing the free forms of MCPDs is consecutively extracted with ethyl acetate, and extracted analytes are derivatised with phenylboronic acid. The measurement of the analytes is performed by gas chromatography mass spectrometry in single ion monitoring mode. Quantification is done internal standardisation with the stable isotope labelled analogues of 2-MCPD and 3-MCPD.

4. Instruments and glassware

4.1. GC-MS system

4.1.1 Autosampler
Capable of injecting 1 µl of sample

4.1.2 Injection port
Split/splitless injection port with low bleed septum and glass insert suitable for splitless injection

4.1.3 GC column
5% diphenyl, 95% dimethyl polysiloxane, 30 m x 0.25 mm internal diameter and 0.25 µm film thickness, or equivalent

4.1.4 Mass spectrometer
Single quadrupole mass spectrometer operating in electron ionisation mode at 70 eV and capable of performing single ion monitoring (SIM)

4.1.5 Data acquisition and analysis system
Suitable data collection and evaluation software

4.2. Calibrated analytical balance with a readability of 0.01 mg

4.3. Calibrated positive displacement variable-volume pipettes

Capacity 0.1 ml, 1 ml, 5 ml and 10 ml

4.4. Wrist arm shaker

Eight position, with adjustable shaking frequency and timer

4.5. Ultrasonic bath

Adjustable water level and timer

4.6. Vacuum centrifuge or similar evaporator

Set maximum temperature to 50 °C. Apply evaporation condition for aqueous solvent

4.7. Glassware

4.7.1 Volumetric flasks with glass stoppers
Volume 25 ml, 50 ml, 100 ml etc., according to ISO 1042

4.7.2 Single use glass vials
Volume of 10 ml with screw caps and PTFE lined septa

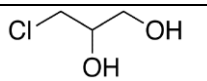
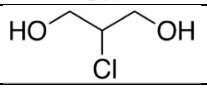
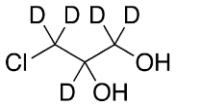
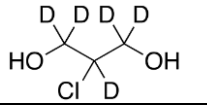
4.7.3 Single use 2 ml autosampler vials with glass inserts

4.7.4 Glass beakers of different size
With the exemption of autosampler vials and 10 ml screw cap glass vials which are applied as supplied, all reusable glass ware is firstly cleaned in a laboratory dish washer and then thoroughly rinsed with methanol and *n*-hexane.

5. Reagents and standards

Chemicals should be of high purity. Standard solutions may be prepared from neat materials or from commercial solutions in appropriate solvents.

5.1 Neat MCPDs
Supplied in screw cap vials with minimum content of 50-100 mg and 2.5-10 mg for native and labelled analytes respectively.

Analytes	Acronym	CAS No.	Structure	Purity (%)
3-Chloro-1,2-propanediol	3-MCPD	96-24-2		98
2-Chloro-1,3-propanediol	2-MCPD	497-04-1		98
3-Chloro-1,2-propanediol-d5	3-MCPD-d5	342611-01-2		97 Isotopic purity 98.7
2-Chloro-1,3-propanediol-d5 (Major)	2-MCPD-d5			98

5.2 Phenylboronic acid, Reagent grade (95 % purity).
Prepare a solution of phenylboronic acid (PBA) in diethyl ether (0.4 g/ ml) on a regular interval.

5.3 Methanol, GC-MS grade

5.4 *n*-Hexane, GC-MS grade

5.5 Acetone, GC-MS grade

5.6 *n*-Hexane-Acetone solution
Prepare a solution of *n*-hexane-acetone in the ratio 1:1 (v/v). Mix thoroughly and store in screw capped vessel of 250 ml.

5.7 Ethyl acetate, GC-MS grade

5.8 Diethyl ether, GC-MS grade

5.9 Isooctane, GC-MS grade

5.10 Deionised water,e.g. water of LC-MS quality

6. Standard solutions

The concentrations given below are just indicative. Therefore, the concentration levels and consecutively the specified volumes of the standard solutions shall be adapted to the actual situation.

Note: All standard solutions are stored refrigerated. Allow standard solutions to get to ambient temperature before further use. The shelf life of the stock standard solutions is at least 3 months.

6.1. Stock standard solution of native and labelled MCPDs in methanol

Transfer all the analytes from the vials supplied by the producer (e.g. 100, 50, 10, and 2.5 mg for 3-MCPD, 2-MCPD, 3-MCPD-d5, 2-MCPD-d5 respectively) into suitable individual volumetric flask, and make up to volume with methanol (5.3) to get stock solutions of 2000, 1000, 400, and 100 µg/ml for 3-MCPD, 2-MCPD, 3-MCPD-d5, 2-MCPD-d5 respectively. Rinse the vials with methanol and combine the rinsing solution with the analytes. The exact amount of transferred substance is determined by differential weighing (weighing of the vials before transfer of the analytes, and after transfer). This solution can be stored at below 10°C for at least 3 months.

Note: The small amount of neat MCPD standards renders difficulties to pipette exact volumes of the neat liquid. Therefore, the microliter syringes (separate syringes for each neat MCPD) are only used as a tool for the transfer of the neat substance onto the inner surface of the volumetric flask. The amount transferred is determined gravimetrically. It is recommended to rinse the inner glass wall of the volumetric flask with a few hundreds microliter of methanol, after recording of the transferred amount of neat MCPDs. This prevents potential losses during filling up with solvent.

6.2. Intermediate standard solution of native MCPDs in methanol (12 µg/ml)

Transfer 600 µl of the stock solution of 3-MCPD in methanol (6.1) (2000 µg/ml) and 1200 µl of the stock solution of 2-MCPD in methanol (6.1) (2000 µg/ml) to a 100 ml volumetric flask and make up to volume with methanol.

6.3. Intermediate standard solution of stable isotope labelled MCPDs in methanol (12 µg/ml)

Transfer 3000 µl of the stock solution of 3-MCPD-d5 in methanol (6.1) (400 µg/ml) and 12000 µl of the stock solution of 2-MCPD-d5 in methanol (6.1) (100 µg/ml) to a 100 ml volumetric flask and make up to volume with methanol.

6.4. Working standard solution of stable isotope labelled MCPDs in methanol (0.6 µg/ml)

Transfer 5000 µl of the intermediate standard solution of stable isotope labelled MCPDs in methanol (6.3) (12 µg/ml) to a 100 ml volumetric flask and make up to volume with methanol (5.3).

6.5. Calibration standards

Note: The given concentration levels are only indicative and refer to the concentration of the native MCPDs in the methanol solution. The real concentrations have to be calculated from the actual concentrations of the stock standard solutions and intermediate standard solutions.

Prepare ten different native calibration standard solutions containing 10, 25, 50, 100, 200, 500, 750, 1000, 1200, 2400 ng/ml for both 3-MCPD and 2-MCPD by pipetting appropriate volumes of intermediate native MCPD standard solution into 10 ml volumetric flasks and make up to volume with methanol (5.3).

7. Procedure

7.1. Test sample preparation

Weigh 1 g of sample with accuracy over 0.01 g into a 10 ml glass vial with screw cap and PTFE lined septum. Transfer with a pipette 5 ml of *n*-hexane-acetone solution (5.6). Add with a pipette (4.3) 100 µl of the working standard solution of stable isotope labelled MCPD in methanol (6.4).Shake the sample on a vortex shaker for 30 seconds and then place into an ultrasonic bath for sonication for 5 minutes under room temperature. After sonication, centrifuge the 10 ml vials for 10 minutes at 4000 rpm at room temperature. Transfer the extract solution to a new 10 ml vial and discard the solid residues. Add 1 ml of water (5.10) and shake vigorously for 30 seconds on a vortex shaker. To speed up the phase separation centrifuge the 10 ml vials for 10 minutes at 4000 rpm at room temperature. After phase separation discard the upper layer. Evaporate the remaining aqueous phase containing the free form of MCPDs by placing the 10 ml vials into a vacuum evaporator (4.6). The evaporation temperature was set to maximum 50 °C. After complete evaporation add 1.8 ml of ethyl acetate (5.7) with a suitable calibrated pipette to dissolve the dried residue in 10 ml vial. Add small amount of anhydrous sodium sulphate and vortex for few second to avoid presence of water in solution. Add 150 µl of PBA solution (5.2) and shake vigorously for 30 seconds and further place into ultrasonicator for 5 minute at room temperature. Evaporate the solvent by placing the 10 ml vials into an evaporator maintained at 40 °C under mild N_2 stream. Extract the derivatised residue by adding 300 µl of isooctane (5.9) to the 10 ml vial. Vortex for 30 seconds and if necessary sonicate for 5 minutes before collecting the extract into the autosampler vial (4.7.3). To eliminate solid residues centrifugate the autosampler vials for 5 min at 4000 rpm and transfer the clear solvent layer into a new vial and analyse by GC-MS in selected ion monitoring mode.

Procedural blank sample

The procedural blank sample consists of 100 µl of working standard solution of stable isotope MCPDs in methanol (6.4) and 5000 µl of *n*-hexane-acetone solution (5.6). This sample is then extracted as described before (see 7.1) and analysed by GC-MS.

7.2. Calibration

Pipette 100 µl of calibration standard solution of native MCPDs in methanol (6.5) and 100 µl of working standard solution of stable isotope MCPDs in methanol (6.4) into a 10 ml vial. Add small amount of sodium sulphate and vortex for 10 seconds. Evaporate the solvent to dryness by placing the 10 ml vials into evaporator maintained at 40 °C under mild N_2 stream. Add 1.8 ml of ethyl acetate with a suitable calibrated pipette to dissolve the dried residue in 10 ml vial. Add 150 µl of PBA solution and shake vigorously for 30 seconds and further place into ultrasonicator for 5 minute at room temperature. Evaporate the solvent by placing the 10 ml vials into evaporator maintained at 40 °C under mild N_2 stream. Extract the derivatised residue by adding 300 µl of isooctane to the 10 ml vial. Vortex for 30 seconds and if necessary sonicate for 5 minutes before collecting the extract into the autosampler vial. To eliminate solid residues centrifugate the autosampler vials for 5 min at 4000 rpm and transfer the clear solvent layer into a new vial and analyse by GC-MS in selected ion monitoring mode.

7.3. GC-MS determination

7.3.1 GC-MS conditions

The following GC parameters were successfully applied for the determination of free MCPDs in several food matrices

GC parameters	
GC Column	DB 5 MS, 30 m x 0.25 mm i.d., 0.25 µm d.f.
Carrier gas	He, 1.2 ml/min constant flow
Temperature programme	60.0 °C/1 min - 6.0 °C/min - 150.0 °C/2 min - 30 °C/min - 300.0 °C/10 min
GC-inlet	Split/splitless injection port with low bleed septum;
Inlet conditions	Mode: Pulsed Splitless, Initial temp: 250 'C (On), Pressure: 71.7 kPa, Pulse pressure: 200 kPa, Pulse time: 0.30 min, Purge flow: 30.0 ml/min, Purge time: 2.00 min, Total flow: 33.9 ml/min
Injection volume	1.0 µl
Injection mode	Splitless for 1.5 min
Total run time	33 min
MS parameters	
Mass filter	Quadrupole
Ionisation	Electron Ionisation, 70 eV
Operation mode	Selected ion monitoring (SIM)
Solvent delay	6 min
Recorded ions (Mass, Dwell time in seconds)	(147.00, 80) (150.00, 80) (196.00, 80)
	(198.00, 60) (201.00, 80) (203.00, 60)
GC-Interface temperature	300 °C
Ion source temperature	250 °C
MS Quad temperature	150 °C

Retention times of native and stable isotope labelled MCPDs and m/z-ratios of native MCPDs used for quantification (confirmation of identity).

Compounds	Retention time	Quantifier ion (m/z)	Qualifier ion (m/z)
3-MCPD	17.209	147	196
2-MCPD	17.991	196	198
3-MCPD-d5	17.124	150	201
2-MCPD-d5	17.891	201	203

7.3.2 Analysis sequence

Inject at the beginning of each sequence at least twice isooctane (5.9), in order to clean the system. Afterwards the test samples, procedural blank samples, quality control samples, and calibration standards are injected in random order. Inject after maximum 10 samples isooctane (5.9) to identify potential carry over.

8. Identification and calculation of results

The peak identity is confirmed by comparison of the peak ratios of quantifier ion and qualifier ion from sample extracts and standard solutions. The ratios should not differ more than ± 20 % from those obtained for standard solutions.

Calibration by internal standardisation is applied for the determination of native MCPDs. A calibration graph is constructed in which the ratio of the areas of the peaks of native MCPDs and the areas of the peaks of the corresponding stable isotope labelled MCPDs (see above) is plotted against the ratio of the concentrations of native MCPDs and stable isotope labelled MCPDs in the respective calibration solution. The calibration function is determined by linear regression. Equation 1 gives the relation between peak areas and concentrations of native and stable isotope labelled compounds.

$$\frac{A_{native}}{A_{labelled}} = a * \frac{C_{native}}{C_{labelled}} + b \qquad \text{Equation 1}$$

where

A_{native}	is the area of the quantifier ion of the native MCPD peaks
$A_{labelled}$	is the area of the corresponding stable isotope labelled MCPD peaks
a	is the slope of the calibration function
C_{native}	is the concentration of native MCPDs
$C_{labelled}$	is the concentration of corresponding stable isotope labelled MCPDs
b	is the intercept of the calibration function

Calculate for each sample the amount of native MCPD that was extracted from the sample (X_{native}) using the following equation:

$$X_{native} = \frac{\frac{\left(\frac{A_{native}}{A_{labelled}} - b\right)}{a} * X_{labelled} * V_{labelled}}{W_{sample}} \qquad \text{Equation 2}$$

where

X_{native}	is the concentration of native MCPDs (in µg/kg) in the sample.
A_{native}	is the area of the native MCPD peak of the test sample
$A_{labelled}$	is the area of the corresponding stable isotope labelled MCPD peak
$X_{labelled}$	is the concentration (in **µg/ml)** of the working standard solution of stable isotope labelled MCPDs in methanol (6.7)
Vlabelled	is the volume (**in ml)** of the working standard solution of stable isotope labelled MCPDs in methanol (6.7)
a	is the slope of the calibration function
B	is the intercept of the calibration function
W_{sample}	is the weight of extracted sample (**in kg**)

Calculate according to Equation 2 the MCPDs content in the procedural blank samples and subtract the average content of the procedural blank samples from the results of the test samples.
The results of the test samples are reported corrected for the background contamination to three significant figures. The reporting unit is µg/kg.

9. Quality control

For each batch of samples the following controls are used:

9.1. Laboratory reference materials

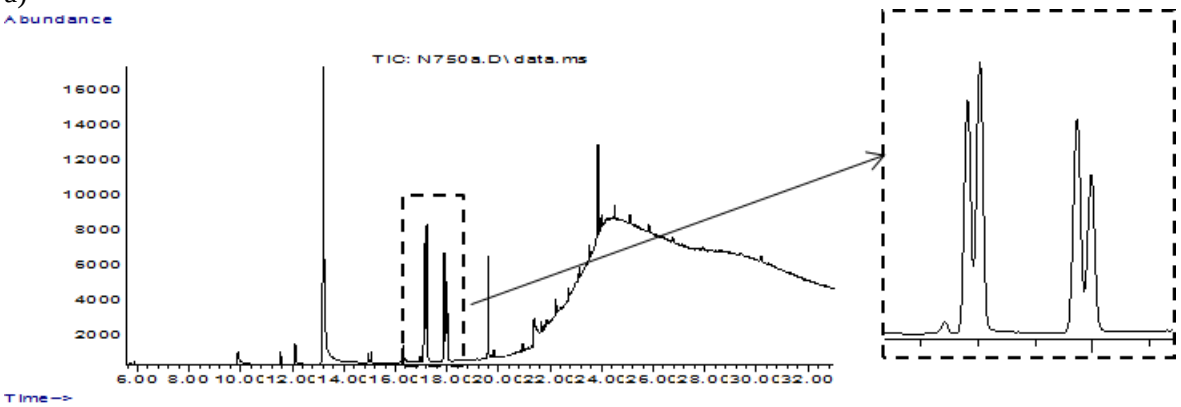
Properly spiked smoked meat sample or other suitable control samples are recommended for use as internal quality control (IQC) materials. The MCPD content of the IQC material should be between 0.05 mg/kg and 0.50 mg/kg.

The results of the IQC samples are plotted in QC charts. Set up and interpretation of QC charts should follow internationally agreed guidelines such as the IUPAC Harmonised Guidelines for Internal Quality Control in Analytical Chemistry Laboratories (Pure & Appl. Chem., Vol. 67, No. 4, pp. 649-666, 1995).

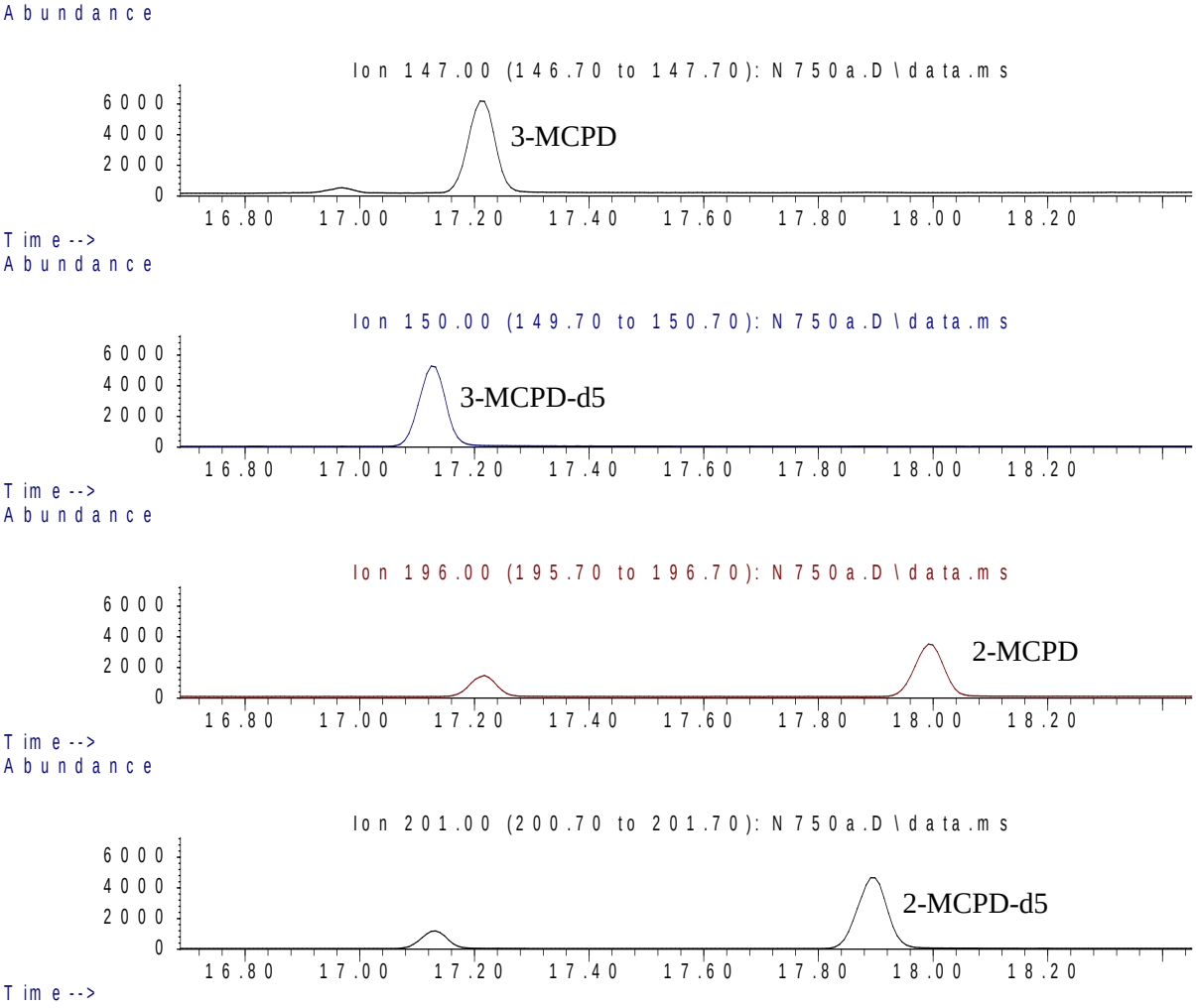
Annexes

Annex 1: Example of GC-MS chromatogram of a spike sample: a) total ion chromatogram (TIC); b) selected ion monitoring (SIM)

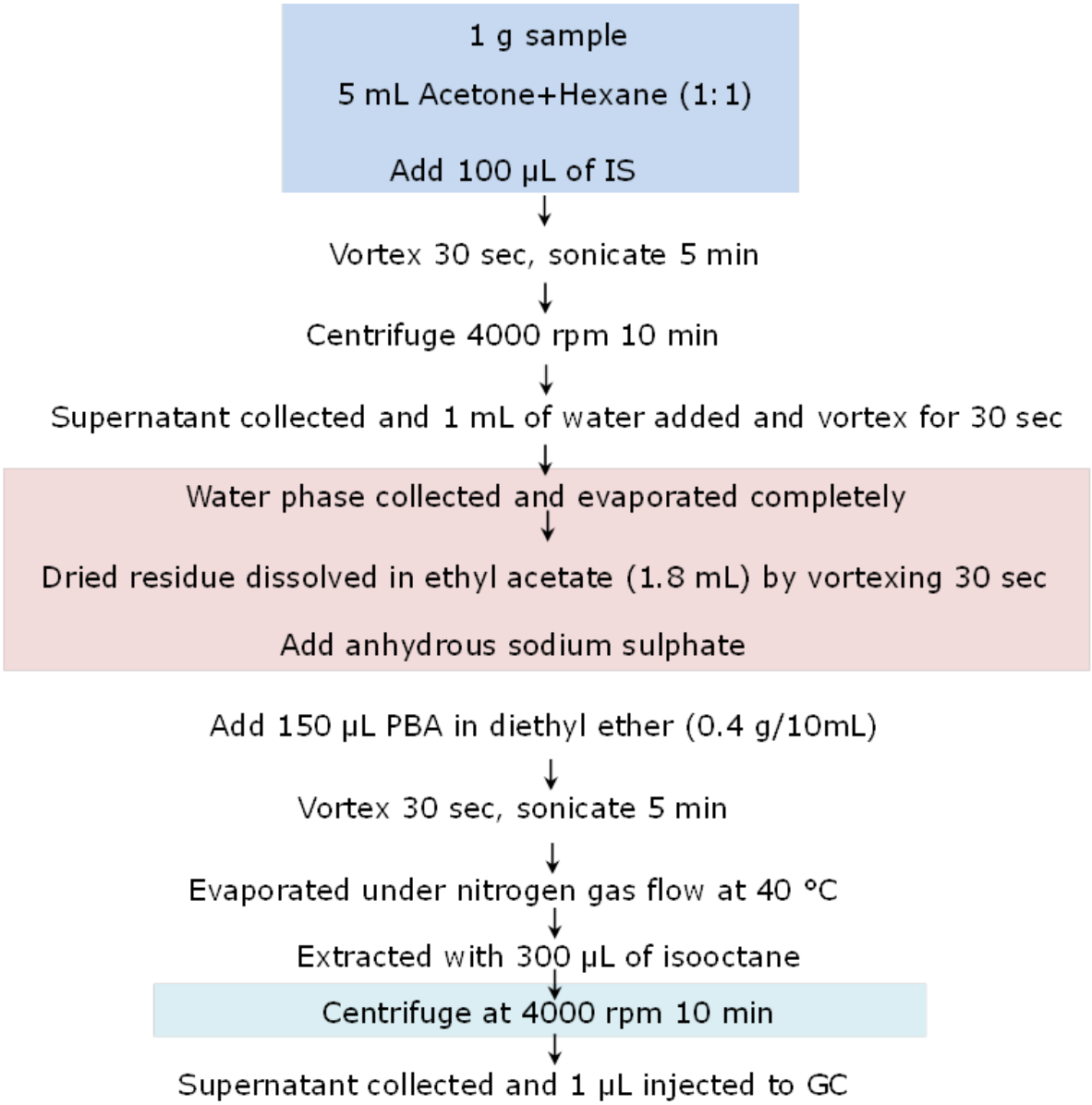
a)



b)



Annex 2: Analysis scheme



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Author(s): Thomas Wenzl, Vasilios Samaras, Anupam Giri, Gerhard Buttinger, Lubomir Karasek, Zuzana Zelinkova

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