Collaborative Study for the Determination of 3-MCPD- and 2-MCPD-Fatty Acid Esters in Fat Containing Foods

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First Collaborative Study Part II
Method Validation and Proficiency Test

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1 Summary

The BfR organised a collaborative trial to “determine 3-MCPD and 2-MCPD fatty acid esters (FAE) in fat containing foods”, which was conducted in line with the provisions of the harmonised protocol in accordance with ISO/IUPAC/AOAC (Horwitz, 1995). A total of 29 laboratories participated in the collaborative trial, whereby it was possible to statistically evaluate the results of 28 of them. As some of the labs analysed the sample using several methods, a total of 36 data sets were available for evaluation.

The current collaborative trial builds on the 2nd collaborative trial Part I (2 RT Part I) to determine 3-MCPD FAE in edible fat and oils, and it constitutes an expansion of the methods with regard to an additional extraction step with which the fat and the MCPD FAE dissolved in it are extracted from the food prior to further analysis. In the 2nd collaborative trial Part I, “BfR Method 9” was successfully validated and 25 of the 36 data sets of the current collaborative trial are based on the use of this method in combination with a preceding extraction process. In addition to this, the participants used in-house methods which differ from BfR Method 9 in several ways.

To estimate the efficiency of the methods used, they were sorted into groups with regard to characteristic common parameters. All of the laboratories that used BfR Method 9 are summarised in Method Groups 1-3. The subdividing classification depends on the extraction step. The remaining laboratories were subdivided and classified with regard to the saponification step.

Method Group 1 comprises all laboratories which used BfR Method 9 and a standardised extraction step by means of ASE (BfR Method 22). This method achieves good results as far as the relative standard deviation of reproducibility is concerned. The determined HorRat values lie between 0.5 and 1.0 for 3-MCPD and between 0.6 and 1.0 for 2-MCPD. The recovery rates of BfR Method 22 vary between 85 and 135 % for 3-MCPD FAE in a spiked sample. Accordingly, this method satisfies the required performance criteria and can be used for the determination of 3- and/or 2-MCPD FAE.

Although the relative standard deviation of reproducibility increases for method group 2 (combined evaluation of the results using BfR method 9 and Soxhlet or ASE extraction), the concentrations determined for Method Groups 1 and 2 are comparable and the method achieves the generally standard performance characteristics. It must be assumed that a standardisation of the extraction protocol of the Soxhlet method would further improve results.

Method Group 3 comprises all of the laboratories which used BfR Method 9 in combination with extraction methods which can be summarised under the term “cold extraction processes”. The results achieved using this method allow the conclusion that a) a standardisation of the extraction step would improve the reproducibility and b) the extraction of the MCPD FAE would not be quantitative for several of the cold extraction methods used here.

One of the samples analysed in the collaborative trial contained glycidyl FAE in addition to 3-MCPD FAE (analysed according to Kuhlmann 2011). The evaluation of the method validation study shows that the determination of 3-MCPD and glycidyl FAE as a sum parameter can be excluded for the majority of the laboratories. It has to be assumed that a specific determination of MCPD FAE can be guaranteed through chloride-free analysis.

A comparison of the statistical characteristics achieved for each method group with the data of the previous BfR collaborative trial or comparably conducted collaborative trial proves that the extraction step does not significantly influence the reproducibility of the methods. There is

no indication that the extraction steps used have an influence on the specificity of the methods, i.e. there is nothing to suggest that 3-MCPD is formed or destroyed by the extraction step.

The evaluation of the 36 data sets as a proficiency test shows that on average 76 % of all submitted results were regarded as satisfactory for the determination of 3-MCPD ($|z| < 2$), 6 % of the results as questionable ($2 \leq |z| \leq 3$) and 18 % as unsatisfactory ($|z| > 3$). For the determination of 2-MCPD, 78 % of the results were evaluated as satisfactory, 7 % as questionable and 18 % as unsatisfactory. The results of the proficiency test match up with the results of similarly conducted PTs.

2 Introduction

2.1 Background

Also known as chloropropanols, 3-Monochloro-1,2-propanediol (3-MCPD) and its isomer 2-Monochloro-1,3-propanediol (2-MCPD) belong to the group of process contaminants. In animal experiments, 3-MCPD produced renal tumors in rats via a non-genotoxic mechanism and was classified as a possible human carcinogen (IARC Group 2B). A tolerable daily intake (TDI) of 2 µg per kg body weight was determined for free 3-MCPD (SCF, 2001; JECFA, 2001) by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the EU Scientific Committee on Food (SCF). While the problem of free 3-MCPDs in foods has been known for quite some time, 3-Monochloro-1,2-propanediol fatty acid esters (3-MCPD FAE) were also detected in higher concentrations a few years ago in certain refined edible oils and fat containing foods (Hamlet et al. 2004; Divinova et al. 2004; Svejkovska et al. 2004; Zelinkova et al. 2006). One of the decisive aspects of toxicological evaluation is whether and to what extent ester-bound 3-MCPD is released by the human digestive system. The latest research results confirm the assumption that the esterified form becomes bioavailable in the body to a similar extent as after the consumption of foods containing free 3-MCPD [Abraham et al. 2012]. Based on the 3 MCPD FAE concentrations determined in infant formula by the CVUA Stuttgart, complete ester cleavage would result in the exceedance of the TDI value of non-breastfed babies (Weisshaar 2011. BfR 2007a, BfR 2009, BfR 2012).

2-MCPD is a constitutional isomer of 3-MCPD which is formed concurrently during the food manufacturing process. Due to a lack of data on occurrence, no risk assessment is currently available for 2-MCPD or 2-MCPD fatty acid ester (2-MCPD FAE). Reference substances for 2-MCPD have been commercially available since 2010, which is why 2-MCPD FAE were also determined in addition to 3-MCPD FAE in the current collaborative trial (1st Collaborative trial Part II; fat containing foods).

The BfR was commissioned by the Federal Ministry of Food, Agriculture and Consumer Protection (BMELV) in December 2007 to establish the “Analysis work group for the determination of 3-MCPD fatty acid esters in refined fats and fat containing foods” and validate an analysis method for the determination of 3-MCPD fatty acid esters (1st Collaborative trial Part I; edible fats and oils). The basis for the validation study was to be the method published by R. Weißhaar (Weisshaar 2008), whereby the participants were also allowed to use in-house laboratory methods.

During this collaborative trial it was ascertained that 3-MCPD is formed in several samples during analysis with the result that increased 3-MCPD concentrations are determined when working with analysis methods that use NaCl. It was concluded from this that a sum parameter of 3-MCPD and 3-MCPD-forming substances was determined with these methods.

According to the latest level of knowledge, the 3-MCPD-forming substances are glycidyl fatty acid esters, which are converted into 3-MCPD during alkaline-catalysed fatty acid alcoholysis in the presence of chloride ions. Due to the different toxicological potential of glycidol and 3-MCPD, the determination of the sum parameter is not sufficient from the point of view of risk assessment. For this reason, the BfR was commissioned by the “Analysis work group for the determination of 3-MCPD fatty acid esters in refined fats and fat containing foods” to develop an analysis method for the exclusive determination of 3-MCPD FAE and validate it in a second collaborative trial (2nd Collaborative trial Part I; edible fats and oils). In this study, participants were to analyse the samples with the three methods developed by the BfR or with their in-house laboratory methods. In this collaborative trial, “BfR Method 9” produced reproducible results, which means that a validated method for the detection of 3-MCPD FAE in edible fats and oils is available.

To determine 3-MCPD FAE (or 2-MCPD FAE) in fat containing foods, a further extraction step is required prior to analysis in which the fat is extracted from the food in question. To this end, an extraction step suitable for various matrices – so-called ASE (accelerated solvent extraction) – was integrated at the BfR into the BfR methods tested in the previous collaborative trial. The validity of these expanded methods had then to be tested by means of the collaborative trial conducted for fat containing foods (1st Collaborative trial Part II).

2.2 Goal of the Study

The goal of this study is the validation of an analysis method for the specific determination of 3-MCPD and 2-MCPD fatty acid esters in fat containing food. In order to check the methods used for possible systematic errors, the collaborative trial was set up in such a way that the participants were allowed to use their own laboratory methods in addition to those validated in-house at the BfR. This study design is of particular advantage because all of the methods used are based on indirect detection and no certified reference material with defined analyte concentrations is available. One sample was spiked with 3-MCPD FAE to estimate recovery. In addition to this, all submitted results were evaluated as a proficiency test.

3 Conducting of the Collaborative trial

3.1 Selection of Participants

The members of the "Work Group for the Analysis of 3-MCPD Fatty Acid Esters" and the participants in the 1st and 2nd collaborative trials to determine 3-MCPD fatty acid esters in edible fats and oils were invited to take part in the collaborative trial. A total of 29 laboratories registered for the study. There was no preselection of participants and all interested parties were admitted. Participation in the collaborative trial was free of charge for all laboratories. The participating laboratories are listed in Appendix 3.

3.2 Selection of Test Methods

3-MCPD and 2-MCPD fatty acid esters are detected indirectly by hydrolysis of the MCPD fatty acid esters and analysing the free 3- and/or 2-MCPDs, which are derivatised and quantified via internal standardisation by means of gas chromatography-mass spectrometry (GC/MS). One advantage of this indirect detection is that all of the MCPD fatty acid esters contained in the sample are detected, irrespective of which fatty acids are bonded to 3-MCPD or 2-MCPD. A disadvantage of this indirect detection method is the potential formation or destruction of 3- and/or 2-MCPD during analysis.

Two analysis methods for determining 3-MCPD fatty acid esters in fats and oils were successfully tested in the previous collaborative trial before being further developed at the BfR in regard to a suitable extraction step and validated in-house. Both methods were expanded to include fat extraction per ASE. They differ from each other in the hydrolysis of the fatty acid esters in an acidic or alkaline milieu.

3.2.1 BfR Method 22

This method is the same as "BfR Method 9" of 2 RT Part I, expanded to include an ASE stage:

Method description:

Accelerated solvent extraction is achieved with a solvent mixture of petrol ether/isohexane/acetone (2/2/1. v/v) in two extraction cycles at a temperature of 125 °C. The solvent of the extract is evaporated under nitrogen until weight constancy is reached. The fat concentration is then determined by weighing back. An aliquot of the fat extract (100-200 mg) is dissolved in tert-Butyl methyl ether and mixed with internal standard (d_5-labelled 3-MCPD and/or d_5-labelled 1,2-Bis-palmitoyl 3-MCPD and d_5-labelled 2-MCPD). Fatty acid methyl esters and free MCPD are produced from the alkaline-catalysed separation of the ester bond with sodium methylate. The hydrolysis reaction is stopped by a solution of ammonium sulphate and sulphuric acid. Thereafter, the sample is degreased with isohexane and the released MCPD extracted with ethyl acetate. After extraction, the samples are derivatised with phenylboronic acid (PBA). The PBA derivatives are completely dried under nitrogen and dissolved in acetone before an aliquot is analysed with GC/MS.

3.2.2 BfR Method 23

This method is the same as “BfR Method 8” of 2 RT Part I, expanded to include an ASE stage:

Principle of the method:

Accelerated solvent extraction is achieved with a solvent mixture of petrol ether/isohexane/acetone (2/2/1. v/v) in two extraction cycles at a temperature of 125 °C. The solvent of the extract is evaporated under nitrogen until weight constancy is reached. The fat concentration is then determined by weighing back. An aliquot of the fat extract (100-200 mg) is dissolved in tert-Butyl methyl ether and mixed with internal standard (d_5-labelled 3-MCPD and/or d_5-labelled 1,2-Bis-palmitoyl 3-MCPD and d_5-labelled 2-MCPD). Fatty acid methyl esters and free MCPD are produced from the acidic hydrolysis of the ester bond with methanol and sulphuric acid. The hydrolysis is stopped by saturated sodium hydrogen carbonate solution. Thereafter, the sample is degreased with isohexane and the released MCPD derivatised with phenylboronic acid. After extraction of the PBA derivatives with cyclohexane, the sample is completely dried and dissolved in iso-octane before an aliquot is analysed with GC/MS.

To familiarise themselves with the methods and in-house laboratory controls, two test samples (**A**: chocolate spread; **B**: onion lard) with a known 3-MCPD concentration were sent out in advance.

3.3 Study Design

The participants had six weeks to get acquainted with the analysis methods. Prior to the final consignment of the samples, an inquiry was made as to the experiences made with the methods and the results were announced in the letter that accompanied the samples. No subsequent changes to the method description were required. In addition to this, the laboratories had the option of participating in the collaborative trial with their own methods.

Each laboratory was given six samples, each of which had to be analysed on one day. A fourfold determination had to be conducted with each sample. If a laboratory wanted to determine the samples with several methods, additional sample material was sent. The standard substances and internal standards were provided in order to avoid calibration errors (d_5-3-MCPD, 3-MCPD, d_5-2-MCPD, 2-MCPD, d_5-1,2-Bis-palmitoyl 3-MCPD ester, 1,2-Bis-palmitoyl-3-MCPD ester).

4 Sample Material

4.1 Production and Characterisation of Sample Material

To produce the sample material, 31 different fat containing foods were purchased in the market during the period from April-October 2010. To determine concentrations, these foods were analysed several times by various operators using different test methods. Four samples were selected directly. To cover the relevant concentration range, an additional milk powder sample was mixed from various infant formulas. In this way, a concentration range for 3-MCPD of 0.65-4.58 mg/kg fat and a range between <LOD-1.76 mg/kg fat for 2-MCPD were covered. The fat concentrations of the samples varied between approx. 25-85 %. To check the specificity of the methods, an onion lard was selected which, in addition to 3-MCPD FAE, also contained glycidyl FAE in a concentration range of approx. 4 mg/kg fat (Sample 4). An additional sample was spiked with 3-MCPD ester (Sample 6) to estimate recovery.

Table 1 Sample Designations and Parameters

Sample	Matrix	Trade Name	Ingredients as stated by manufacturer	Concentration 3-MCPD [mg/kg fat]*	Concentration 2-MCPD [mg/kg fat]*	Fat content [%]*
P1	Milk powder A	Infant formula	veg. oil veg. fats	1.30	0.64	24.5
P2	Strawberry cream	Sweet spread, strawberry duo creme	veg. fats	0.65	0.29	32.9
P3	Choc. cream	Choc. filling	veg. oil veg. fats	1.94	1.06	34.6
P4	Onion lard	Plant-based onion lard	palm oil, palm kernel fat, palm fat, soya oil	2.54	1.04	83.1
P5	Milk powder B (mixed sample)	Infant formula	veg. oil veg. fats	4.58	1.76	24.6
P6	Mayonnaise (spiked)	Salad mayonnaise	rapeseed oil	1.02	< LOD	52.5

* Mean values from BfR homogeneity determination

Sample P1 was a conventional milk powder that was stored at room temperature and submitted in portions of approx. 15 g.

1.1-1.5 kg respectively from the same batch of **samples P2, P3 and P4** were each combined in a mixing bowl and homogenised with a mixer at room temperature for 30 minutes. The homogenised sample material was transferred to a piping bag, filled into brown glass sampling vials with a snap-on lid and stored at 6 (± 4) °C.

To produce **Sample P5**, various milk powders were strained, mixed together in a small vat and homogenised for two hours in a drum hoop mixer. Thereafter, portions of the sample material each weighing approx. 15 g were filled into aluminium compound bags, sealed with a vacuum heat sealer and stored at room temperature.

Sample P6 was analysed several times prior to spiking. 3-MCPD and/or 2-MCPD concentrations above the detection limit could not be detected in any of the analyses (analysis in accordance with BfR Method 22). A fat content of 50 % was declared on the packaging. 1.2 kg

of mayonnaise from the same batch was put into a mixing bowl and spiked with 1,2-Bis-palmitoyl-3-chloropropane-1,2-diol (3-MCPD ester). To do so, 10 g of a 3-MCPD-free linseed oil was spiked with 610 µl of a 3-MCPD ester standard solution with a concentration of 5.4 mg/ml and added to the mayonnaise. This mayonnaise-linseed oil mixture was then mixed in a mixing bowl for one hour at room temperature. The total concentration of 3-MCPD ester in Sample M6 equates to 3.29 mg in 610 g of fat. With complete ester cleavage, this equates to a concentration of 1 mg 3-MCPD per kg of fat.

4.2 Homogeneity Testing

To check homogeneity, twelve filled samples were drawn at random (from 100 aliquots). The concentrations of 3-MCPD and 2-MCPD in the fat served as the homogeneity parameter (analysis in accordance with BfR Method 22). Each sample was analysed in duplicate. The test results were evaluated with a single factor **an**alysis **o**f **va**riance (ANOVA). On a significance level of 95%, all samples were homogenous and therefore suitable as test material for the study. The analysis results of homogeneity determination are listed in Appendix 4.

4.3 Stability Testing

Aliquots of each sample were kept and analysed during the study period. In order to test stability, analyses were conducted at the time of homogeneity testing, prior to the consignment of samples, and at the beginning and end of the collaborative trial. The results confirm the stability of the samples for this period.

4.4 Consignment of Samples and Transfer of Results

Each lab was given a confidential laboratory code “LC00XX”. Laboratories which participated in the study with various analysis methods were given an independent laboratory code for each method.

The samples were sent to the laboratories with accompanying documentation in February 2011. To transfer the results, the analysis results and each sample weight were entered by the participants into files in Software ProLab, Version 2.12. In addition to this, each participant was given an Excel file in which inquiries were made regarding the details of the extraction and detection methods used.

5 Results

5.1 Evaluation Procedure

The participants gave the analysis results either in relation to fresh substance or in relation to fat. To enable the common statistical evaluation of all results, fat was chosen as the uniform reference value. If a laboratory gave the fat content of a sample, the results that related to fresh substance were converted to fat content. If a laboratory did not give any fat content, the fat content determined by the BfR during homogeneity testing was used for conversion (Table 1). This fat content matched up for all samples with the mean values of the fat contents determined by the other participants (±10%). With labs of this kind, the laboratory code was changed from LC00xx to LC60xx.

Data sets were evaluated in accordance with DIN ISO 5725-2. Because the sample material used was purchased on the open market, no certified 3-MCPD and/or 2-MCPD concentrations were available. After being cleared of outliers, the mean value of the results of the laboratories was calculated and established as a reference value for statistical evaluation.

In compliance with the provisions of the validation protocol, four parallel analyses were required for the calculation of repeatability. As the calculation of repeatability requires a minimum of two parallel tests, only results data sets involving at least two parallel tests could be used to calculate the statistical parameters. Results which showed “< LOQ” or “< LOD” were not taken into account.

The laboratory results were checked with the help of numerical outlier tests and the calculation of the compatibility parameters h and k in accordance with Mandel on a significance level of 1%. The standard deviations of the laboratories were checked for outliers in every material in accordance with Cochran. When outliers were identified, the individual results of the laboratory in question were checked for individual outliers within the laboratory using the Grubbs test. Values identified as outliers were eliminated. Thereafter, the standard deviations and laboratory mean values were checked once again. The standard laboratory deviation which proved to be significantly deviant from the standard deviations of the other laboratories in the Cochran Test and in Mandel’s k-statistic was marked and the laboratory eliminated. All laboratory mean values were checked for deviations from the overall mean value by means of the Grubbs Test and Mandel’s h-statistic. Laboratory mean values which showed significant deviations from the overall mean value of the laboratories in both tests were marked and eliminated. By making inquiries at the laboratories, it was clarified that these values were not arithmetical or transfer errors and the remaining outliers were eliminated.

The HorRat value (Horwitz Ratio) was used to evaluate the performance of the analysis methods. The HorRat is the quotient of the standard deviation of reproducibility and the standard deviation according to Horwitz. HorRat values between 0.5 and 1.5 prove that the performance characteristics of the method comply with the generally encountered performance characteristics, and values < 2 are considered acceptable (Horwitz and Albert 2006).

5.2 Submitted Results

A total of 29 laboratories submitted results, whereby one laboratory withdrew its results due to subsequently established analysis problems. Five laboratories analysed the samples using two methods. One laboratory submitted four data sets because the samples were analysed with two methods additionally using different internal standards. In this way, a total of 36 data sets from 28 laboratories were available for the evaluation of the proficiency test. When cal-

culating the statistical parameters for method validation, several data sets per laboratory were only used when the methods used by the labs differed. Accordingly, a total of 29 data sets were available for the evaluation of the method validation study.

5.3 Validation of BfR Method 9 + ASE (BfR Method 22)

5.3.1 Classification of results

To facilitate understanding of the classification, you are reminded in advance that BfR Method 22 is the same as BfR Method 9 of the previous collaborative trial *expanded to include an extraction step (ASE).*

Most participants used BfR Method 9 of 1 RV Part II in combination with a preceding extraction process (19 of the 29 data sets). BfR Method 9 contained a defined reaction for ester cleavage by means of sodium methylate (NaMeO) in alkaline solution followed by derivatisation by means of PBA. All work stages were carried out NaCl-free.

Fat was extracted at the laboratories either by means of ASE (BfR Method 22. [3.2.1]), Soxhlet or another method. Only when using ASE were the extraction agent and extraction duration prescribed, i.e. laboratories that used Soxhlet or another extraction method were able to use different solvents and vary the duration of extraction.

Nine laboratories (10 data sets) used a method which differed from BfR Method 22 in several ways.

The submitted data sets were classified into five groups for statistical evaluation. Groups 1-3 contained all of the laboratories which used BfR Method 9 with a preceding extraction step, while all other laboratories were allocated to Groups 4 and 5.

Because a detailed protocol was only available for BfR Method 22, only this method can be tested for validity.

- **Method Group 1** consisted of all data sets for BfR Method 9 + ASE [BfR Method 22]
  Criteria: ASE extraction; alkaline ester cleavage, derivatisation by means of PBA
  6 data sets for 3-MCPD, 5 data sets for 2-MCPD

The allocation of all other methods into method groups serves the testing of accuracy.

- **Method Group 2** comprised all data sets for the use of BfR Method 9 + ASE. In addition to this, it included all of the laboratories which used the Soxhlet extraction method instead of ASE.
  Criteria: hot extraction; alkaline ester cleavage, derivatisation by means of PBA
  6 + 3 data sets, 5 + 2 data sets for 2-MCPD

- **Method Group 3** comprised the data sets of all laboratories which used BfR Method 9 plus a “cold extraction method” to extract fat, e.g. solvent plus sample in combination with vortex or ultrasonic extraction or stirring.
  Criteria: “cold extraction”; alkaline ester cleavage, derivatisation by means of PBA
  10 data sets for 3-MCPD, 10 data sets for 2-MCPD

- **Method Group 4** comprised the results of laboratories that used in-house methods and whose common criterion was alkaline ester cleavage (but no standardised procedure). Where fat extraction is concerned, the methods vary between no preceding extraction and

hot or cold extraction. Derivatisation was either done with PBA or HFBA.
6 data sets for 3-MCPD, 5 data sets for 2-MCPD

- **Method Group 5** comprised the results of laboratories that used in-house methods and whose common criteria was acidic ester cleavage (standardised procedure in accordance with Divinova et al. 2004). Where fat extraction is concerned, methods involving ASE or cold extraction were used. Derivatisation was either done with PBA, a ketone reagent or HFBI.
4 data sets for 3-MCPD, 4 data sets for 2-MCPD

This means that only in Method Group 1 it was possible to classify laboratories which analysed the samples in accordance with a precisely defined protocol.

A summary of the statistical parameters of the above-mentioned method groups is given in Table 2 for the determination of 3-MCPD and in Table 3 for 2-MCPD. A detailed presentation of the statistical parameters and the individual results per method group are listed in Tables 6 – 15 in Appendix 2 for both analytes.

The results of all laboratories were also evaluated in the Proficiency Test (5.4).

Table 2: Statistical Parameters for 3-MCPD Compiled by Method Group

	Lab. after outlier elim. [Outliers]	Mean value [mg/kg fat]	Repeatability CV [%]	Reproducibility CV [%]	HorRat
Method Group1:					
M1: Milk powder	6 [0]	1.22	5.87	15.65	1.0
M2: Strawb. cream	5 [1]	0.71	7.79	17.18	1.0
M3: Choc. cream	5 [1]	2.20	4.47	11.74	0.8
M4: Onion lard	5 [1]	2.60	5.17	10.07	0.7
M5: Milk powder	6 [0]	4.22	3.58	6.61	0.5
M6: Mayonnaise	6 [0]	1.04	4.90	16.34	1.0
Method Group 2:					
M1: Milk powder	6 [1]	1.22	5.87	15.65	1.0
M2: Strawb. cream	8 [1]	0.77	10.63	23.72	1.4
M3: Choc. cream	9 [0]	2.17	12.37	20.19	1.4
M4: Onion lard	8 [1]	2.57	10.34	14.69	1.1
M5: Milk powder	9 [0]	4.14	3.76	7.12	0.6
M6: Mayonnaise	8 [1]	1.02	5.01	16.00	1.0
Method Group 3:					
M1: Milk powder	6 [1]	1.01	6.03	44.09	2.8
M2: Strawb. cream	10 [0]	0.72	6.34	54.21	3.2
M3: Choc. cream	10 [0]	1.72	8.02	32.16	2.2
M4: Onion lard	9 [1]	2.24	4.68	14.23	1.0
M5: Milk powder	10 [0]	3.16	3.55	48.16	3.6
M6: Mayonnaise	9 [1]	0.81	3.80	36.87	2.2
Method Group 4:					
M1: Milk powder	4 [1]	0.87	3.16	67.88	4.2
M2: Strawb. cream	6 [0]	0.81	7.36	28.38	1.7
M3: Choc. cream	5 [1]	1.90	3.75	6.46	0.4
M4: Onion lard	6 [0]	2.83	4.47	29.91	2.2
M5: Milk powder	6 [0]	3.56	8.33	36.49	2.8
M6: Mayonnaise	6 [0]	1.00	9.98	12.99	0.8
Method Group 5:					
M1: Milk powder	4 [0]	1.30	5.08	11.46	0.8
M2: Strawb. cream	4 [0]	0.74	4.83	19.72	1.2
M3: Choc. cream	4 [0]	2.04	4.16	22.18	1.5
M4: Onion lard	4 [0]	2.57	2.97	10.19	0.7
M5: Milk powder	4 [0]	4.51	5.14	9.09	0.7
M6: Mayonnaise	4 [0]	1.01	7.49	8.32	0.5

Table 3: Statistical Parameters for 2-MCPD Compiled by Method Group (Sample 6 did not contain any 2-MCPD).

	Lab. after outlier elim. [Outliers]	Mean value [mg/kg fat]	Repeatability CV [%]	Reproducibility CV [%]	HorRat
Method Group 1:					
M1: Milk powder	5 [0]	0.58	7.15	13.66	0.8
M2: Strawb. cream	5 [0]	0.35	8.71	17.92	1.0
M3: Choc. cream	5 [0]	1.12	4.29	8.84	0.6
M4: Onion lard	5 [0]	0.96	5.32	10.38	0.7
M5: Milk powder	5 [0]	1.71	4.03	14.36	1.0
Method Group 2:					
M1: Milk powder	6 [0]	0.59	6.71	12.82	0.7
M2: Strawb. cream	6 [1]	0.34	9.99	18.19	1.0
M3: Choc. cream	6 [1]	1.09	4.71	9.94	0.6
M4: Onion lard	6 [1]	0.93	5.59	12.44	0.8
M5: Milk powder	7 [0]	1.78	3.97	17.08	1.2
Method Group 3:					
M1: Milk powder	6 [1]	0.45	3.03	77.66	4.3
M2: Strawb. cream	8 [1]	0.25	3.70	44.04	2.2
M3: Choc. cream	9 [1]	0.82	2.74	44.50	2.7
M4: Onion lard	10 [0]	0.74	9.73	37.53	2.2
M5: Milk powder	10 [0]	1.25	3.80	48.34	3.1
Method Group 4:					
M1: Milk powder	4 [0]	0.45	5.18	56.27	3.1
M2: Strawb. cream	3 [1]	0.30	4.98	5.78	0.3
M3: Choc. cream	5 [0]	0.98	8.35	15.87	1.0
M4: Onion lard	4 [1]	0.90	4.36	20.34	1.3
M5: Milk powder	4 [0]	1.08	6.69	38.43	2.4
Method Group 5:					
M1: Milk powder	4 [0]	0.62	6.15	10.16	0.6
M2: Strawb. cream	4 [0]	0.32	8.11	10.33	0.5
M3: Choc. cream	4 [0]	1.01	6.76	18.38	1.2
M4: Onion lard	4 [0]	0.92	5.88	7.36	0.5
M5: Milk powder	4 [0]	1.67	7.64	7.77	0.5

Very good values in regard to rel. standard deviation of reproducibility and the HorRat values were determined for **Method Group 1** (BfR Method 22) when determining 3 and/or 2-MCPD. The rel. standard deviation of reproducibility varies between 7-17 % for the analysis of 3-MCPD (equates to HorRat values between 0.5 and 1.0) and between 9-18 % for the analysis of 2-MCPD (equates to HorRat values between 0.6 and 1.0).

Statistical significance is restricted by the limited number of participants (6 results for 3-MCPD and 5 for 2-MCPD). According to the harmonised ISO/IUPAC/AOAC protocol, 7 remaining laboratories are required after the elimination of outliers, whereby a maximum of 2 out of 9 laboratories may be eliminated. Only in exceptional circumstances can participant numbers from 5 laboratories be accepted.

In the 1st collaborative trial Part II, in which BfR Method 9 was validated in fats and oils without a preceding extraction step a reproducibility between 13-23 % were determined, which equates to HorRat values of between 0.9-1.4. The rel. standard deviation was 55 % for one material that contained 3-MCPD concentrations in the range of the quantification limit (equates to a HorRat value of 2.9). The results of materials 2 to 6 permit the conclusion that the additional extraction stage does not significantly increase the rel. standard deviation of the method.

In **Method Group 2,** the data of the laboratories that conducted a procedure in accordance with Soxhlet (no defined protocol) were included in addition to those of the laboratories that extracted fat per ASE in line with a defined protocol. Both of these methods are combined under the term “hot extraction methods”. The reproducibility decreases for the 3- as well as the 2-MCPD analysis for this method group (see Table 2 and 3). The HorRat values achieved for both analytes lie between 0.7 and 1.7, thus proving that the standard performance characteristics are reached. The use of both extraction types results in comparable sample concentrations.

Method Group 3 encompasses the results of all laboratories that also used BfR Method 9 (just like method groups 1 and 2), but did not conduct classical fat extraction, such as ASE or Soxhlet. The processes used here, e.g. stirring, shaking or ultrasound, were summarised under the term “cold extraction methods”. The reproducibility and HorRat values decrease for both analytes and all collaborative trial materials. The determined reproducibility lies between 14-44 % and 78 % for infant formula (Milk powder A), which equates to HorRat values of between 1.0-4.3. The immense difference between the repeatability and reproducibility is conspicuous. The repeatability is usually approx. ½ to ⅔ of the reproducibility (Horwitz and Albert 2006). This is only approx. ¼ or even less for Method Group 3, however (see Table 2 and 3). As the repeated standard deviation in this method group is comparable with the other method groups, it has to be assumed that in this case the extraction has a strong influence on the reproducible standard deviation and that a standardisation of the extraction step would have a positive effect on the analysis. The 3- and 2-MCPD mean values of the sample concentrations for Method Group 3 are approx. 20 % lower than those of the other groups (see Fig. 1 and 2).

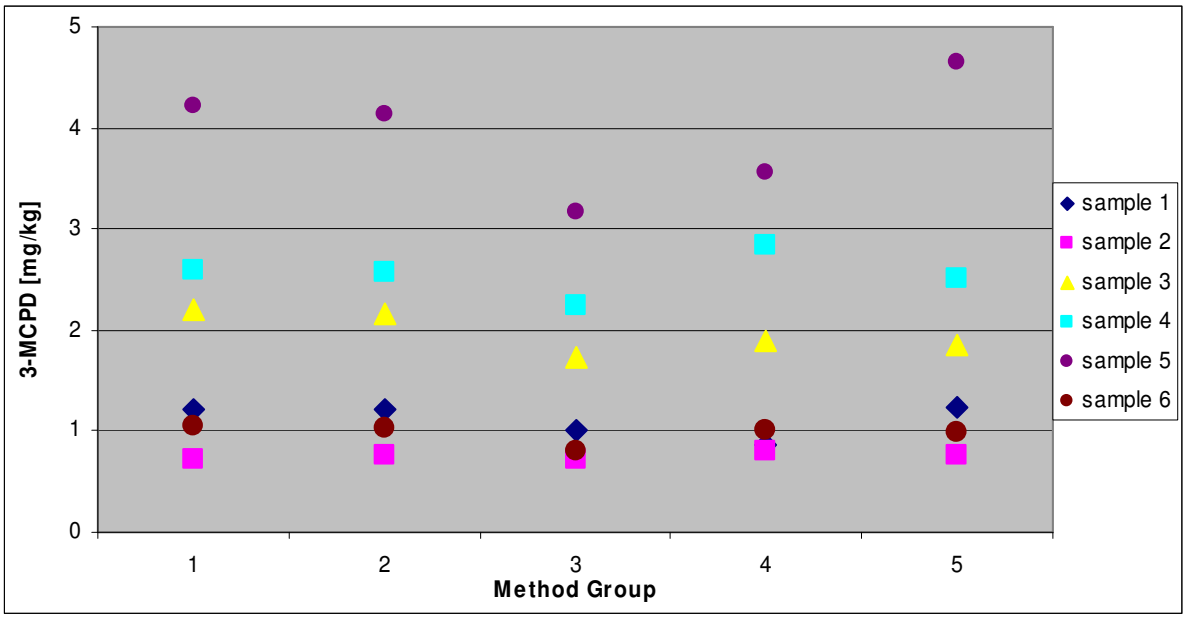

Fig. 1: 3-MCPD Mean Values per Method Group in Collaborative trial Samples 1-6

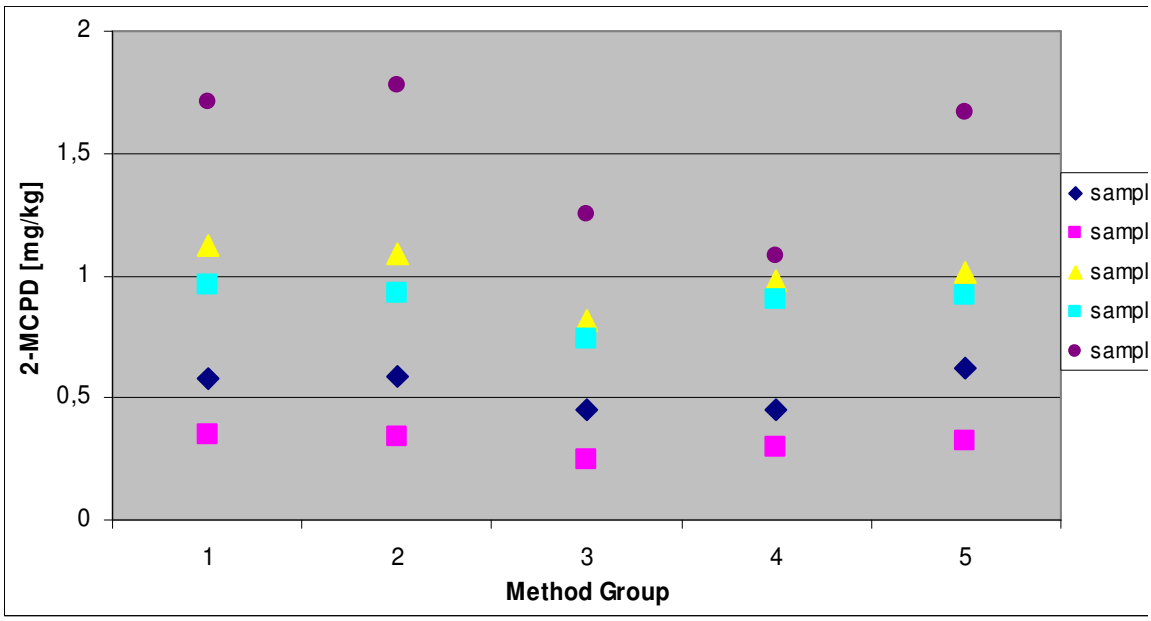

Fig. 2: 2-MCPD Mean Values per Method Group in Collaborative trial Samples 1-5

This indicates that the extraction yield tends to be lower in comparison with the other extraction methods used. This aspect is dealt with again in the determination of the recovery rate of 3-MCPD (5.3.3).

The results of the laboratories from Method Group 3 regarding the extraction method used are presented in Fig. 3. Fig. 3 also makes it clear that it cannot be generally concluded for the “cold extraction methods” employed that a stirring step by means of ultrasound, for example, leads in general to lower sample concentrations, or a longer extraction duration to generally higher sample concentrations.

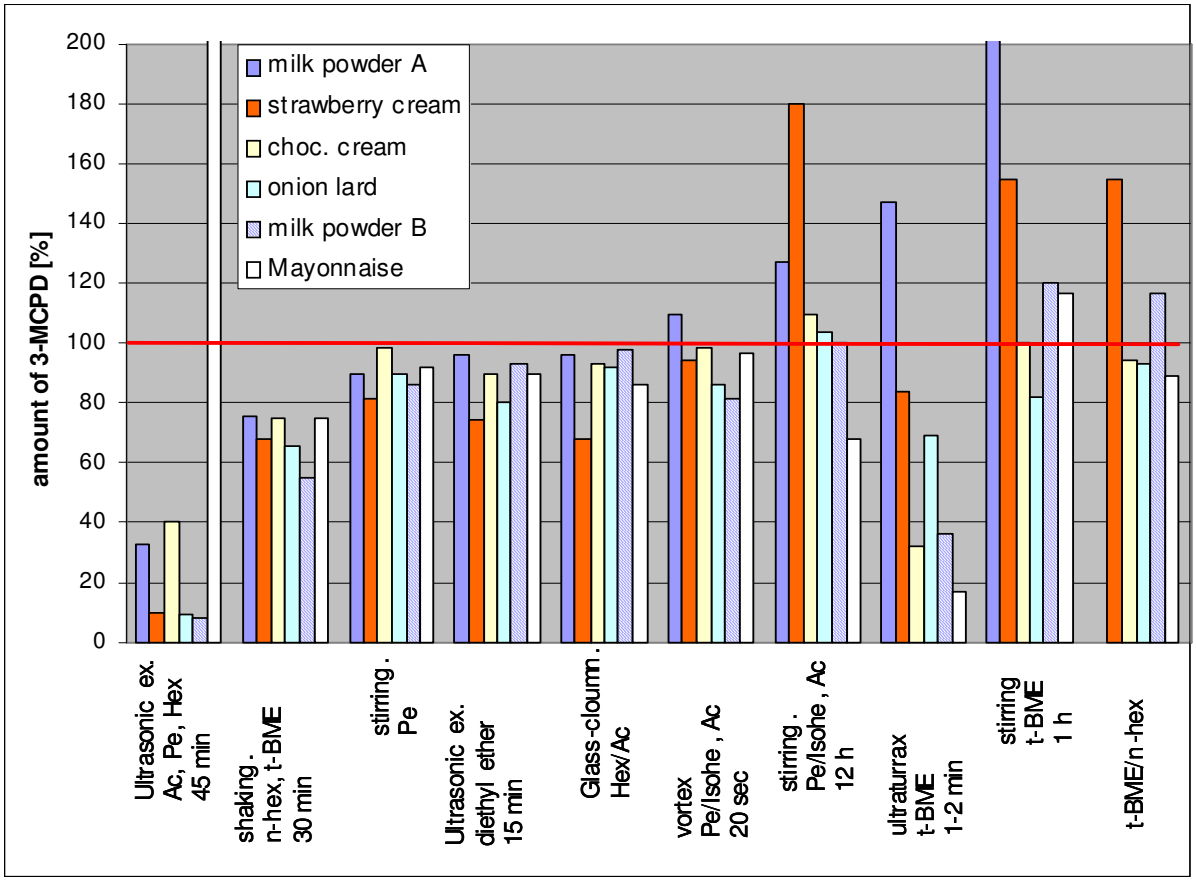

Fig. 3: Presentation of the percentages of 3-MCPD concentrations in relation to the mean sample value of all laboratories (100%), depending on the "cold extraction" method used [Pe=petroleum ether, Ac=acetone, Hex=hexane].

As classification into method groups in the manner shown here ultimately has the result that not only different extraction methods but also different laboratories are compared with one another, it cannot be clarified whether the increase in the rel. standard deviation of reproducibility and the tendency towards a lower sample yield can be attributed solely to the extraction step.

In **Method Group 4**, the results of the laboratories that used an in-house method which differed from BfR Method 22 in regard to the analytical procedure but which had in common the cleavage of the esters in an alkaline solution were evaluated. Comparable mean values were calculated although the data sets of only six laboratories were available.

Method Group 5 comprises the results of all laboratories that used in-house methods , but which had in common the cleavage of the esters in an acidic solution (Divinova et al. 2004). Comparable mean values were calculated although the data sets of only four laboratories were available and it has to be assumed that methods that involve acidic ester cleavage produce results that are comparable with methods that involve alkaline ester cleavage (see Table 2 and 3).

In synopsis, it is ascertained for all method groups and all materials that lower HorRat values are achieved in the analysis of 2-MCPD than in the simultaneously conducted 3-MCPD analysis. As both analytes are subjected to the same processing steps and, due to their constitutional isomerism, are also detected on the same m/z value, it has to be assumed that

2-MCPD has a sharper elution profile or that in general, less matrix impairs quantification at this retention time.

5.3.2 Specificity

During pretesting, collaborative trial sample 6 was identified as MCPD-free prior to spiking. Accordingly, the mayonnaise sample only contained 3-MCPD FAE after spiking and was able to be used as blank material for 2-MCPD. In 9 out of 33 data sets, it was stated that 2-MCPD concentrations were above the detection limit, whereby in 9 of the 10 data sets only a low 2-MCPD concentration in the range of 10-350 µg/kg fat was declared. As it cannot be excluded that the carried out clean up procedures caused a percentage of the 3-MCPD to convert into 2-MCPD so that the sample solutions actually did contain 2-MCPD, it cannot be concluded in general that the method is not specific in the lower ppb range. No common characteristics in the methodical procedure to which a conversion could be traced were recognisable.

In previous studies, the question was raised as to whether the methods for determining 3-MCPD fatty acid esters are specific or whether they cover a sum parameter made up of 3-MCPD fatty acid esters and 3-MCPD-forming substances, such as glycidyl fatty acid esters.

In this collaborative trial, all of the participants examined an onion lard sample (Sample 4) which contained 3-MCPD FAE in a concentration range of approx. 2.5 mg/kg fat (measured values for homogeneity determination) and glycidyl FAE in a concentration range of approx. 4 mg/kg fat (Kuhlmann 2011). 3-MCPD concentrations of between 0.3 and 4.4 mg/kg fat were determined for this sample (see Table 6–14 in Appendix 2). A graphic display of the determined 3-MCPD concentrations depending on the analysis method used is shown in Fig. 4.

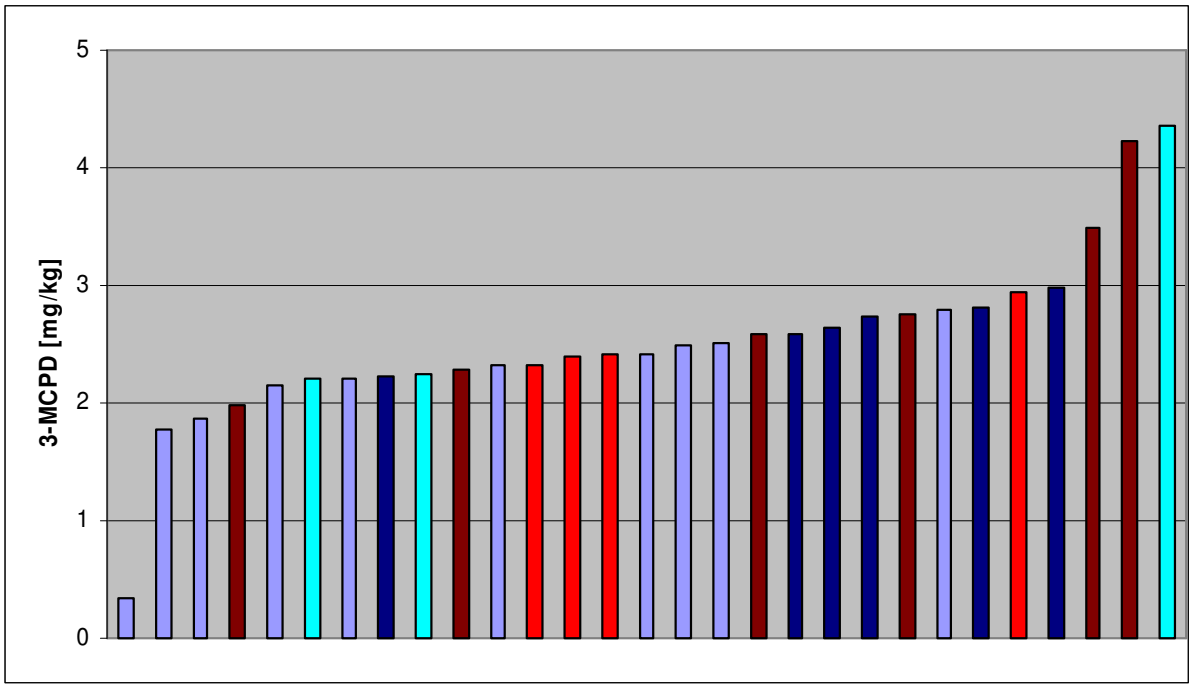

Fig. 4: 3-MCPD concentrations in onion lard (sample 4). The classification of participants into method groups outlined in Item 5.2 is depicted by the different colours (see figure 5)

The laboratory with the lowest 3-MCPD concentrations declared values which were often identified as outliers. It is therefore assumed that this laboratory made a methodical or calculation error. If this laboratory were omitted, the detected 3-MCPD concentrations would lie between 1.7 and 4.4 mg/kg fat. The mean value of all laboratories for 3-MCPD was 2.7 mg/kg fat.
It can be concluded from this that the recording of 3-MCPD and glycidyl FAE as a sum parameter can be excluded for the majority of the laboratories.

5.3.3 Determination of Recovery

To estimate the recovery of the method, Sample 6, a mayonnaise, was spiked with 1,2-Bis-palmitoyl-3-chloropropane-1,2-diol (3-MCPD concentration with complete ester cleavage: 1.0 mg/kg fat). The recovery of the result data sets is shown in Fig. 5 by method group.

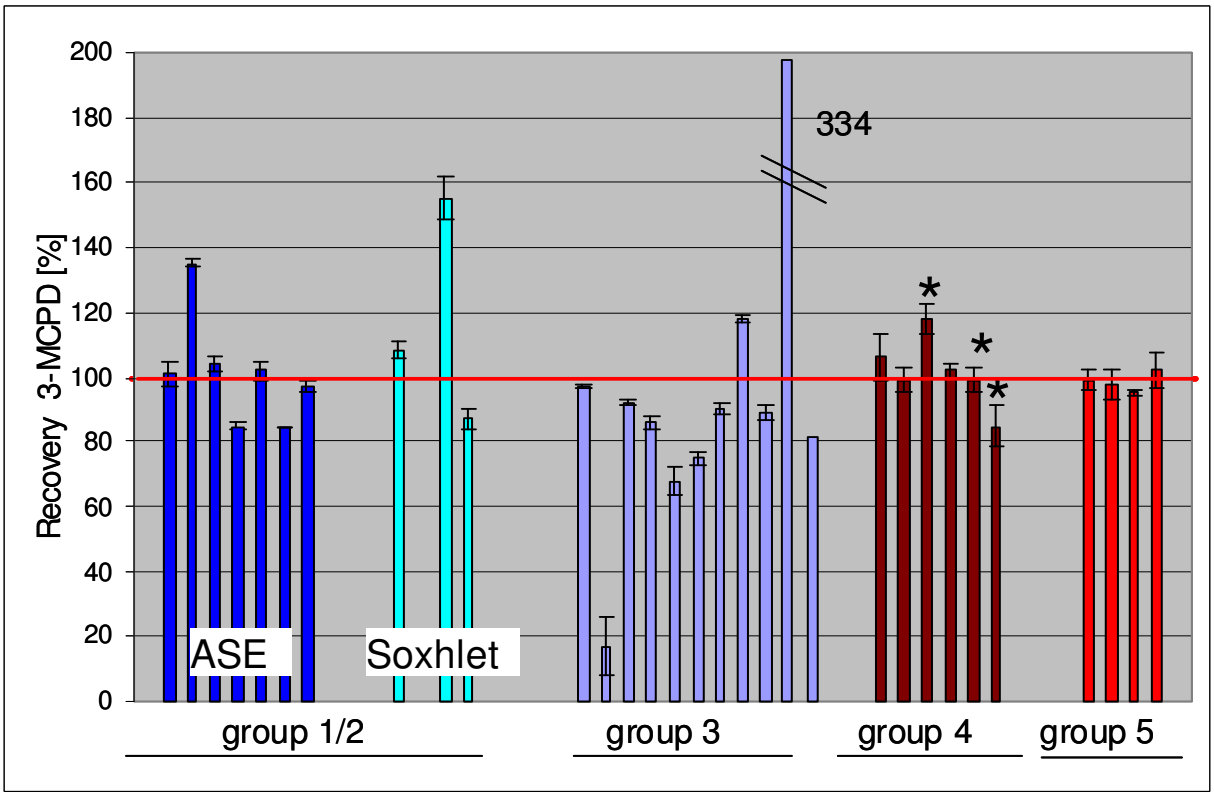

Fig. 5. Recovery of 3-MCPD in Sample 6 – spiked mayonnaise. The rel. standard deviation of the four sample processings is shown for each laboratory. The classification into method groups outlined in Item 5.2 is depicted by the different colours. The analysis methods marked with an asterisk do not involve any preceding fat extraction.

As can be seen from Fig. 5, satisfactory recovery is achieved in almost all method groups. The internal isotopically labeled standards were only added after extraction with the exception of the methods marked with an asterisk. In this way, the extraction yield of the methods was not corrected via the internal standard and it can be concluded that – at least for the mayonnaisee sample – the extraction step for 3-MCPD was done quantatively in almost all method groups. The methods of Group 4, in which 3-MCPD was determined directly from the fresh substance without any preceding fat extraction, achieved recovery rates of between 81 and 118%. As described previously in 5.3, the 3-MCPD concentrations determined within Method Group 3 tend to be lower than those of the other method groups. The average re-

covery rate was 81 % (RR = 338 % was not included), so it has to be assumed that the extraction step for the fat and/or 3-MCPD FAE was not quantitative.

5.3.4 Selection of the Internal Standard for the Determination of 3-MCPD FAE

All participating laboratories used an internal standard to determine the 3-MCPD FAE, whereby 30 data sets were determined using free deuterated 3-MCPD and 6 results through the use of deuterated 3-MCPD fatty acid esters (d_5-3-MCPD-1,2-Bis-palmitoyl ester).

Almost all of the laboratories added the internal standard after fat extraction, which meant that possible errors during extraction could not be corrected through the internal standard. If free d5-3-MCPD is used as the internal standard, losses during clean up and faults in detection can be compensated. If ester-bound d5-MCPD is used, faults in the saponification step through which the ester-bound MCPD is transferred to free MCPD can be compensated additionally.

The 3-MCPD concentrations determined by the participants with dependence on the internal standard are shown in Fig. 6. Whereas comparable 3-MCPD concentrations are achieved for samples 2, 3, 4 and 6 for both internal standards, the medians of the two groups differ in the two milk powder samples (samples 1 and 5). When free MCPD is used, concentrations of 1.18 and 4.05 mg/kg fat are determined in Sample 1 and Sample 5 respectively, whereas higher concentrations of 1.35 and 4.82 mg/kg fat are detected when bound MCPD is used. This difference could be an indication of potential difficulties with the hydrolysis step in milk powder which was corrected through the the ester-bound internal standard. In this way, the use of ester-bound MCPD as an internal standard serves as a control of ester cleavage and is to be recommended.

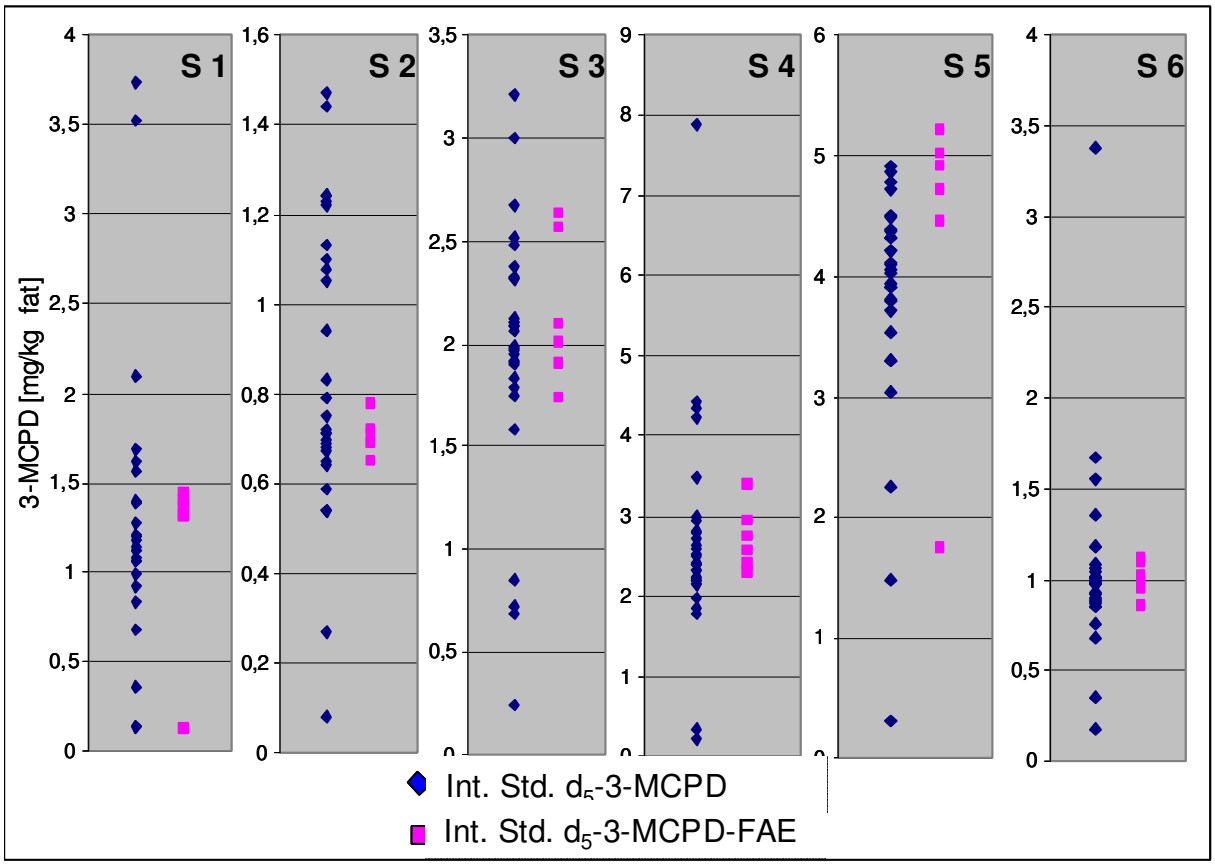

Fig. 6: 3-MCPD concentrations in collaborative trial samples P1-P6. Concentrations with which free d_5-3-MCPD was used as the internal standard are marked in blue. Values determined using ester-bound d_5-3-MCPD bispalmitate as the internal standard are shown in magenta.

5.4 Proficiency Test (PT)

5.4.1 General Procedure

The results of all laboratories, irrespective of the analysis method used, were evaluated jointly as a proficiency test. The statistical parameters were determined using the robust process in accordance with DIN 38402-45.

The robust mean value, which was formed in line with an estimation procedure from Hampel on the basis of the mean values of the laboratories, served as the target value (SW). The repeatability (s_r) and reproducibility (s_R) were determined using the Q Method with which extreme values which deviate from the mean value by more than 4.5 times the reproducibility are not taken into account. Because values with a deviation between $3*s_R$ and $4.5*s_R$ are capped, extremely deviant values do not have to be checked for outliers and eliminated prior to the calculation of the statistical parameters. The advantage of the robust method is that it has a breakdown point of 50 %, which means that reliable statistical results are achieved even if 49 % of the individual values would have to be classified as outliers. It can also be used when there is no normal distribution of the measured values (Hampel, 1980). Results "<BG" and/or "<NG" were not taken into account in the evaluation. The performance of the laboratory is estimated on the basis of the z-scores.

$$z = \frac{x - SW}{Horw}$$

with x *mean value of the individual analyses of the laboratory [mg/kg]*

SW target value calculated as a robust mean value from the mean values of the involved laboratories [mg/kg]
*Horw Horwitz standard deviation calculated in accordance with (x*sH)/100% [mg/kg]*

The performance of the laboratory is appraised in line with the provisions of ISO 13528. Accordingly, at

$|z| < 2$ the result is satisfactory
$2 \leq |z| \leq 3$ the result is questionable and at
$|z| > 3$ the result is unsatisfactory.

5.4.2 Results of the Proficiency Test

The results of the statistical evaluation of the PT for 3 and 2-MCPD are shown in Table 4.

Table 4: Statistical Parameters of the Proficiency Test

	3-MCPD						2-MCPD				
Sample	P1	P2	P3	P4	P5	P6	P1	P2	P3	P4	P5
Mean value [mg/kg]	**1.15**	**0.80**	**2.08**	**2.56**	**4.03**	**0.98**	**0.52**	**0.32**	**1.04**	**0.91**	**1.52**
Repeatability [mg/kg]	0.05	0.05	0.11	0.15	0.16	0.06	0.03	0.02	0.04	0.05	0.07
Reproducibility [mg/kg]	0.43	0.22	0.44	0.57	0.74	0.21	0.21	0.10	0.19	0.17	0.37
Repeatability [%]	**4.34**	**5.98**	**5.40**	**5.69**	**4.07**	**6.38**	**6.71**	**7.77**	**3.69**	**5.58**	**4.61**
Reproducibility [%]	**37.20**	**27.15**	**21.03**	**22.17**	**18.5**	**21.57**	**40.16**	**31.88**	**18.43**	**18.64**	**24.20**
No. of participants	33	36	36	36	36	36	31	32	33	33	33
No of individual values	108	129	127	131	128	131	102	117	118	122	118
Standard errors MW [mg/kg]	0.07	0.04	0.07	0.09	0.12	0.03	0.04	0.02	0.03	0.03	0.06
Percentage E [%] *	39.4	27.8	16.7	25.0	16.7	19.4	25.8	21.9	21.2	18.2	27.3

* Percentage E – relative percentage of laboratories with extremely deviant mean values on the total number of participants

As can be seen from Table 4, reproducibilities ranging between 19 and 31 % were calculated for both analytes in samples 2-6. In Milk Powder A (Sample 1), the reproducibility of approx. 40 % for both analytes was significantly higher than in Milk Powder B (Sample 5) and in comparison with the other samples. In Collaborative trial 1 Part II, which was also evaluated as a PT, reproducbilities of 14-23 % were calculated for the determination of 3-MCPD in four samples and 56 % for one sample. The concentration range examined was comparable with that of the current collaborative trial.

In further collaborative trials to determine 3-MCPD, rel. standard deviations averaging between 11-38 % were calculated, whereby the examined concentration range lay between 1-10 mg/kg. For one sample whose 3-MCPD concentration was in the range of the quantitation limit, the rel. standard deviation increased to >100 % (Fiebig 2011). Accordingly, the PT conducted here, in which almost all of the laboratories carried out an additional extraction step, achieved comparable results from which the conclusion can be drawn that the additional extraction step does not significantly increase the rel. standard deviation of the methods.

The z-scores achieved by the laboratories for all materials and both analytes are shown graphically in **Fehler! Verweisquelle konnte nicht gefunden werden.** to **Fehler! Verweisquelle konnte nicht gefunden werden.**. A synopsis of the z-scores achieved for the individual samples is shown in Table 5 .

Table 5: Evaluation of the Results of the Proficiency Test for 3 and 2-MCPD

	3-MCPD						2-MCPD				
Laboratory	P1	P2	P3	P4	P5	P6	P1	P2	P3	P4	P5
Results mit $\|z\| < 2$ in [%]	61	72	83	75	83	81	74	78	79	82	82
Results mit $2 \leq \|z\| \leq 3$ in [%]	12	6	3	8	3	3	6	9	6	3	3
Results mit $\|z\| > 3$ in [%]	27	22	14	17	14	17	19	13	15	15	15

It can be summarised for the determination of 3-MCPD that an average of 76 % of all submitted results were evaluated as sufficient ($|z| < 2$), 6 % as questionable ($2 \leq |z| \leq 3$) and 18 % as unsatisfactory ($|z| > 3$). For the determination of 2-MCPD, 78 % of the results were evaluated as sufficient, 7 % as questionable and 15 % as unsatisfactory.

In a proficiency test conducted by the Joint Research Centre in 2009 to determine 3-MCPD, an average of 70 % of all participants achieved sufficient results (Karasek et al. 2011).

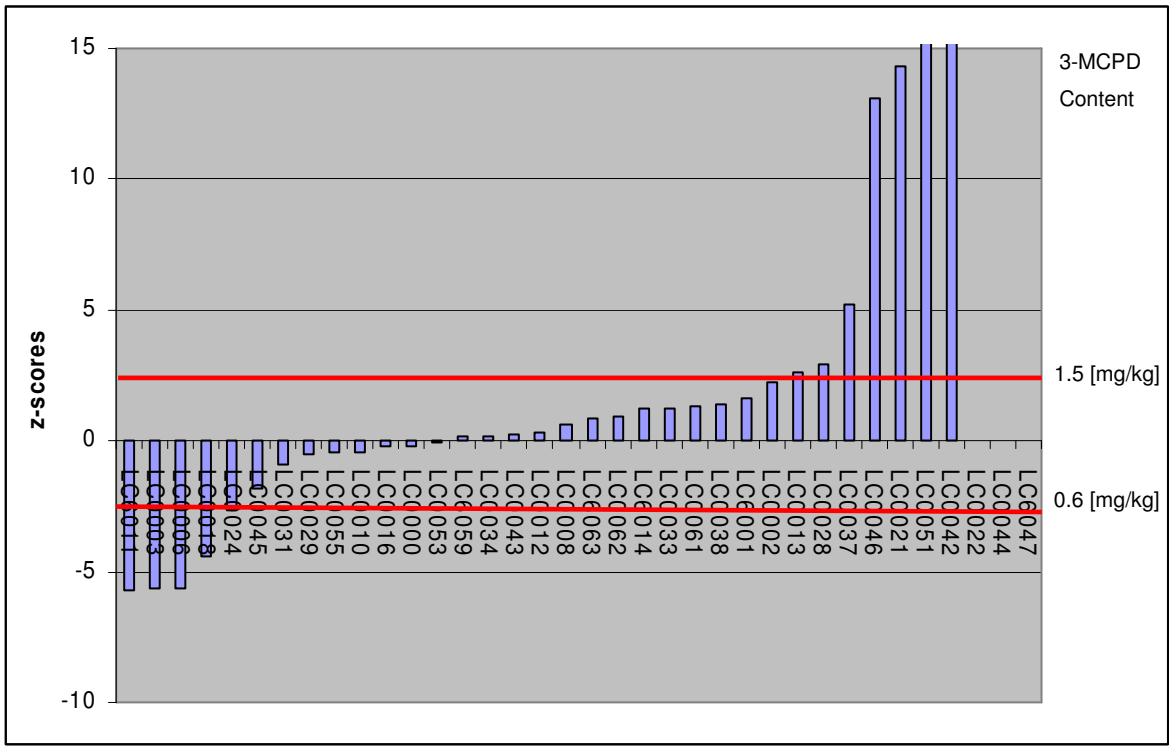

Fig. 7: Z-scores of all Participants for 3-MCPD in Milk Powder A (Sample 1).

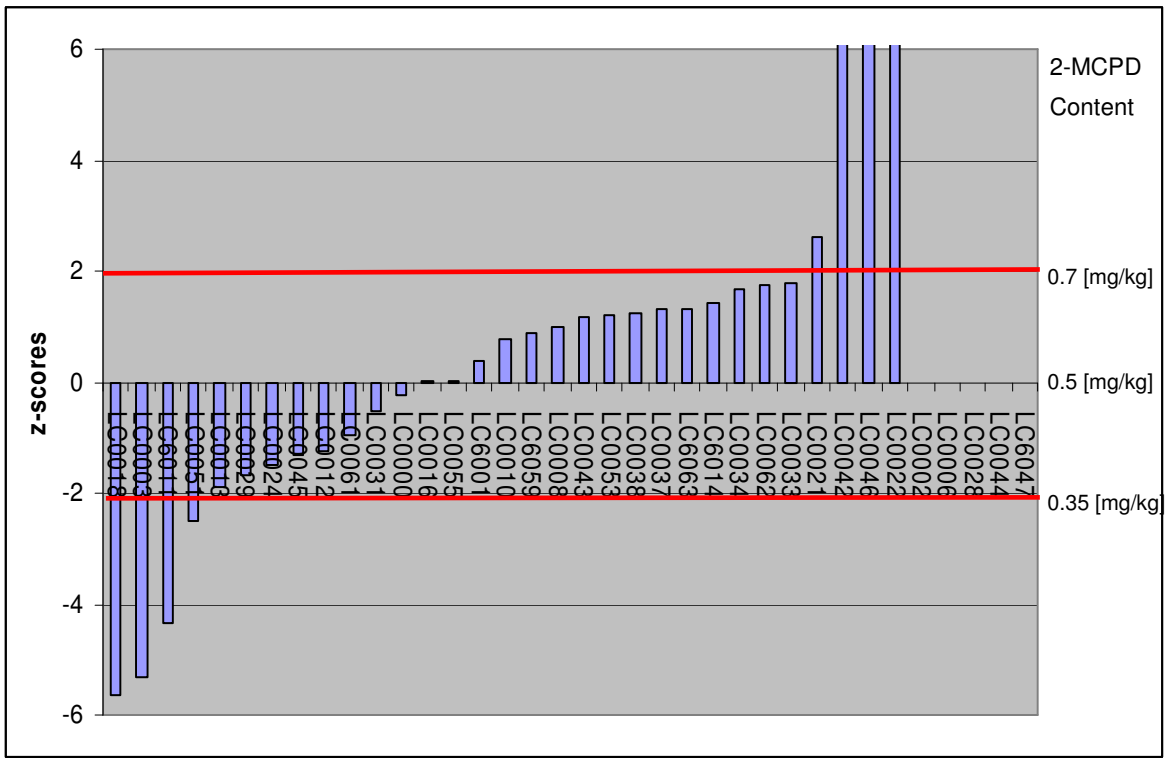

Fig. 8: Z-scores of all Participants for 2-MCPD in Milk Powder A (Sample 1).

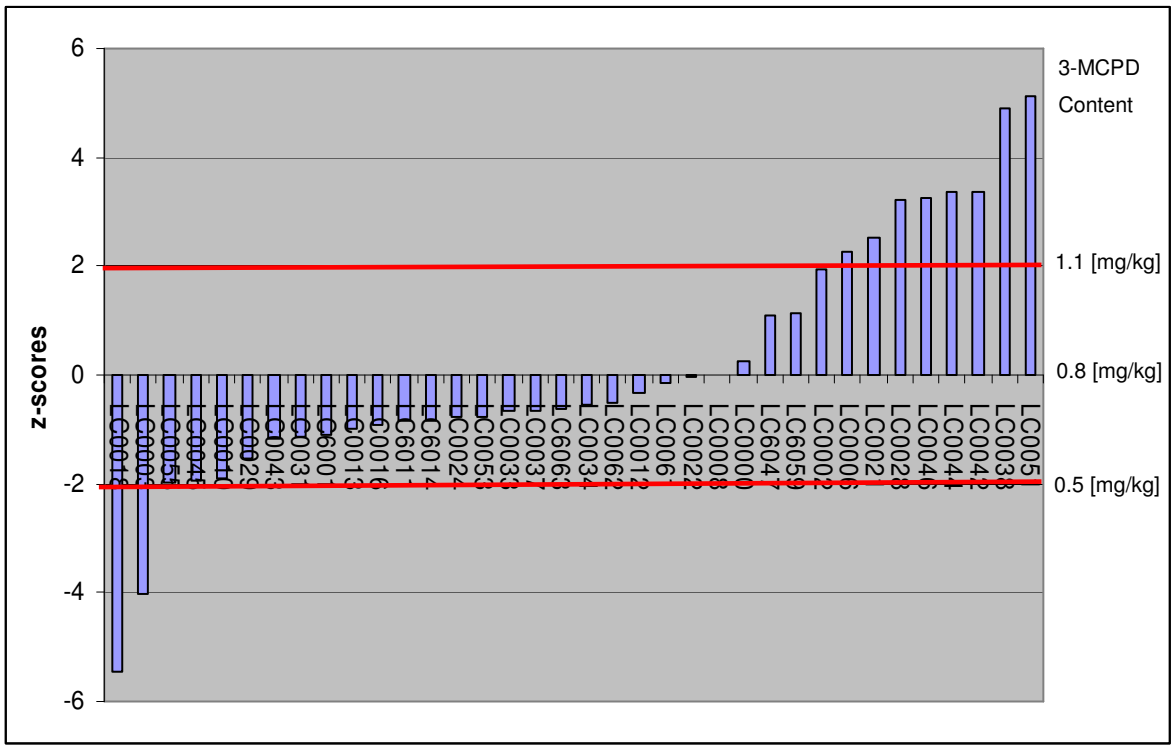

Fig. 9: Z-scores of all Participants for 3-MCPD in Strawberry Cream (Sample 2).

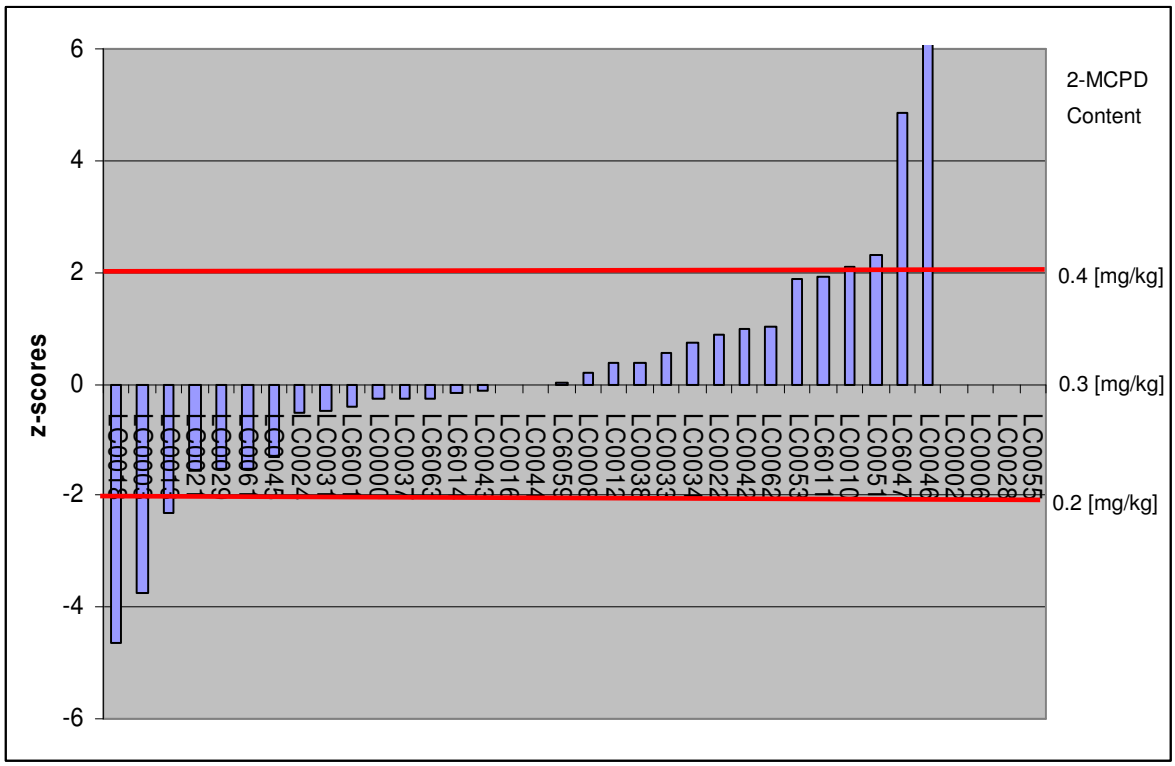

Fig. 10: Z-scores of all Participants for 2-MCPD in Strawbwerry Cream (Sample 2).

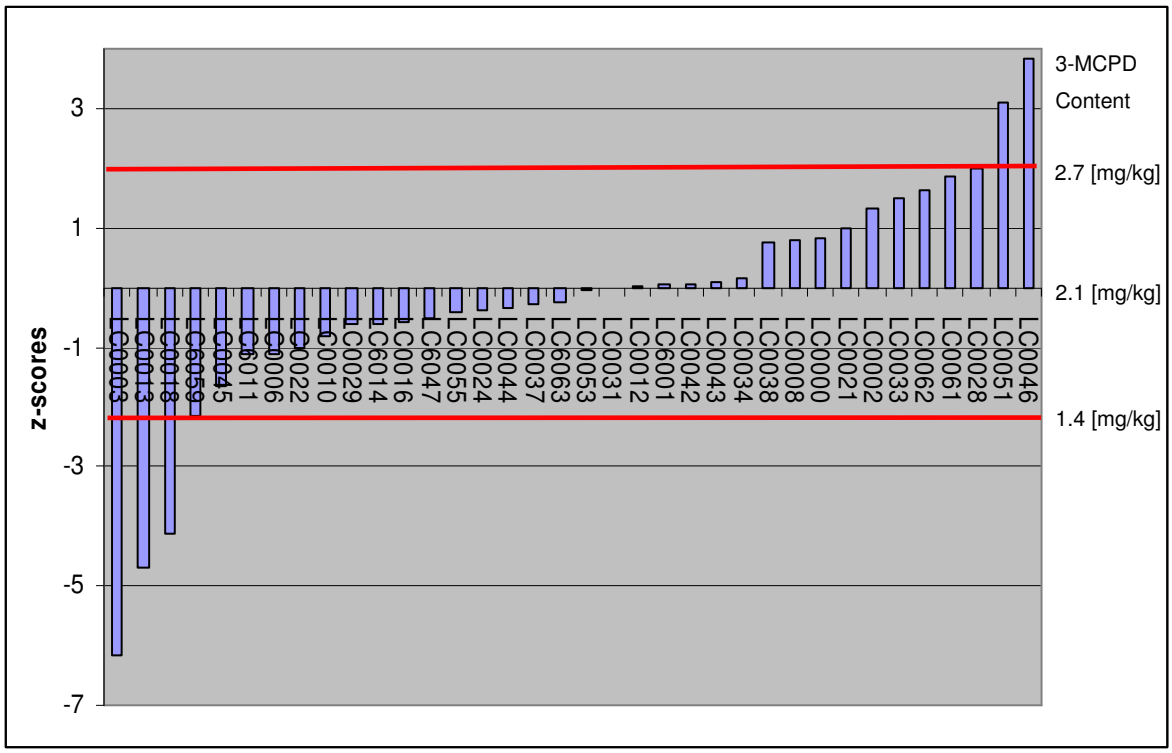

Fig. 11: Z-scores of all Participants for 3-MCPD in Chocolate Cream (Sample 3).

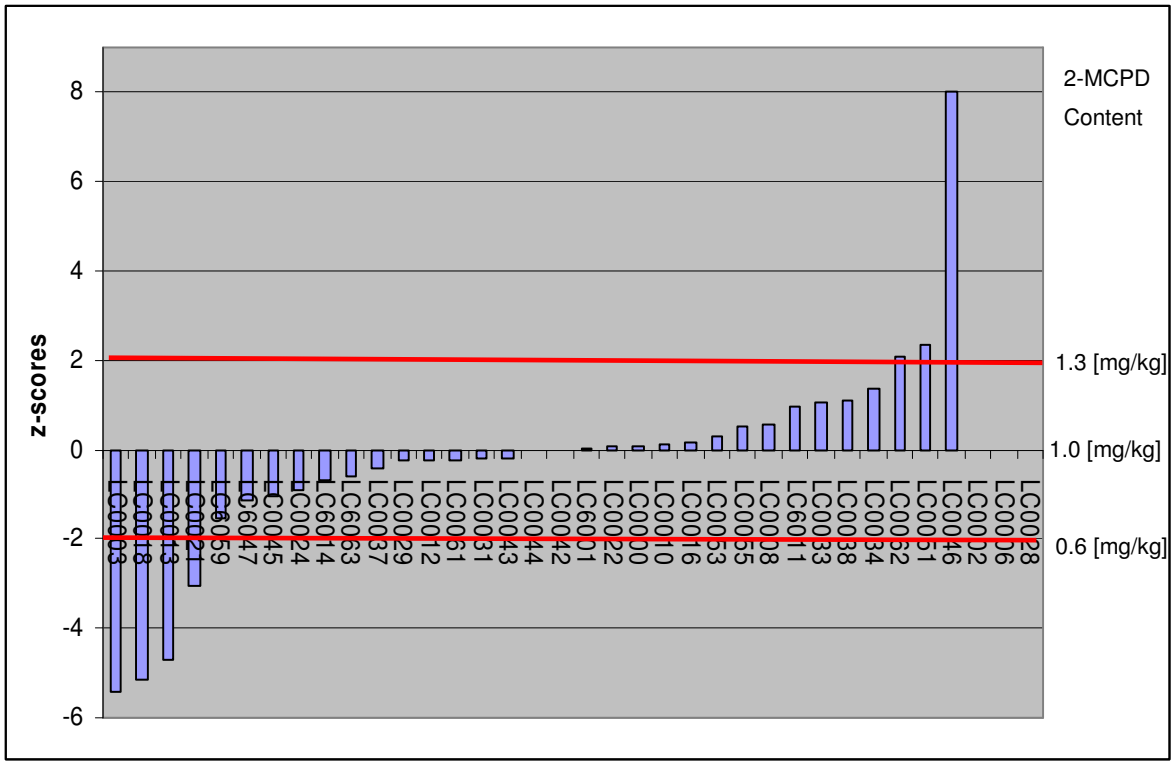

Fig. 12: Z-scores of all Participants for 2-MCPD in Chocolate Cream (Sample 3).

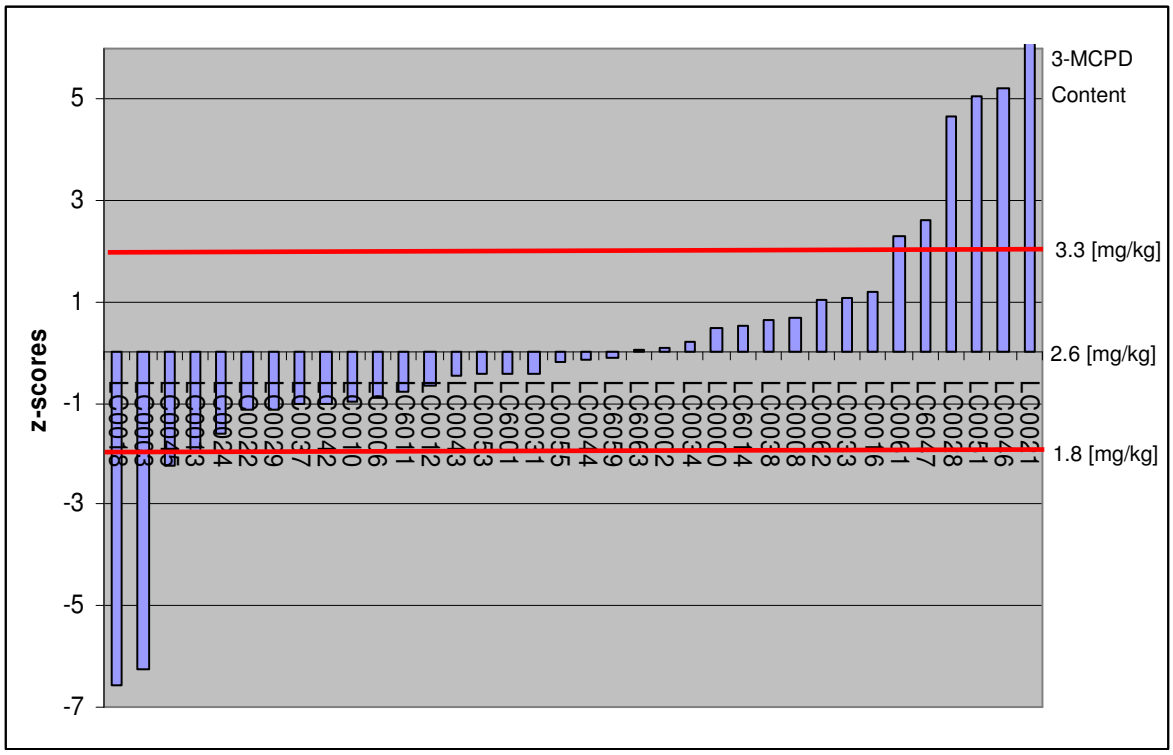

Fig. 13: Z-scores of all Participants for 3-MCPD in Onion Lard (Sample 4).

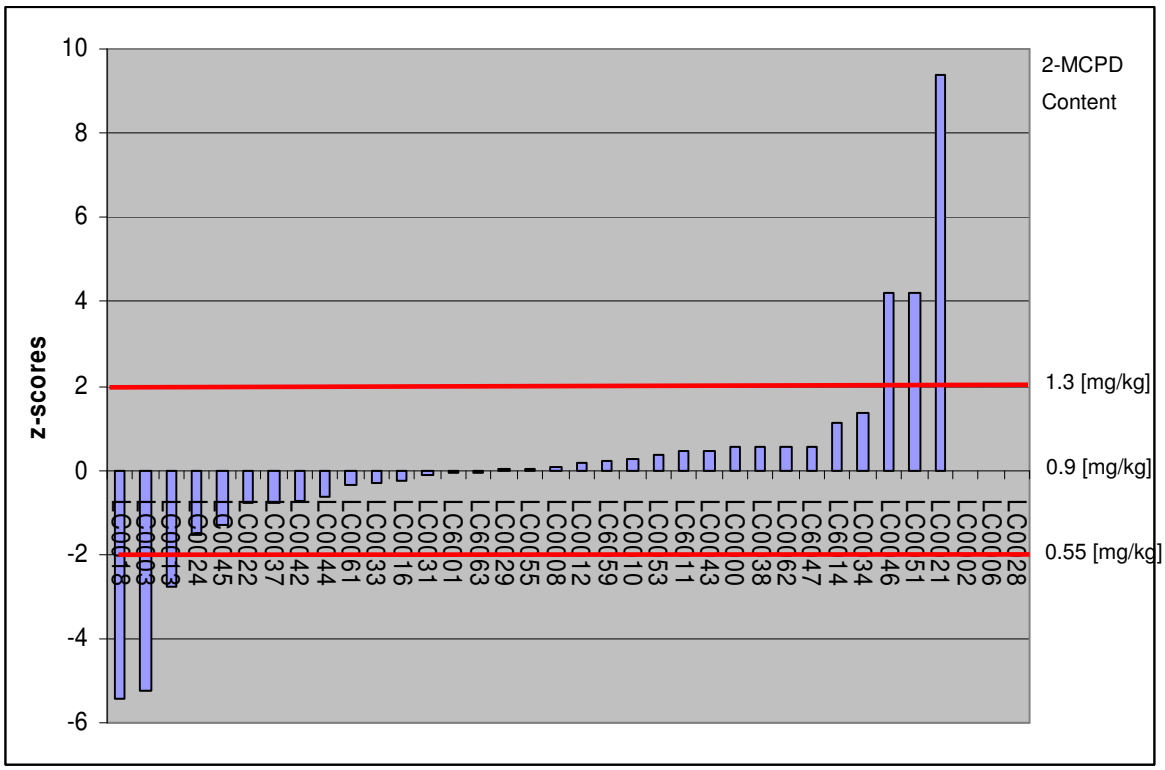

Fig. 14: Z-scores of all Participants for 2-MCPD in Onion Lard (Sample 4).

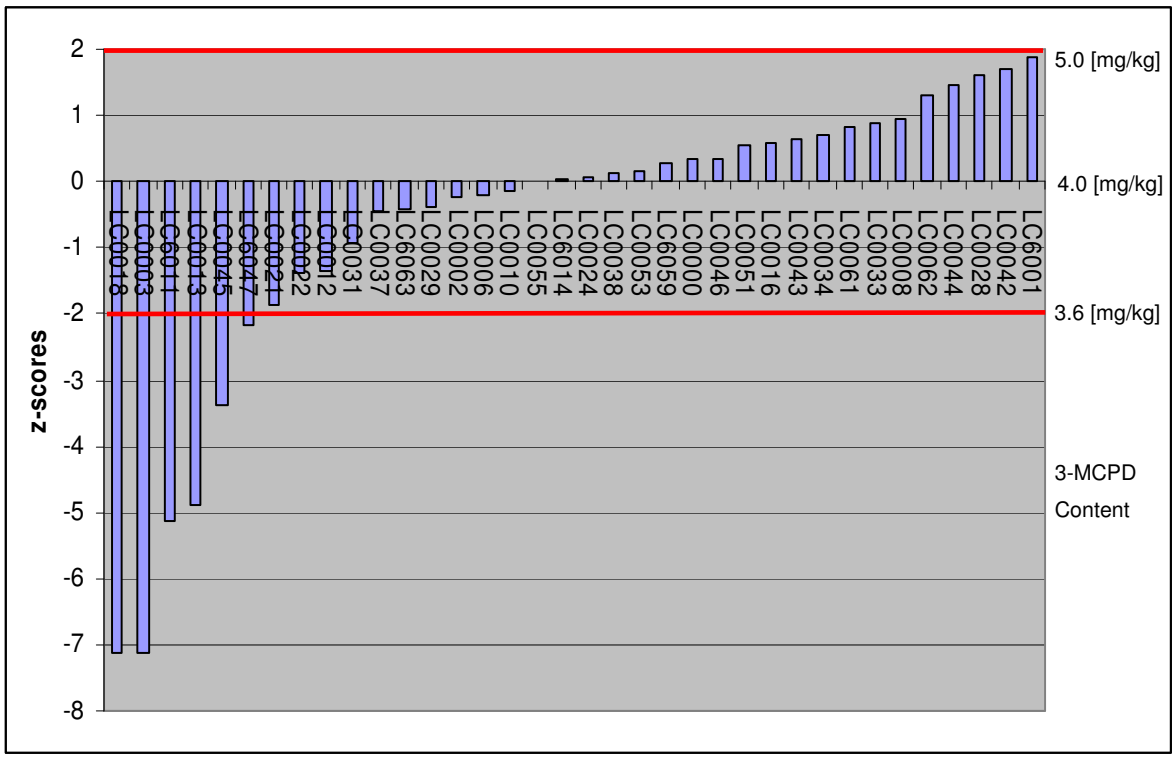

Fig. 15: Z-scores of all Participants for 3-MCPD in Milk Powder B (Sample 5).

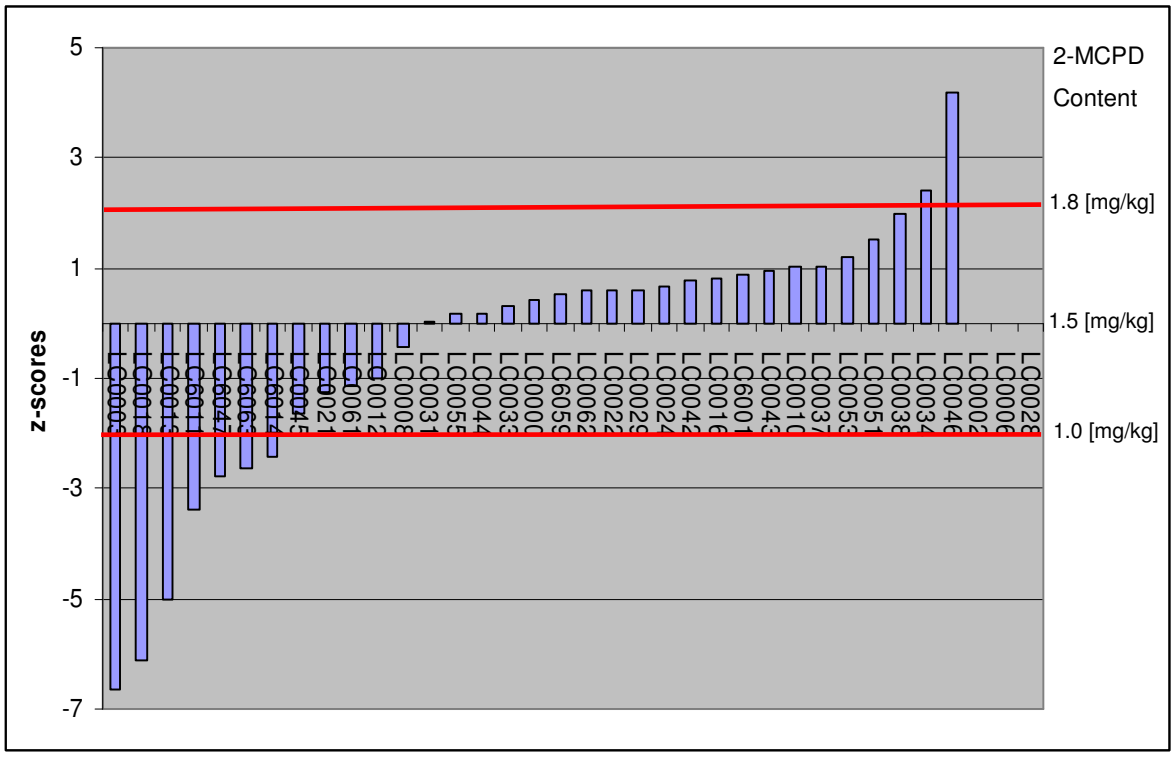

Fig. 16: Z-scores of all Participants for 2-MCPD in Milk Powder B (Sample 5).

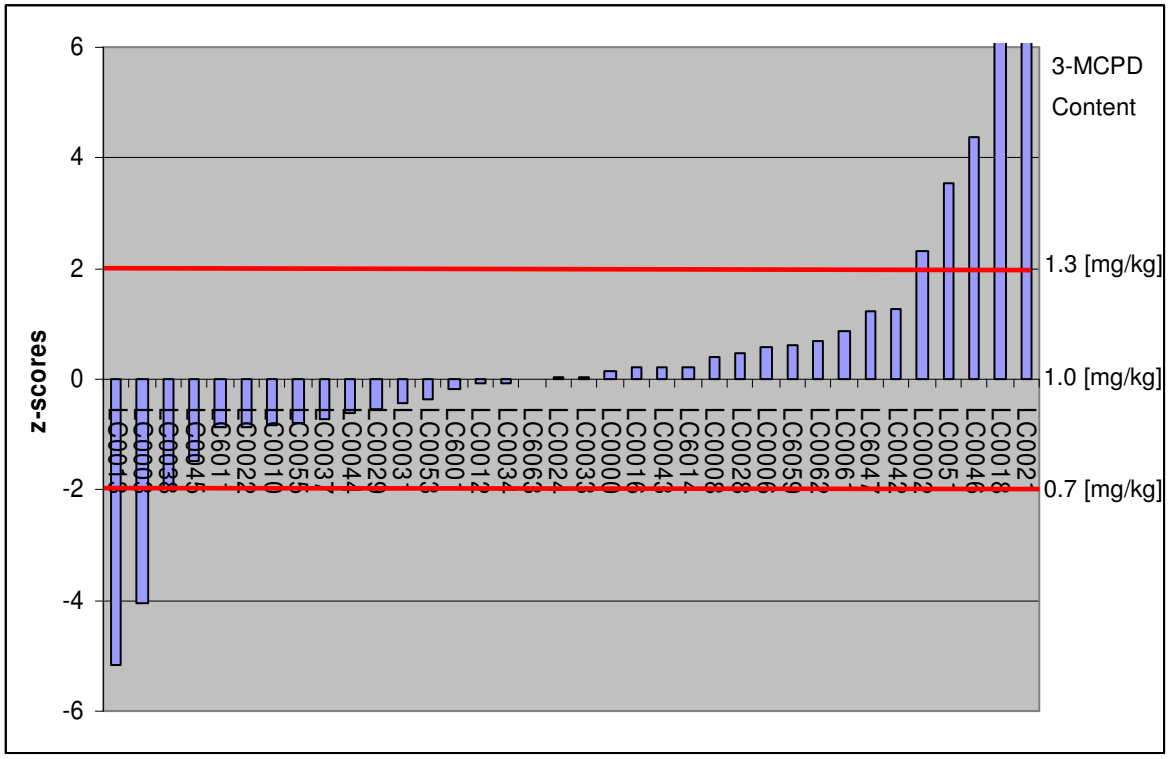

Fig. 17: Z-scores of all Participants for 3-MCPD in Mayonnaise (Sample 6).

The results of the proficiency test comply with the results of comparably conducted PTs. Sample A – a commercially available milk powder – appears to pose special problems as, compared to the other samples, there is a significant increase in the number of laboratories with extreme results as well as a decrease reproducibility for both analytes (Table 4). The high number of positive extreme values is conspicuous. Fat extraction from dairy products often causes problems because the milk fat is enveloped in a protein layer through phospholipids and this protein layer has to be separated in order to extract the fat. Although the fat in milk powder used as infant formula is mainly adjusted by adding refined vegetable fat, it also appears to cause difficulties during extraction. If the inefficient extraction of the fat or a coextraction of the fat with other matrix compounds is assumed, a laboratory would have to achieve low readings for the analytes in question. High readings tend to indicate that for this sample, a matrix compound was analysed which simulates a higher 3-MCPD concentration.

5.5 Summary of Results

The collaborative trial for determining 3- and/or 2-MCPD FAE in fat containing foods produced very good results for BfR Method 22 (BfR Method 9 of Collaborative trial 1 Part II + ASE) in regard to reproducibility and the resultant HorRat values. The data sets of six laboratories were included in the statistical evaluation. The calculated HorRat values lay between 0.5 and 1.0 for 3-MCPD and between 0.6 and 1.0 for 2-MCPD. The recovery rates of BfR Method 22 varied between 85 and 135 % for 3-MCPD FAE in a spiked sample. The use of ASE including the applied protocol for fat extraction respectively the extraction of the MCPD FAE produced results for all tested matrices which indicate very good repeatability within the laboratories as well as very good reproducibility between the laboratories.

If laboratories which used the Soxhlet method to extract fat instead of ASE (these labs work without a standardised extraction protocol) are additionally included in the calculation of the statistical parameters of BfR Method 22, although the reproducibility decreases for the method, the established concentrations are comparable and the method achieves generally common performance characteristics. A standardisation of the extraction protocol of the Soxhlet method would further improve results.

The results of the laboratories which used BfR Method 9 in combination with extraction methods other than ASE or Soxhlet allow the conclusion that a) a standardisation of the extraction would improve the reproducibility of the method and b) the extraction of the MCPD FAE is not done quantitatively for several of the cold extraction methods used here.

The specificity of the methods used was tested by means of a sample which contained glycidyl FAE in addition to 3-MCPD FAE. The results of the collaborative trial show that the recording of 3-MCPD and glycidyl FAE as a sum parameter can be excluded for the majority of the laboratories.

A comparison of the statistical parameters established for the method groups with the data of BfR Collaborative trial 1 Part II or similarly conducted collaborative trials proves that the extraction step does not significantly worsen the reproducibility of the methods. It can also be ascertained that there is no indication that the extraction steps used have an influence on the specificity of the methods, i.e. there is no indication that 3-MCPD is formed or destroyed through the extraction step.
Evaluation with regard to the use of internal standards gives indications that the internal standard should be selected in such a way that the ester cleavage step can be monitored and/or corrected. The use of deuterated MCPD FAE in place of free MCPD could guarantee this.

The proficiency test showed that for the determination of 3-MCPD, an average of 76 % of all submitted results were evaluated as satisfactory ($|z| < 2$), 6 % as questionable ($2 \leq |z| \leq 3$) and 18 % as unsatisfactory ($|z| > 3$). For the determination of 2 MCPD, 78 % of the results were evaluated as sufficient, 7 % as questionable and 15 % as unsatisfactory. The results of the proficiency test match up with the results of PTs conducted in a comparable manner.

6 Appendices

Appendix 1 – Literature

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Appendix 2 – Data

Table 6: Statistical Parameters for 3-MCPD for Method Group 1

	P1	P2	P3	P4	P5	P6
Laboratory	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]
LC0000	1.12	0.83	2.32	2.73	4.21	1.01
LC0002	1.56	1.05	2.48	2.59	3.91	1.35
LC0008	1.27	0.8	2.31	2.81	4.51	1.05
LC0010	1.07	0.54	1.83	2.22	3.95	0.85
LC0016	1.12	0.68	1.91	2.99	4.33	1.02
LC0034	1.18	0.72	2.12	2.64	4.39	0.97
Mean Value	**1.22**	**0.71**	**2.2**	**2.6**	**4.22**	**1.04**
Horwitz STD	0.19	0.12	0.31	0.36	0.54	0.17
Reproducibility	0.19	0.12	0.26	0.26	0.28	0.17
Repeated STD	0.07	0.06	0.1	0.13	0.15	0.05
Rel. Horwitz STD	15.52%	16.83%	14.21%	13.86%	12.88%	15.90%
Reproducibility	**15.65%**	**17.18%**	**11.74%**	**10.07%**	**6.61%**	**16.34%**
Rel. repeated STD	**5.87%**	**7.79%**	**4.47%**	**5.17%**	**3.58%**	**4.90%**
HorRat	**1.0**	**1.0**	**0.8**	**0.7**	**0.5**	**1.0**
Reproducibility Limit, R (2.80 X sR)	0.54	0.34	0.72	0.73	0.78	0.48
Repeat Limit, r (2.80 X sr)	0.2	0.16	0.28	0.38	0.42	0.14
Number of participating laboratories after the elimination of outliers	6	5	5	5	6	6
Number of individual values without outliers	23	20	19	19	24	24
Mandel's h (0.01)						

Table 7: Statistical Parameters for 2-MCPD for Method Group 1

	P1	P2	P3	P4	P5
Laboratory	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]
LC0000	0.5	0.3	1.05	0.99	1.61
LC0002					
LC0008	0.61	0.33	1.13	0.92	1.42
LC0010	0.59	0.44	1.06	0.94	1.75
LC0016	0.52	0.32	1.06	0.87	1.71
LC0034	0.67	0.36	1.27	1.1	2.08
Mean value	**0.58**	**0.35**	**1.12**	**0.96**	**1.71**
Horwitz STD	0.1	0.07	0.18	0.16	0.25
Reproducibility	0.08	0.06	0.1	0.1	0.25
Repeatability	0.04	0.03	0.05	0.05	0.07
Rel. Horwitz STD	17.36%	18.74%	15.74%	16.09%	14.75%
Reproducibility	**13.66%**	**17.92%**	**8.84%**	**10.38%**	**14.36%**
Repeatability	**7.15%**	**8.71%**	**4.29%**	**5.32%**	**4.03%**
HorRat	**0.8**	**1.0**	**0.6**	**0.6**	**1.0**
Reproducibility Limit, R (2.80 X sR)	0.22	0.18	0.28	0.28	0.69
Repeat Limit, r (2.80 X sr)	0.12	0.09	0.13	0.14	0.19
Number of participating laboratories after the elimination of outliers	5	5	5	5	5
Number of individual values without outliers	19	20	20	20	20

Table 8: Statistical Parameters for 3-MCPD for Method Group 2

	P1	P2	P3	P4	P5	P6
Laboratory	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]
LC0000	1.12	0.83	2.32	2.73	4.21	1.01
LC0002	1.56	1.05	2.48	2.59	3.91	1.35
LC0006		1.1	1.75	2.25	3.92	1.08
LC0008	1.27	0.8	2.31	2.81	4.51	1.05
LC0010	1.07	0.54	1.83	2.22	3.95	0.85
LC0016	1.12	0.68	1.91	2.99	4.33	1.02
LC0034	1.18	0.72	2.12	2.64	4.39	0.97
LC0037	**2.09**	0.71	1.99	2.2	3.78	0.87
LC0046		**1.23**	3.21	**4.42**	4.21	1.67
Mean value	**1.22**	**0.77**	**2.17**	**2.57**	**4.14**	**1.02**
Horwitz STD	0.19	0.13	0.31	0.36	0.54	0.16
Reproducibility	0.19	0.18	0.44	0.38	0.29	0.16
Repeatability	0.07	0.08	0.27	0.27	0.16	0.05
Rel. Horwitz STD	**15.52%**	**16.63%**	**14.24%**	**13.88%**	**12.92%**	**15.95%**
Reproducibility	**15.65%**	**23.72%**	**20.19%**	**14.69%**	**7.12%**	**16.00%**
Repeatability	5.87%	10.63%	12.37%	10.34%	3.76%	5.01%
HorRat	1.0	1.4	1.4	1.1	0.6	1.0
Reproducibility Limit, R (2.80 X sR)	0.54	0.51	1.23	1.06	0.83	0.46
Repeat Limit, r (2.80 X sr)	0.20	0.23	0.75	0.74	0.44	0.14
Number of participating laboratories after the elimination of outliers	6	8	9	8	9	8
Number of individual values without outliers	23	29	31	29	32	30

Mandel's h (0.01)
Mandel's k (0.01)

Table 9: Statistical Parameters for 2-MCPD for Method Group 2

	P1	P2	P3	P4	P5
Laboratory	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]
LC0000	0.5	0.3	1.05	0.99	1.61
LC0002					
LC0006					
LC0008	0.61	0.33	1.13	0.92	1.42
LC0010	0.59	0.44	1.06	0.94	1.75
LC0016	0.52	0.32	1.06	0.87	1.71
LC0034	0.67	0.36	1.27	1.1	2.08
LC0037	0.64	0.3	0.97	0.79	1.76
LC0046		1.2	2.37	1.52	2.48
Mean value	**0.59**	**0.34**	**1.09**	**0.93**	**1.78**
Horwitz STD	0.1	0.06	0.17	0.15	0.26
Reproducibility	0.08	0.06	0.11	0.12	0.3
Repeatability	0.04	0.03	0.05	0.05	0.07
Rel. Horwitz STD	**17.31%**	**18.81%**	**15.79%**	**16.16%**	**14.67%**
Reproducibility	**12.82%**	**18.19%**	**9.94%**	**12.44%**	**17.08%**
Repeatability	6.71%	9.99%	4.71%	5.59%	3.97%
HorRat	0.7	1.0	0.6	0.8	1.2
Reproducibility Limit, R (2.80 X sR)	0.21	0.17	0.3	0.33	0.85
Repeat Limit, r (2.80 X sr)	0.11	0.1	0.14	0.15	0.2
Number of participating laboratories after the elimination of outliers	6	6	6	6	7
Number of individual values without outliers	23	24	24	24	26

Mandel's h (0.01)

(x) Only one measured value submitted

Table 10: Statistical Parameters for 3-MCPD for Method Group 3

	P1	P2	P3	P4	P5	P6
Laboratory	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]
LC0012	1.21	0.75	2.08	2.33	3.31	0.97
LC0013	1.62	0.67	0.68	1.86	1.48	0.17
LC0018	0.36	0.08	0.85	0.23	0.31	3.38
LC0029	1.06	0.59	1.9	2.16	3.82	0.9
LC0031	0.99	0.65	2.08	2.42	3.54	0.92
LC0038	1.4	1.44	2.31	2.8	4.09	0.68
LC0042	8.41	1.24	2.1	2.21	4.92	1.18
LC0044		1.24	1.98	2.51	4.79	0.89
LC0045	0.83 (x)	0.54	1.58	1.77	2.26	0.75
LC0055	1.06 (x)	0.54	1.95	2.49	4.03	0.86
Mean value	1.01	0.72	1.72	2.24	3.16	0.81
Horwitz STD	0.16	0.12	0.25	0.32	0.43	0.13
Reproducibility	0.44	0.39	0.55	0.32	1.52	0.3
Repeatability	0.06	0.05	0.14	0.1	0.11	0.03
Rel. Horwitz STD	15.98%	16.79%	14.74%	14.16%	13.45%	16.52%
Reproducibility	**44.09%**	**54.21%**	**32.16%**	**14.23%**	**48.16%**	**36.87%**
Repeatability	**6.03%**	**6.34%**	**8.02%**	**4.68%**	**3.55%**	**3.80%**
HorRat	**2.8**	**3.2**	**2.2**	**1.0**	**3.6**	**2.2**
Reproducibility Limit, R (2.80 X sR)	1.24	1.1	1.55	0.89	4.27	0.83
Repeat Limit, r (2.80 X sr)	0.17	0.13	0.39	0.29	0.31	0.09
Number of participating laboratories after the elimination of outliers	6	10	10	9	10	9
Number of individual values without outliers	18	36	34	33	36	31

Mandel's h (0.01)

(x) Only one measured value submitted

Table 11: Statistical Parameters for 2-MCPD for Method Group 3

	P1	P2	P3	P4	P5
Laboratory	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]
LC0012	0.41	0.34	1.0	0.93	1.29
LC0013	0.34	0.18	0.26	0.5	0.38
LC0018	0.0034	0.04	0.19	0.11	0.12
LC0029	0.36	0.23	1.0	0.91	1.66
LC0031	0.47	0.29	1.01	0.89	1.53
LC0038	0.63	0.34	1.23	0.98	1.98
LC0042	1.1	0.38	1.04	0.8	1.7
LC0044		0.32	1.03	0.81	1.56
LC0045	0.4 (x)	0.24	0.87	0.71	1.15
LC0055	0.52 (x)	< 0.50	1.13	0.91	1.56
Mean value	**0.45**	**0.25**	**0.82**	**0.74**	**1.25**
Horwitz STD	0.08	0.05	0.14	0.12	0.19
Reproducibility	0.35	0.11	0.37	0.28	0.6
Repeatability	0.01	0.01	0.02	0.07	0.05
Rel. Horwitz STD	18.03%	19.74%	16.47%	16.73%	15.47%
Reproducibility	**77.66%**	**44.04%**	**44.50%**	**37.53%**	**48.34%**
Repeatability	**3.03%**	**3.70%**	**2.74%**	**9.73%**	**3.80%**
HorRat	**4.3**	**2.2**	**2.7**	**2.2**	**3.1**
Reproducibility Limit, R (2.80 X sR)	0.98	0.3	1.03	0.78	1.69
Repeat Limit, r (2.80 X sr)	0.04	0.03	0.06	0.2	0.13
Number of participating laboratories after the elimination of outliers	6	8	9	10	10
Number of individual values without outliers	18	31	30	37	36

Mandel's k (0.01)

(x) Only one measured value submitted

Table 12: Statistical Parameters for 3-MCPD for Method Group 4

	P1	P2	P3	P4	P5	P6
Laboratory	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]
LC0024	0.68	0.7	1.97	1.99	4.06	0.99
LC0028	1.68	1.22	2.67	4.22	4.87	1.06
LC6011	0.12	0.69	1.74	2.29	1.35	0.85
LC6014	1.37	0.68	1.9	2.75	4.04	1.02
LC6047	< 0.81	0.94	1.92	3.49	2.88	1.18
LC6063	1.31	0.71	2.01	2.58	3.81	0.98
Mean value	**0.87**	**0.81**	**1.9**	**2.83**	**3.56**	**1**
Horwitz STD	0.14	0.13	0.28	0.39	0.47	0.16
Reproducibility	0.59	0.23	0.12	0.85	1.30	0.13
Repeatability	0.03	0.06	0.07	0.13	0.30	0.1
Rel. Horwitz STD	16.34%	16.50%	14.52%	13.68%	13.21%	16.00%
Reproducibility	**67.88%**	**28.38%**	**6.46%**	**29.91%**	**36.49%**	**12.99%**
Repeatability	**3.16%**	**7.36%**	**3.75%**	**4.47%**	**8.33%**	**9.98%**
HorRat	**4.2**	**1.7**	**0.4**	**2.2**	**2.8**	**0.8**
Reproducibility Limit, R (2.80 X sR)	1.65	0.65	0.34	2.37	3.64	0.36
Repeat Limit, r (2.80 X sr)	0.08	0.17	0.2	0.35	0.83	0.28
Number of participating laboratories after the elimination of outliers	4	6	5	6	6	6
Number of individual values without outliers	16	22	18	22	22	22

Mandel's h (0.01)

Mandel's k (0.05)

Table 13: Statistical Parameters for 2-MCPD for Method Group 4

	P1	P2	P3	P4	P5
Laboratory	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]
LC0024	0.38	0.29	0.89	0.68	1.68
LC0028					
LC6011	0.12	0.43	1.2	0.97	0.75
LC6014	0.65	0.31	0.93	1.07	0.97
LC6047		0.61 (x)	0.85	0.98	0.89 (x)
LC6063	0.64	0.3	0.94	0.9	0.92
Mean value	**0.45**	**0.3**	**0.98**	**0.9**	**1.08**
Horwitz STD	0.08	0.06	0.16	0.15	0.17
Reproducibility	0.25	0.02	0.15	0.18	0.41
Repeatability	0.02	0.01	0.08	0.04	0.07
Rel. Horwitz STD	18.05%	19.20%	16.06%	16.26%	15.82%
Reproducibility	**56.27%**	**5.78%**	**15.87%**	**20.34%**	**38.43%**
Repeatability	**5.18%**	**4.98%**	**8.35%**	**4.36%**	**6.69%**
HorRat	**3.1**	**0.3**	**1.0**	**1.3**	**2.4**
Reproducibility Limit, R (2.80 X sR)	0.71	0.05	0.43	0.51	1.16
Repeat Limit, r (2.80 X sr)	0.07	0.04	0.23	0.11	0.20
Number of participating laboratories after the elimination of outliers	4	3	5	4	4
Number of individual values without outliers	16	12	18	14	16

(x) Only one measured value submitted

Mandel's h (0.01)

Mandel's k (0.01)

Table 14: Statistical Parameters for 3-MCPD for Method Group 5

	P1	P2	P3	P4	P5	P6
Laboratory	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]
LC0033	1.38	0.71	2.52	2.94	4.49	0.99
LC0043	1.2	0.64	2.1	2.4	4.37	1.02
LC6001	1.44	0.65	2.09	2.41	5.01	0.96
LC6059	1.18	0.95	1.44	2.53	4.17	1.08
Mean value	**1.30**	**0.4**	**2.04**	**2.57**	**4.51**	**1.01**
Horwitz STD	0.20	0.12	0.29	0.36	0.58	0.16
Reproducibility	0.15	0.15	0.45	0.26	0.41	0.08
Repeatability	0.08	0.04	0.08	0.08	0.23	0.08
Rel. Horwitz STD	15.38%	16.75%	14.37%	13.88%	12.75%	15.97%
Reproducibility	**11.46%**	**19.72%**	**22.18%**	**10.19%**	**9.09%**	**8.32%**
Repeatability	**5.80%**	**4.83%**	**4.16%**	**2.97%**	**5.14%**	**7.49%**
HorRat	**0.7**	**1.2**	**1.5**	**0.7**	**0.7**	**0.5**
Reproducibility Limit, R (2.80 X sR)	0.42	0.41	1.27	0.73	1.15	0.5
Repeat Limit, r (2.80 X sr)	0.21	0.1	0.24	0.21	0.65	0.24
Number of participating laboratories after the elimination of outliers	4	4	4	4	4	4
Number of individual values without outliers	16	16	16	16	16	16

Table 15: Statistical Parameters for 2-MCPD for Method Group 5

	P1	P2	P3	P4	P5
Laboratory	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]
LC0033	0.68	0.35	1.22	0.86	1.6
LC0043	0.63	0.31	1.01	0.98	1.74
LC6001	0.56	0.29	1.04	0.9	1.72
LC6059	0.60	0.32	0.79	0.94	1.64
Mean value	**0.62**	**0.32**	**1.01**	**0.92**	**1.67**
Horwitz STD	0.11	0.06	0.16	0.15	0.25
Reproducibility	0.06	0.03	0.19	0.07	0.13
Repeatability	0.04	0.03	0.07	0.05	0.13
Rel. Horwitz STD	17.21%	19.01%	15.96%	16.20%	14.80%
Reproducibility	**10.16%**	**10.33%**	**18.38%**	**7.36%**	**7.77%**
Repeatability	**6.15%**	**8.11%**	**6.76%**	**5.88%**	**7.64%**
HorRat	**0.6**	**0.5**	**1.2**	**0.5**	**0.5**
Reproducibility Limit, R (2.80 X sR)	0.18	0.09	0.52	0.19	0.36
Repeat Limit, r (2.80 X sr)	0.11	0.07	0.19	0.15	0.36
Number of participating laboratories after the elimination of outliers	4	4	4	4	4
Number of individual values without outliers	16	16	16	16	16

Table 16: Z-scores of all Laboratories for the Determination of 3 and/or 2-MCPD

	3-MCPD						2-MCPD				
Laboratory	P1	P2	P3	P4	P5	P6	P1	P2	P3	P4	P5
LC0000	-0.18	0.26	0.82	0.47	0.35	0.14	-0.23	-0.28	0.07	0.53	0.41
LC0002	2.24	1.93	1.34	0.08	-0.23	2.30					
LC0003	-5.67	-4.02	-6.18	-6.26	-7.11	-4.05	-5.32	-3.73	-5.41	-5.22	-6.63
LC0006	-5.67	2.27	-1.11	-0.90	-0.21	0.57					
LC0008	0.65	0	0.79	0.69	0.93	0.38	0.99	0.18	0.56	0.07	-0.44
LC0010	-0.44	-1.92	-0.82	-0.97	-0.16	-0.82	0.78	2.09	0.13	0.26	1.02
LC0012	0.32	-0.32	0.01	-0.67	-1.36	-0.09	-1.24	0.39	-0.25	0.16	-1.00
LC0013	2.59	-0.97	-4.69	-1.97	-4.88	-5.18	-1.90	-2.31	-4.72	-2.78	-5.01
LC0016	-0.20	-0.90	-0.57	1.19	0.59	0.21	0.01	-0.03	0.15	-0.27	0.81
LC0018	-4.40	-5.45	-4.13	-6.57	-7.12	15.19	-5.63	-4.65	-5.17	-5.42	-6.11
LC0021	14.30	2.52	0.99	14.96	-1.88	74.01	2.63	-1.56	-3.07	9.40	-1.25
LC0022		-0.04	-1.00	-1.13	-1.37	-0.85	55.56	0.88	0.06	-0.79	0.61
LC0024	-2.63	-0.76	-0.38	-1.60	0.07	0.03	-1.49	-0.53	-0.90	-1.56	0.68
LC0028	2.92	3.22	1.98	4.65	1.62	0.48					
LC0029	-0.53	-1.52	-0.61	-1.13	-0.40	-0.54	-1.68	-1.52	-0.26	0.02	0.61
LC0031	-0.89	-1.12	-0.01	-0.41	-0.93	-0.44	-0.51	-0.49	-0.20	-0.10	0.03
LC0033	1.25	-0.65	1.49	1.06	0.89	0.05	1.79	0.55	1.07	-0.28	0.32
LC0034	0.17	-0.55	0.15	0.21	0.70	-0.08	1.68	0.72	1.36	1.35	2.42
LC0037	5.20	-0.65	-0.27	-1.03	-0.46	-0.73	1.32	-0.28	-0.41	-0.79	1.04
LC0038	1.40	4.89	0.77	0.66	0.13	-1.96	1.24	0.39	1.11	0.53	2.00
LC0042	40.19	3.36	0.07	-1.00	1.71	1.25	6.34	0.97	0	-0.74	0.78
LC0043	0.24	-1.16	0.09	-0.45	0.65	0.22	1.18	-0.11	-0.20	0.48	0.95
LC0044		3.35	-0.34	-0.15	1.46	-0.62		0	-0.04	-0.63	0.18
LC0045	-1.79	-1.94	-1.66	-2.24	-3.39	-1.47	-1.30	-1.32	-1.05	-1.30	-1.64
LC0046	13.12	3.26	3.82	5.22	0.35	4.36	36.57	14.62	8.00	4.19	4.17
LC0051	34.40	5.12	3.11	5.05	0.56	3.56	-2.50	2.30	2.37	4.19	1.51
LC0053	-0.05	-0.76	-0.05	-0.43	0.17	-0.36	1.21	1.88	0.28	0.38	1.21
LC0055	-0.48	-1.97	-0.43	-0.20	0	-0.79	0.02		0.53	0.03	0.16
LC0061	1.34	-0.16	1.85	2.29	0.83	0.87	-0.94	-1.52	-0.23	-0.33	-1.14
LC0062	0.93	-0.53	1.62	1.04	1.32	0.68	1.73	1.01	2.10	0.53	0.59
LC6001	1.62	-1.10	0.04	-0.43	1.88	-0.19	0.39	-0.40	0.01	-0.06	0.88
LC6011	-5.72	-0.84	-1.12	-0.77	-5.12	-0.87	-4.35	1.92	0.96	0.45	-3.38
LC6014	1.21	-0.84	-0.61	0.52	0.04	0.22	1.43	-0.15	-0.70	1.13	-2.43
LC6047		1.10	-0.53	2.60	-2.18	1.24		4.87	-1.12	0.53	-2.76
LC6059	0.13	1.14	-2.14	-0.11	0.28	0.60	0.88	0.01	-1.53	0.21	0.51
LC6063	0.87	-0.61	-0.23	0.04	-0.41	0	1.32	-0.28	-0.59	-0.06	-2.62

Appendix 3 – List of Participants

Institution	City	Country
ANALYTEC - Laboratory für Lebensmitteluntersuchung und Umweltanalytik	Salzburg	Germany
Arotop Food & Environment	Mainz	Germany
Bayrisches Landesamt für Gesundheit und Lebensmittelsicherheit	Erlangen	Germany
Bilacon	Berlin	Germany
Bundesinstitut für Risikobewertung, Fachgruppe 82 Kontaminanten	Berlin	Germany
Bureau of Chemical Safety, Health Products and Food Branch	Ottawa	Canada
CHELAB	Hemmingen	Germany
Chemisches Laboratory Dr. Wirts und Partner	Hanover	Germany
Chemisches und Veterinäruntersuchungsamt Stuttgart	Fellbach	Germany
Chemisches Untersuchungsamt der Stadt Hagen	Hagen	Germany
Chemisches Untersuchungsinstitut Leverkusen	Leverkusen	Germany
Deutsches Institut für Lebensmitteltechnik e.V.	Quakenbrück	Germany
Ferrero S.p.A.	Alba (CN)	Italy
Institut Dr. Appelt Hilter	Hilter a. T.W.	Germany
Institut für Qualitätsforschung in der Süßwarenwirtschaft e.V.	Cologne	Germany
Institut Kirchhoff Berlin GmbH	Berlin	Germany
Institut Nehring GmbH	Brunswick	Germany
Institut Prof. Dr. Georg Kurz GmbH	Cologne	Germany
Laboratory für Umweltanalytik GmbH	Schwerin	Germany
Laboratory Kneißler	Burglengenfeld	Germany
Landeslaboratory Schleswig-Holstein (Lebensmittel- Veterinär- und Umweltuntersuchungsamt)	Lübeck	Germany
Landesuntersuchungsamt Sachsen	Dresden	Germany
Landesuntersuchungsamt Rheinland-Pfalz, Institut für Lebensmittelchemie	Trier	Germany
LAVES Lebensmittelinstitut Braunschweig	Brunswick	Germany
Nestlé Deutschland AG, NQAC Weiding	Polling-Weiding	Germany
Nofalab	Schiedam	Netherlands
SGS Belgium-IAC	Antwerp	Belgium
SGS Germany GmbH, Consumer Testing Services Food	Hamburg	Germany
Zentrales Institut des Sanitätsdienstes der Bundeswehr	Koblenz	Germany

Appendix 4 – Results of the Homogeneity Determination of the Sample Material

Measured Results				
Sample	3-MCPD		2-MCPD	
	MV (mg/kg)	VK (%)	MV (mg/kg)	VK (%)
Milk powder A	1.30	1.90	0.64	2.21
Strawb. cream	0.65	2.18	0.29	1.66
Choc. cream	1.94	6.95	1.06	2.35
Onion lard	2.54	3.91	1.04	3.65
Milk powder B	4.58	3.75	1.76	2.13
Mayonnaise	1.02	2.82		

ANOVA						
Sample	3-MCPD			2MCPD		
	F	F-crit	Sample homogeneous	F	F-crit	Sample homogeneous
Milk powder A	0.75	4.41	yes	0.52	4.41	yes
Strawb. cream	1.63	4.41	yes	1.76	4.49	yes
Choc. cream	0.11	4.41	yes	0.20	4.49	yes
Onion lard	2.77	4.41	yes	0.05	4.41	yes
Milk powder B	2.19	4.41	yes	3.90	4.41	yes
Mayonnaise	4.02	4.41	yes			yes

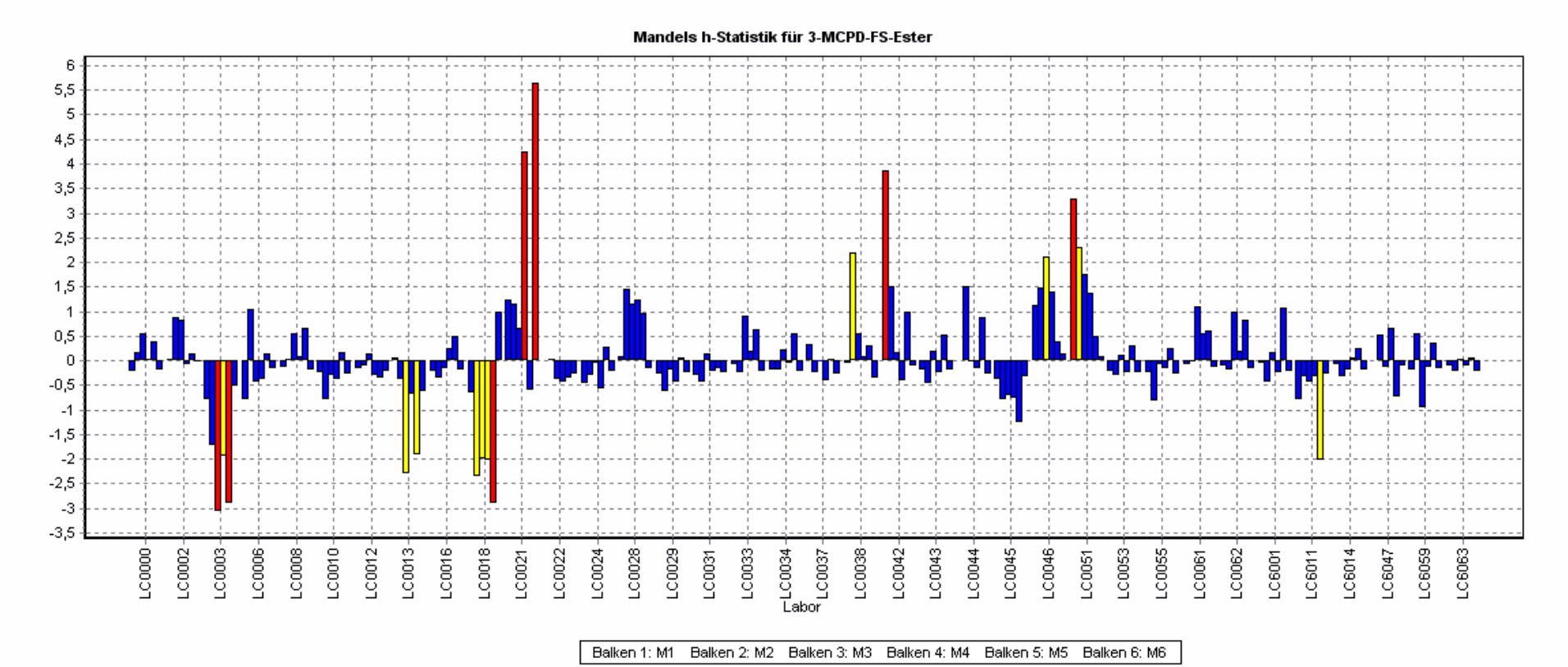

Fig. 18: Mandel's h Statistic for 3-MCPD FAE

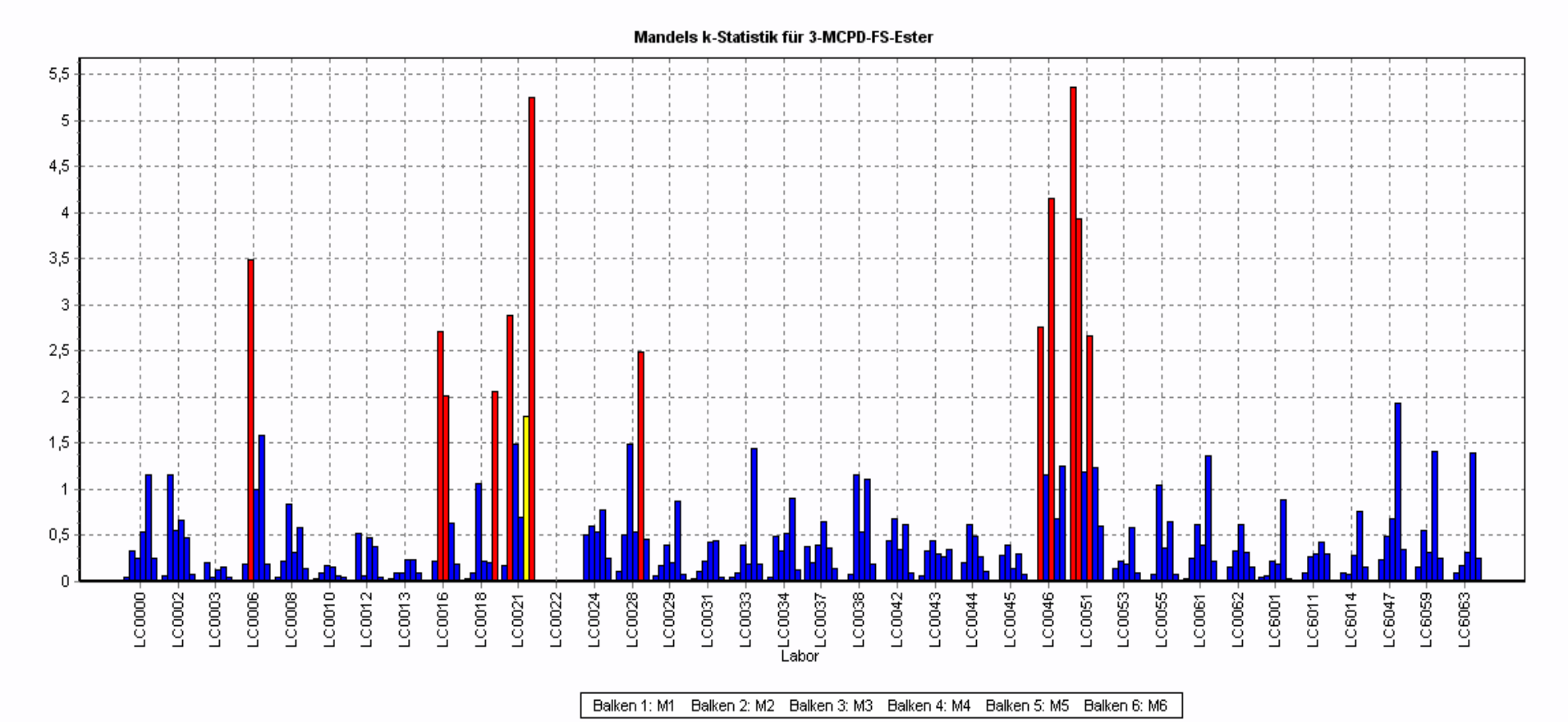

Fig. 19: Mandel's k Statistic for 3-MCPD FAE

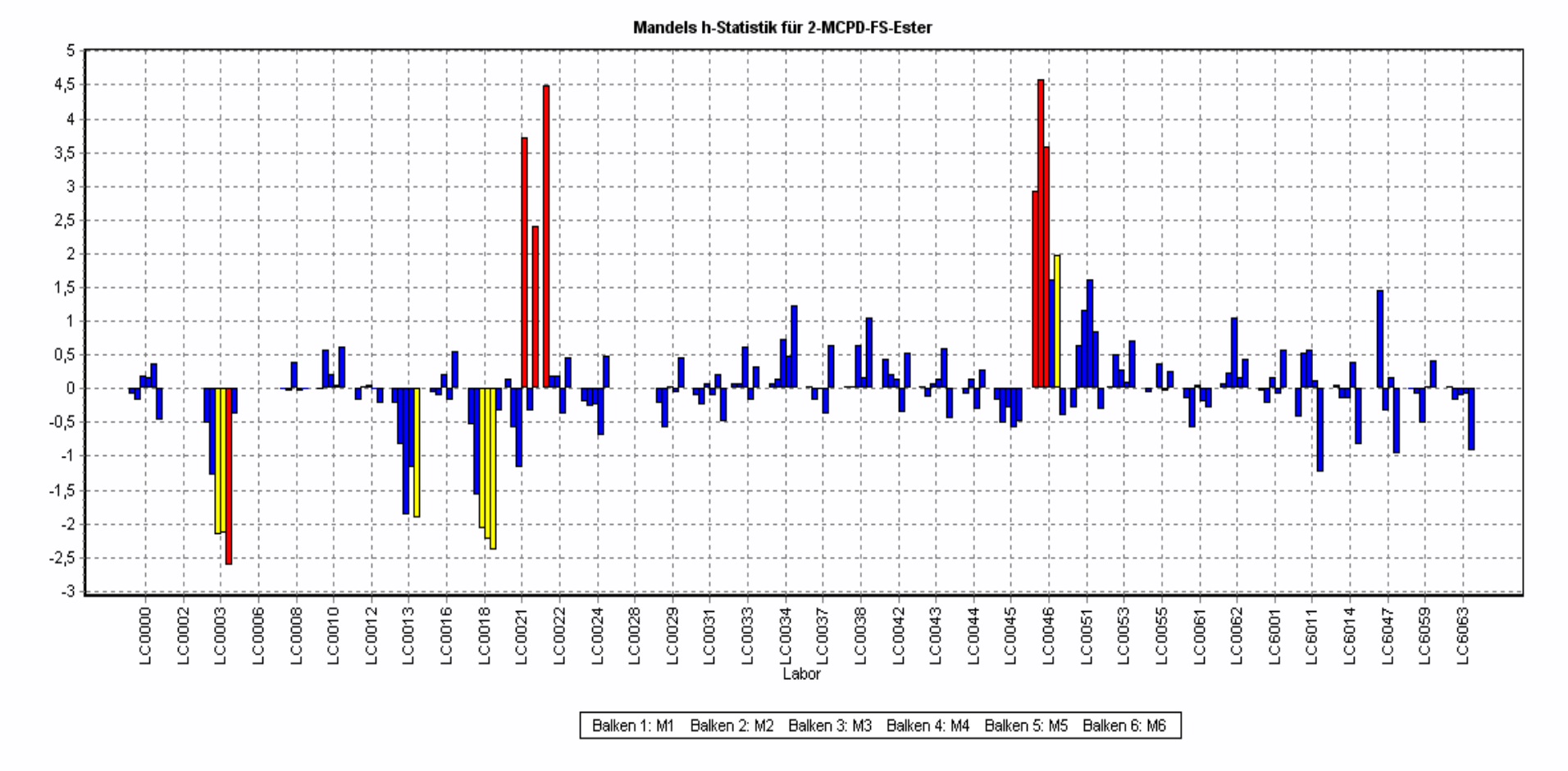

Fig. 20: Mandel's h Statistic for 2-MCPD FAE

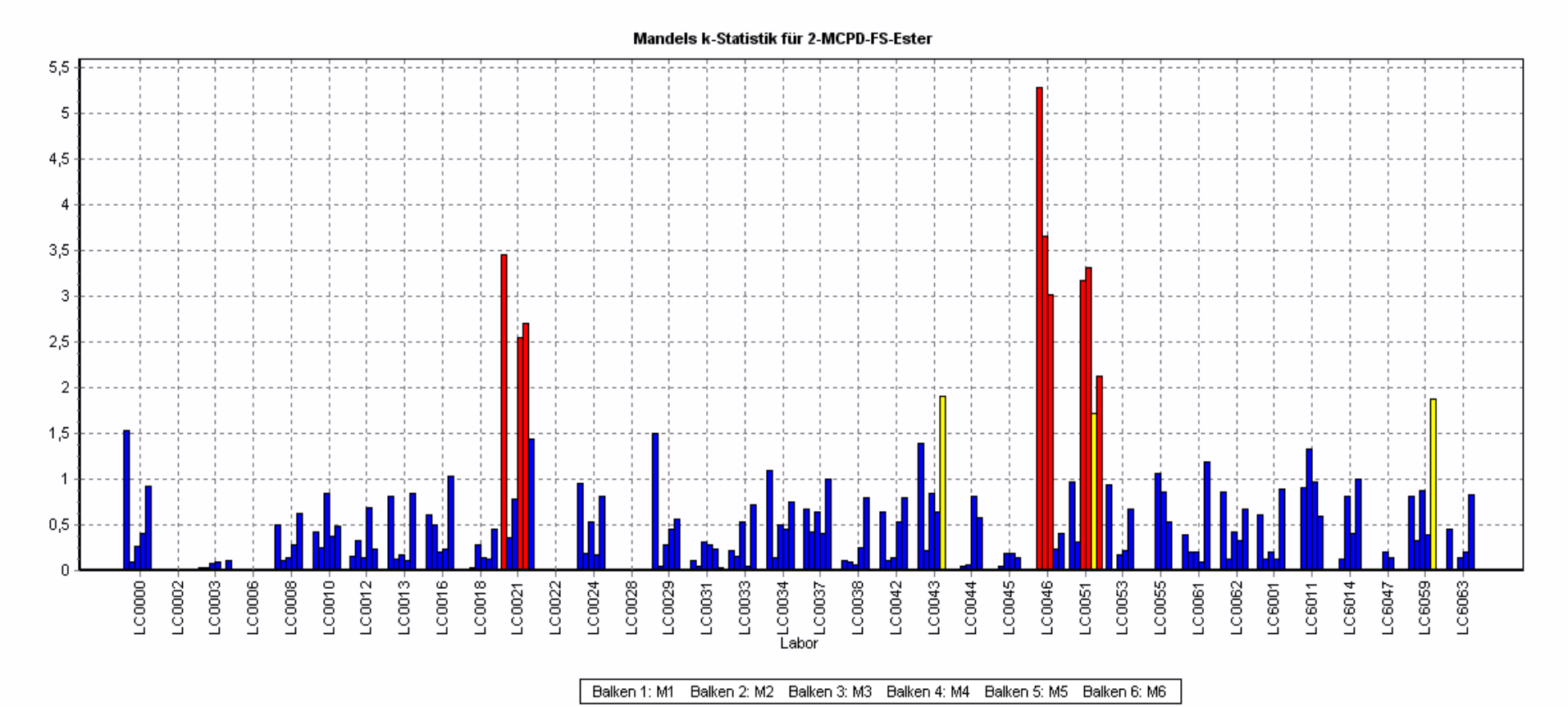

Fig. 21: Mandel's k Statistic for 2-MCPD FAE

Appendix 5 – List of Illustrations

Appendix 6 – List of Tables

BfR Method-22
Version 1

Determination of 3-MCPD- and 2-MCPD-Fatty Acid Esters in fat-containing foods with GC/MS

Fat extraction with **ASE**
An indirect Determination by Detection of free 3-MCPD and 2-MCPD released from MCPD-esters by **Alkaline Saponification**
Derivatization by **Phenylboronic Acid**

NOTE: Potential modifications of this method will be published on the homepage of the Federal Institute of Risk Assessment (BfR).

This method is revised on some points in comparison to the method distributed for the collaborative study.
Revisions are labelled in grey.

1. In the meantime standard substances of 2-MCPD-Esters are available.
2. It is strongly recommended to use the esters of 2-MCPD and 3-MCPD for quantification purposes. The values for precision obtained over an extended period of time, by different operators and using different equipment are better using the esters than the free substances.

7 Scope of Application

This method describes the determination of ester-bound 3-chloropropane-1,2-diol (3-MCPD-fatty acid esters) and 2-chloropropane-1,3-diol (2-MCPD-fatty acid esters) in fat-containing foods by means of gas chromatography-mass spectrometry.

8 Principle of Method

To analyses MCPD-fatty acid esters in fat-containing foods in a first step the samples must be extracted. Therefore accelerated solvent extraction (ASE) with a solvent mixture of petroleum ether / isohexane / acetone (2/2/1; v/v) in two extraction cycles and a temperature of 125 °C is used. After extraction solvent of the ASE extract is dried under a stream of nitrogen, the remaining fat residue is weighed to consistency, and the extraction yield determined.

An appropriate amount of fat extract (100 - 200 mg) is weighed out and dissolved in t-BME and the internal standards (d_5-labeled 3-MCPD or d_5-labeled 3-MCPD ester, d_5-labeled 2-MCPD or d_5-labeled 2-MCPD ester) are added. Cleavage of the ester bond is performed by alkaline hydrolysis with a sodium methylate solution; as a result fatty acid methyl esters and free 3-MCPD respectively 2-MCPD are formed. The reaction is stopped with a solution of ammonium sulphate and sulphuric acid. The sample is defatted with isohexane and subsequently the released 3-MCPD respectively 2-MCPD is extracted with ethyl acetate, derivatized with phenylboronic acid and the extract evaporated to complete dryness. The residue is dissolved in acetone and an aliquot is taken for analysis by GC-MS.

Warning and Safety Precautions

- When handling acids, bases, organic solvents and standard substances (pure substances and solutions) gloves must be used. Use solvents in places provided with a fume hood.
- Attention is drawn to the information contained in the Safety Data Sheet (SDS) and the regulations, which specify the handling of reagents and solvents.
- All crucial steps in this method are marked by ***Note.***

9 Reagents and Products

Note: *The names of manufacturers have been mentioned for information purposes only.*

9.1 Reference Substances

9.1.1	3-MCPD	3-chloropropane-1,2-diol (e.g. FLUKA)
9.1.2	d_5-3-MCPD	3-chloropropane-1,2-diol-d_5 (e.g. CIL)
9.1.3	2-MCPD	2-chloropropane-1,3-diol (e.g. Toronto Research Chemicals)
9.1.4	d_5-2-MCPD	2-chloropropane-1,3-diol-d_5 (e.g. TRC)
9.1.5	3-MCPD ester	1,2-bis-palmitoyl-3-chloropropane-1,2-diol (e.g. TRC)
9.1.6	d_5-3-MCPD ester	1,2-bis-palmitoyl-3-chloropropane-1,2-diol- d_5 (e.g. TRC)
9.1.7	2-MCPD-Ester	1,3-Distearoyl-2-chloropropanediol (e.g. TRC)
9.1.8	d_5-2-MCPD-Ester	1,3-Distearoyl-2-chloropropanediol-d_5 (e.g TRC)

All 3-MCPD and 2-MCPD stock solutions are prepared in methanol; 3-MCPD-Ester and 2-MCPD-Ester stock solutions are prepared in ethyl acetate and stored at +6 (±4) °C in the dark.

The working solutions of standard substances are prepared by diluting the stock solutions with methanol respectively ethyl acetate and are stored in the same way.

The working solutions of internal standards are prepared by diluting the stock solutions with methanol respectively ethyl acetate and are stored in the same way.

9.1.9 **Stock Solutions:**

9.1.9.1 3-MCPD stock solution (S0-solution):

Weigh 10 (±0.1) mg standard substance (3.1.1) into a 10 mL volumetric flask (11.8) and fill up to the mark by adding methanol (3.2.9) (c = 1 mg/mL).

9.1.9.2 d_5-3-MCPD stock solution (S0-solution):

Weigh 100 (±0.1) mg standard substance (3.1.2) into a 100 mL volumetric flask (11.8) and fill up to the mark by adding methanol (3.2.9) (c = 1 mg/mL).

9.1.9.3 2-MCPD stock solution (S0-solution):

Weigh 25 (±0.1) mg standard substance (3.1.3) into a 25 mL volumetric flask (11.8) and fill up to the mark by adding methanol (3.2.9) (c = 1 mg/mL).

9.1.9.4 d_5-2-MCPD stock solution (S0-solution):

Weigh 100 (±0.1) mg standard substance (3.1.4) into a 100 mL volumetric flask (11.8) and fill up to the mark by adding methanol (3.2.9) (c = 1 mg/mL).

9.1.9.5 3-MCPD-ester stock solution (SV0-solution)

Weigh 10.8 (±0.1) mg standard substance (3.1.5) into a 2 mL volumetric flask (11.8) and fill up to the mark by adding ethyl acetate (3.2.6) (c = 5.4 mg/mL).

9.1.9.6 d_5-3-MCPD-ester stock solution (SV0-solution)

Weigh 10.8 (±0.1) mg standard substance (3.1.6) into a 2 mL volumetric flask (11.8) and fill up to the mark by adding ethyl acetate (3.2.6) (c = 5.4 mg/mL).

9.1.9.7 2-MCPD-ester stock solution (SV0-solution)

Weigh 11.6 (±0.1) mg standard substance (9.1.7) into a 2 mL volumetric flask (11.8) and fill up to the mark by adding ethyl acetate (3.2.6) (c = 5.8 mg/mL).

9.1.9.8 d_5-2-MCPD-ester stock solution (SV0-solution)

Weigh 11.6 (±0.1) mg standard substance (9.1.8) into a 2 mL volumetric flask (11.8) and fill up to the mark by adding ethyl acetate (3.2.6) (c = 5.8 mg/mL).

9.1.10 **3-MCPD standard solution for the calibration function**

9.1.10.1 S2 standard solution:

Pipet 100 µL stock solution S0 (9.1.9.1) into a 10 mL volumetric flask (11.8) and fill up to the mark by adding methanol (3.2.9) (c = 10 µg/mL).

9.1.10.2 S3 standard solution:

Pipet 1 mL S2-standard solution (9.1.10.1) into a 10 mL volumetric flask (11.8) and fill up to the mark by adding methanol (3.2.9) (c = 1 µg/mL).

9.1.11 **2-MCPD standard solution for the calibration function**

9.1.11.1 S2 standard solution:

Pipet 100 µL stock solution S0 (9.1.9.3) into a 10 mL volumetric flask (11.8) and fill up to the mark by adding methanol (3.2.9) (c = 10 µg/mL).

9.1.11.2 S3 standard solution:

Pipet 1 mL S2-standard solution (9.1.11.1) into a 10 mL volumetric flask (11.8) and fill up to the mark by adding methanol (3.2.9) (c = 1 µg/mL).

9.1.12 **3-MCPD-Ester standard solution for the calibration function**

9.1.12.1 SV2 standard solution:

Pipet 100 µL stock solution SV0 (9.1.9.5) into a 10 mL volumetric flask (11.8) and fill up to the mark by adding ethyl acetate (3.2.6) (c = 54 µg/mL).

9.1.12.2 SV3 standard solution:

Pipet 1 mL SV2-standard solution (9.1.12.1) into a 10 mL volumetric flask (11.8) and fill up to the mark by adding ethyl acetate (3.2.6) (c = 5.4 µg/mL).

9.1.13 **2-MCPD-Ester standard solution for the calibration function**

9.1.13.1 SV2 standard solution:

Pipet 100 µL stock solution SV0 (9.1.9.7) into a 10 mL volumetric flask (11.8) and fill up to the mark by adding ethyl acetate (3.2.6) (c = 58 µg/mL).

9.1.13.2 SV3 standard solution:

Pipet 1 mL SV2-standard solution (9.1.13.1) into a 10 mL volumetric flask (11.8) and fill up to the mark by adding ethyl acetate (3.2.6) (c = 5.8 µg/mL).

9.1.14 **d_5-3-MCPD working solution as internal standard solution**

9.1.14.1 S2 working solution d_5-3-MCPD:

Pipet 1 mL stock solution S0 (9.1.9.2) into a 100 mL volumetric flask (11.8) and fill up to the mark by adding methanol (3.2.9) (c = 10 µg/mL).

9.1.15 **d_5-2-MCPD working solution as internal standard solution**

9.1.15.1 S2 working solution d_5-2-MCPD:

Pipet 1 mL stock solution S0 (9.1.9.4) into a 100 mL volumetric flask (11.8) and fill up to the mark by adding methanol (3.2.9) (c = 10 µg/mL).

9.1.16 **d_5-3-MCPD-Ester working solution as internal standard solution**

9.1.16.1 SV2 working solution d_5-3-MCPD-Ester:

Pipet 100 µL stock solution SV0 (9.1.9.6) into a 10 mL volumetric flask (11.8) and fill up to the mark by adding ethyl acetate (3.2.6) (c = 54 µg/mL).

9.1.17 **d_5-2-MCPD-Ester working solution as internal standard solution**

9.1.17.1 SV2 working solution d_5-2-MCPD-Ester:

Pipet 100 µL stock solution SV0 (9.1.9.8) into a 10 mL volumetric flask (11.8) and fill up to the mark by adding ethyl acetate (3.2.6) (c = 58 µg/mL).

9.2 Reagents

If not otherwise specified, all reagents shall have at least p.a. quality.

9.2.1 Acetone, for residue analysis (e.g. Merck 100012)

9.2.2 Ammonium sulphate (e.g. Merck 101217)

9.2.3 Distilled water

9.2.4 Diethyl ether (e.g. Promochem SO1187)

9.2.5 Ethanol absolute (e.g. Merck 100983)

9.2.6 Ethyl acetate (e.g. Promochem SO1191)

9.2.7 Isohexane, for residue analysis (e.g. Promochem SO1251)

9.2.8 Isolute HM-N (e.g. I.S.T. 9800-1000)

9.2.9 Methanol (e.g. Merck 106035)

9.2.10 Methyl tert-buthyl ether (t-BME), for GC (e.g. Merck 101995)

9.2.11 Petroleum ether (e.g. Promochem)

9.2.12 Phenylboronic acid > 95 %, for residue analysis (e.g. Fluka 78181)

9.2.13 Sodium methylate ≥ 97 % (e.g. Fluka 71750)

9.2.14 Sulphuric acid 98 % (e.g. Merck 112080)

9.3 Solutions

9.3.1 Extracting reagent:

Prepare a mixture of 2:2:1 (v/v) petroleum ether (3.2.11), isohexane (3.2.7) und acetone (3.2.1) in a pressure resistant bottle.

9.3.2 Saponification reagent (c = 0.5 mol/L):

Dissolve 0.27 (± 0.01) g sodium methylate (3.2.13) in 10 mL methanol (3.2.9).

Note: *Saturated solution, please use only the supernatant**!***

9.3.3 Stop reagent:

Dissolve 10 (± 0.1) g ammonium sulphate (3.2.2) in 25 mL distilled water (3.2.3) and 1.5 ml 25 % sulphuric acid.

Note: *Preparation of 25 % sulphuric acid:*

*Dilute **slowly** 13.6 mL 98 % sulphuric acid (3.2.14) in 73 mL distilled water (3.2.3).*

9.3.4 Derivatization reagent:

Dissolve ca. 0.4 g phenylboronic acid (3.2.12) in 10 mL diethyl ether (3.2.4).

Note: *Saturated solution,* please *use only the supernatant**!***

For the preparation of *the solution 3.3.1 to 3.3.4 the use of an ultrasonic bath is recommend to facilitate dissolution (4.18).*

9.4 Gases

9.4.1 Helium (5.0) (e.g. Air Liquide)

9.4.2 Nitrogen (5.0) (e.g. Air Liquide)

10 Apparatus

Note: *The names of* manufacturers *have been mentioned for information purposes only.*

10.1 11 mm crimp caps with PTFE/silicone/PTFE septa (e.g. Agilent 5181-1211)

10.2 2 mL crimp vials, clear glass (e.g. Agilent 5181-3375) with glass inserts (0.1 mL)

10.3 Accelerated Solvent Extractor ASE 200 with solvent reservoir and compressor modell 6-4 (e.g. Dionex)

10.4 Analytical balance

10.5 ASE 22 mL extraction cell with cell caps (e.g. Dionex)

10.6 ASE vials 60 mL and screw cap with septa (e.g. I-Chem)

10.7 Beaker

10.8 Calibrated volumetric flasks in various volume range

10.9 Cellulose filters for ASE cells d=1.983 cm (e.g. Dionex)

10.10 Centrifuge with cooler (e.g. Thermo Scientific RT7 and Rotor RTH 750)

10.11 Displacement or single channel pipettes

10.12 Drying oven

10.13 Evaporator (e.g. Barkey)

10.14 Manual crimper for 11 mm crimps caps (e.g. Chromacol CR-11)

10.15 Micro tips

10.16 Pasteur pipettes

10.17 Thick-walled test tubes ca. 5 mL (e.g. Hecht Assistant 75x12 mm, No.2775/6)

10.18 Ultrasonic bath

10.19 Vortex test tube shaker (e.g. Scientific Industries)

11 GC-MS System

11.1 Automatic Sampler

11.2 Capillary column (e.g. Agilent DB-5MS, 30 m x 0.25 mm, 0.25 µm)

11.3 Capillary gas chromatograph with an integrated programmable column oven providing a temperature up to at least 300°C (e.g. Agilent – 6890 / 7890A)

11.4 Glass liner, single taper, 4 mm ID, QW (e.g. Agilent 19251-60540)

11.5 Mass selective detector (e.g. Agilent MSD 5973 or 5975C) with ion source for electron-impact ionization

11.6 Pre-column (e.g. Phenomenex, Fused Silica, deaktiviert, 5 m x 0.32 mm)

11.7 Split/split less injector (e.g. Agilent)

12 Sample and Sampling

12.1 Lab samples

Sufficient sample amount should be provided to allow for triple determination at least.
Unequivocal identification of samples must be ensured throughout the process of sampling and sample packaging.

- Please provide for proper packaging, preservation and transport of samples in order to ensure the good condition of the samples so that the analytical results are not affected. Store the samples at +6 (±4) °C in the dark.
- Sufficient sample amount must be provided in order to ensure homogeneity.

12.2 Test samples

- Blank sample
- Reference sample

13 Procedure

13.1 Sample preparation

13.1.1 According to the fat content weigh (fc) 2.5 g (fc < 30%), 2 g (fc 30-60%) or 1.5 g (fc > 60%) sample by means of an analytical balance (4.4) into a beaker (4.7).

13.1.2 Add 4 g dispersing agent such as Isolute (3.2.8) to the sample and homogenize.

Note: *If problems* occur *during the fat extraction of infant formula additionally, add 0.4 mL water* (*3.2.3) per gram sample to dispersing agent before homogenization*.

Therefore, weigh 4 g Isolute into a beaker and mix with water. Subsequently add the infant formula *and homogenize with the dispersing agent.*

13.1.3 A disposable filter (4.9) is installed in the cell before the sample is loaded. The filter prevents blockage of the stainless steel frit in the bottom cap.

13.1.4 Unscrew the top cap from the cell body and place the filter in the cell at a slight angle. Position the insertion tool over the filter and slowly push the insertion tool into the cell. Make sure the filter is in full contact with the cell!

Note: *Always hand-*tighten *the bottom cell cap onto the cell body before installing the filter.*

Do not *place the* filter *in the bottom cap before installing the cap.*

13.1.5 Load the homogenized sample into a 22 mL cell (4.5).

Note: *Being careful to* keep *the threads clean.*

Take up fat residues from the beaker with a tissue and put it inside the cell for extraction.

13.1.6 If desired, fill any void volume in the cell with an inert material such as sea sand. This reduces the amount of solvent needed during the extraction.

13.1.7 Screw the top cap onto the cell body and **hand-tighten**.

Note: ***Do not use*** *a* wrench *or other tool to tighten the cap! This can damage the cell.*

13.1.8 Before starting the fat extraction weigh the empty ASE vials (4.6) and **record the weight**.

13.2 Fat extraction with ASE

13.2.1 Turn on the ASE extractor (4.3).

Note: *Additionally* the *nitrogen gas cylinder (3.4.2) must be open and turn on the compressor.*

13.2.2 Loading filled cells (4.5) into the upper tray slot in numerical order. Hang the cell vertically in the tray slots from their top caps.

13.2.3 Place the 60 mL vials (4.6) into the lower tray slot in numerical order.

13.2.4 Extraction parameter for ASE:

Solvent	Petroleum ether (3.2.11) / Isohexane (3.2.7) / Acetone (3.2.1) (2:2:1, v/v)
Temperature [°C]	125
Pressure [bar]	103.4
Heat time [min]	6
Static time [min]	5
Flush volume [%]	100
Purge time [sec]	60
Cycles	2

Note: *Pressure was* defined *automatic by the Extractor.*

13.2.5 After the sequence, solvent of the extract is completely dried under a stream of nitrogen at 40°C.

13.2.6 Weigh again the ASE vials, **record the weight** and calculate the extraction yield for fat.

Note: *If only a* non-*heatable evaporator is available, evaporation of the solvent can take place at room temperature.*

The fat extracts *can dried additionally in a drying oven (4.12) at 70°C-100°C.*

13.2.7 After use, empty the cells and rinse the cell caps and cell bodies with water or organic solvent (for example ethanol or methanol). Place the pre-cleaned cell caps and cell bodies in a beaker is filled with an organic solvent and sonicate (4.18) for 15 min.

Note: *At regular* intervals *remove the snap ring from the cell cap, remove the cap inserts and clean separately.*

Also replacing *the cell PEEK Seal at regular intervals!*

13.3 Reprocessing the fat extracts

13.3.1 Prior to weighing, bring the fat extracts to room temperature.

13.3.2 Weighing the samples:

Weigh 100 (± 5) mg sample by means of an analytical balance (4.4) into a test tube (4.17) and **record the weight**.

Note: *The sample amount may be raised up to 200 mg, if the 3-MCPD respectively 2-MCPD* concentrations *are expected to be low (< 0.5 mg/kg). Higher sample amounts are not tested.*

13.3.3 Dissolution of samples:

Dissolve the samples in 0.5 mL t-BME (3.2.10). The use of a Vortex (4.19) for 20 s proved to be suitable for this purpose.

Note: *In case* the *sample has already solidified by then, dip the test tube into a hot water bath for a moment and re-dissolve. The water temperature required depends on the melting point of the solid fat.*

13.3.4 Add 30 µL of the internal standard **d_5-3-MCPD** work solution (9.1.14.1) **(Analysis according to annex 2A) or** add 30 µL of the internal standard **d_5-3-MCPD ester** work solution (9.1.16.1) (recommended!) **(Analysis according to annex 2B)**.

Note: *If d_5-3-MCPD ester (*9.1.16.1*) is used as internal standard, it is necessary to prepare* the *calibration standards at the same way as the samples (7.3.3).*

13.3.5 Add 30 µL of the internal standard **d_5-2-MCPD** work solution (3.1.12.1) **or** add 30 µL of the internal standard **d_5-2-MCPD ester** work solution (9.1.17.1) (recommended).

13.4 3-MCPD ester and 2-MCPD calibration standards (Analysis according to annex 2B)

Note: *If d_5-3-MCPD* ester *is used as internal standard, we advise to prepare a* ***matrix calibration*** *to ensure the linear range of the calibration function.*

Therefore, you can use a sample *devoid of analyte e.g.* ***blank oil*** *or the* ***fat extract of a blank matrix.***

Weigh in each case *100 (± 5) mg blank oil into a test tube. Subsequently the blank samples were spiked with the corresponding calibration solutions of the analytes and internal standards (see Table 1).*

13.4.1 Preparation of the calibration standards in test tubes (4.17):

In succession pipet 30 µL, respectively, of d_5-3-MCPD-Ester working solution (9.1.16.1) and of d_5-2-MCPD-Ester working solution (9.1.17.1) into a test tube and add in the same way the respective volumes of the 3-MCPD-Ester standard solution SV2 (9.1.12.1), SV3 (9.1.12.2) and the 2-MCPD-Ester standard solution SV2 (9.1.13.1), SV3 (9.1.13.2) (see Table 1).

Then add 0.5 mL each of t-BME (3.2.10) to all the standards.

Table 1: Pipetting scheme for calibration standards for alkaline saponification

Standard	IS d_5-3-MCPD Ester and d_5-2-MCPD-Ester	3-MCPD Ester and 2-MCPD-Ester	t-BME (3.2.10)	IS d_5-3-MCPD and d_5-2-MCPD	3-MCPD/2-MCPD absolute (µg) in the derivatization preparation	3-MCPD/2-MCPD (mg/kg) per 100 mg sample amount
1	30 µL SV2	25 µL SV3	0.5 mL	0.3 µg abs. in the derivatization preparation or 3 mg/kg sample	0.025	0.25
2		50 µL SV3			0.05	0.5
3		100 µL SV3			0.1	1.0
4		20 µL SV2			0.2	2.0
5		30 µL SV2			0.3	3.0
6		40 µL SV2			0.4	4.0
7		50 µL SV2			0.5	5.0
8		60 µL SV2			0.6	6.0

13.5 Alkaline saponification and subsequent defatting

13.5.1 The ester cleavage is achieved with a solution of 0.5 M sodium methylate in methanol.

Add **0.2 mL** saponification reagent (3.3.2) to the sample. After shaking (10 s Vortex (4.19)) incubate the sample at room temperature for **9-10 min.**

13.5.2 After the incubation period, stop the reaction by adding **0.6 mL** stop reagent (3.3.3) under vigorously shaking at high rotation speed (20 s Vortex (4.19)).

13.5.3 Samples are defatted by adding 1 mL isohexane (3.2.7) and shaking (10 s Vortex (4.19)). The **upper phase** is discarded.

Note: Subsequent *defatting is **not applicable** by the calibration standards (7.4); that does not apply for matrix calibration standards!*

n-hexane may *be used instead of isohexane for defatting.*

If necessary, centrifuge the samples at 207 x g for 2 min at room temperature (4.10) to improve the phase separation.

Repeat defatting of the aqueous phase by adding 1 mL isohexane (3.2.7) and shaking (10 s Vortex (4.10)). The **upper phase** is discarded.

Note: *If necessary, centrifuge the samples at 207 x g for 2 min at room temperature (*4.10*) to improve the phase separation.*

13.6 Extraction of the released 3-MCPD and 2-MCPD

To remove matrix components that could interfere with GC-MS analysis, the released 3-MCPD and 2-MCPD is extracted into a nonpolar organic phase, which is derivatized and carefully dried. Then the analytes are re-dissolved into a polar solvent.

13.6.1 Extraction of the released 3-MCPD and 2-MCPD:

After adding 0.6 mL ethyl acetate (3.2.6) to the sample, shake the mixture for 10 s (Vortex (4.10)). Transfer the **upper organic phase** to a test tube and repeat this extraction of the aqueous phase one time.

Note: *If* necessary*, centrifuge the samples at 207 x g for 2 min at 10 °C (*4.10*) to improve the phase separation.*

If only a non-refrigerated centrifuge is available, centrifugation can take place at room temperature.

During the *extraction with ethyl acetate, take care that no water passed into the organic extracts. To avoid this, it is better to transfer the upper organic phase incompletely; in this case, an additional extraction with ethyl acetate may be performed.*

13.7 3-MCPD and 2-MCPD calibration standards (Analysis according to annex 2A)

It is strongly recommended to use the esters of 2-MCPD and 3-MCPD for quantification purposes.

Preparation of the calibration standards in test tubes (4.17):
In succession pipet 30 µL, respectively, of d_5-3-MCPD working solution (3.1.11.1) and of d_5-2-MCPD working solution (3.1.12.1) into a test tube and add in the same way the respective volumes of the 3-MCPD-standard solution S2 (3.1.8.1), S3 (3.1.8.2) and the 2-MCPD-standard solution S2 (3.1.9.1), S3 (3.1.9.2) (see Table 2).

Then add 1.2 mL each of ethyl acetate (3.2.6) to all the standards.

Table 2: Pipetting scheme for calibration standards for alkaline saponification

Standard	IS d_5-3-MCPD and d_5-2-MCPD	3-MCPD and 2-MCPD	EtoAc (3.2.6)	IS d_5-3-MCPD and d_5-2-MCPD	3-MCPD/2-MCPD absolute (µg) in the derivatization preparation	3-MCPD/2-MCPD (mg/kg) per 100 mg sample amount
1	30 µL	25 µL S3	1.2 mL	0.3 µg abs. in the derivatization preparation or 3 mg/kg sample	0.025	0.25
2		50 µL S3			0.05	0.5
3		100 µL S3			0.1	1.0
4		20 µL S2			0.2	2.0
5		30 µL S2			0.3	3.0
6		40 µL S2			0.4	4.0
7		50 µL S2			0.5	5.0
8		60 µL S2			0.6	6.0

13.8 Derivatization

Note: *The derivatization of the pre-prepared standards (7.4 respectively 7.7) and the samples (7.5) has to be performed simultaneously.*

Add 150 µL each of the derivatization reagent (3.3.4) to all the samples and standards.

13.8.1 The derivatization reaction takes place in the ultrasonic bath (4.18) at room temperature for 2-3 min.

13.9 Subsequent processing of the derivatized samples and calibration standards

13.9.1 Drying of the phenylboronic acid derivatives:

Evaporate the phenylboronic acid derivatives of the samples and standards in an evaporator (4.13) at 40°C under a stream of nitrogen to **complete** dryness. During the drying process, a white precipitate forms at the rim of the glass tube.

Note: *If only a non-heatable evaporator is available, evaporation of the solvent can take place at room temperature.*

13.9.2 Dissolution of the residue:

Dissolve the residue in 300 µL acetone (3.2.1) under shaking (10 s Vortex (4.19)). Note that the white precipitate at the rim of the glass tube remains there. Transfer an aliquot of the residue into a GC crimp vial with a glass insert (4.2) and close with a crimp cap (4.1).

Note: Cyclohexane *or isooctane may be used instead of acetone for the dissolution.*

If necessary*, centrifuge the samples at 207 x g for 2 min at 10°C to improve the phase separation.*

If only a non-refrigerated centrifuge is available, centrifugation can take place at room temperature.

The extracts *can be stored at 4 °C for 3 days prior to GC/MS-analysis.*

14 GC/MS-Analysis

The following specifications are given by way of example.

The GC-MS analysis is based on electron-impact ionization operated in SIM mode with the following parameters:

14.1 Injector conditions

Split/split less injector	
Mode	Pulsed split less
Injector temperature	180 °C
Insert liner	Liner, single taper with glass wool (5.4)

14.2 GC and MS conditions

GC column	DB-5MS (5.2)
Pre-column	Fused Silica, deactivated (5.6)
Flow	1.2 ml/min (constant)
Carrier gas	Helium (3.4.1)
GC oven programme	60 °C (kept constant for 1 min); 6 °C/min up to 190 °C; 30 °C/min up to 280 °C (kept constant for 10 min)
Temperature transfer line	280 °C
Temperature ion source	230 °C
Temperature quadrupole	150 °C

14.2.1 Selected fragment ions of the PBA derivative for SIM method

3-MCPD derivative	Ions (m/z)	Dwell (ms)
	196	80
	147	80
d_5-3-MCPD derivative	Ions (m/z)	Dwell (ms)
	201	80
	150	80
2-MCPD derivative	Ions (m/z)	Dwell (ms)
	196	80
d_5-2-MCPD derivative	Ions (m/z)	Dwell (ms)
	201	80

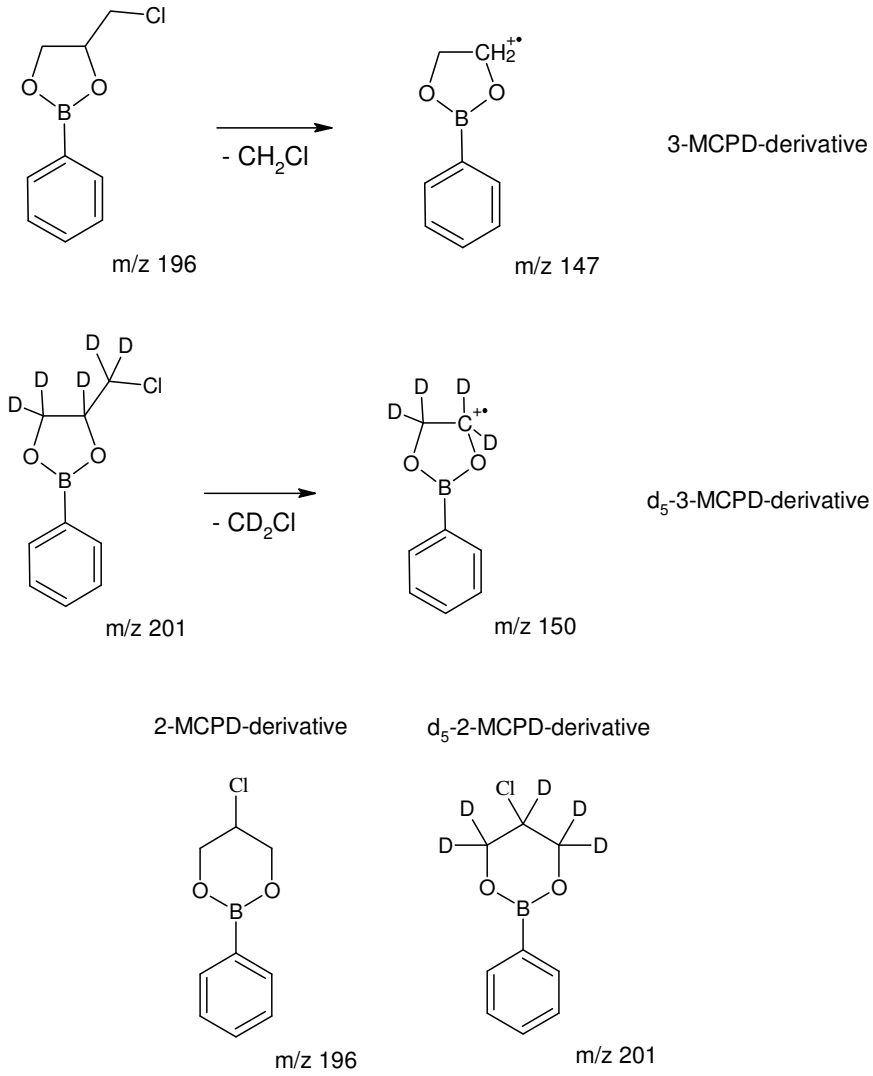

Figure 1: Selected fragment ions for the SIM method

15 Evaluation

15.1 GC/MS Evaluation

15.1.1 Response ratios and calibration function

Ion m/z 196 and ion m/z 147 shall be used as quantifying ions for 3-MCPD and ions m/z 201, 150 for the internal standard d_5-3-MCPD. For the isomer 2-MCPD shall be used ion m/z 196 and ion m/z 201 for the internal standard d_5-2-MCPD.

Based on the calibration standards, determine the areas of the quantifying ions of the phenylboronic acid derivatives of the 3-MCPD respectively 2-MCPD as well as d_5-3-MCPD respectively d_5-2-MCPD internal standard, and form the response ratios of analyte/internal standard.

$$R = \frac{A \quad (m/z \quad 196)}{A \quad (m/z \quad 201)} \qquad \text{respectively} \qquad R = \frac{A \quad (m/z \quad 147)}{A \quad (m/z \quad 150)}$$

R = Response ratio of standard/internal standard
A = Response area

To set up the calibration function, plot the response ratio of the 3-MCPD standard and the d_5-3-MCPD internal standard against the concentration of the 3-MCPD standard (µg). Calculate the calibration function by means of linear regression. Proceed in the same manner for the concentration of the 2-MCPD standard (µg).

$$R = a * m_{3-MCPD} + b$$

R = Response ratio of standard/internal standard
a = Slope of the regression line
b = Intercept of the regression line
$m_{3\text{-}MCPD}$ = Absolute amount of 3-MCPD respectively 2-MCPD (µg) in the derivatization preparation of the standard

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* Interference due to matrix and/or GC-MS properties (such as conditions of the column or of the ion source, altered by age or other) may cause faulty ion traces of the 3-MCPD phenylboronic acid derivatives. Therefore, it is recommended to determine the response ratio of the

ion traces 147 and 196. The response ratios should range between 4.5 and 5.8. Any outlying ratio suggests a disturbance.

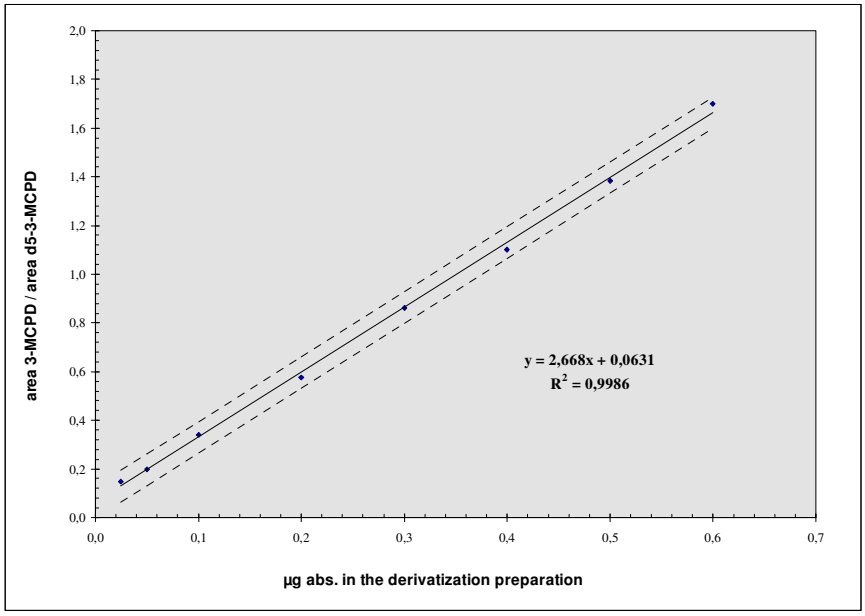


Figure 2: Model for a 3-MCPD calibration line

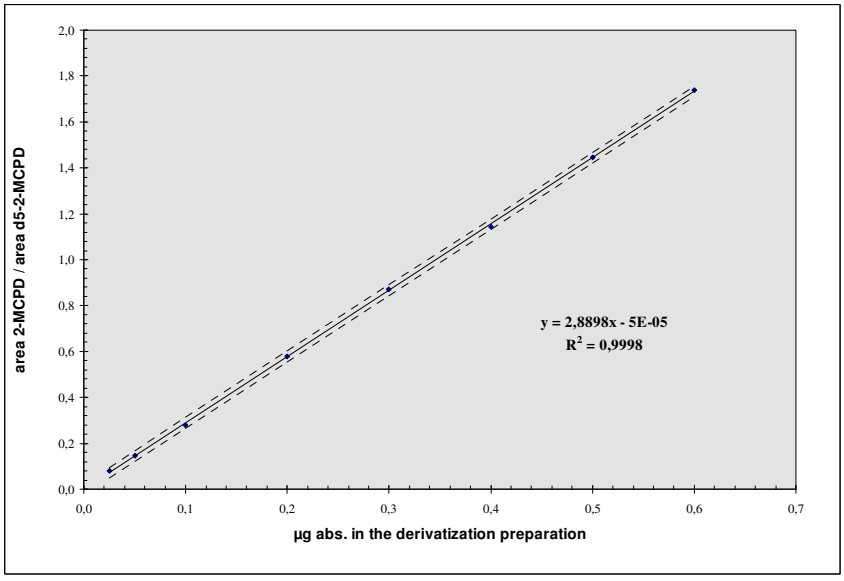


Figure 3: Model for a 2-MCPD calibration line

15.1.2 Calculation of the released 3-MCPD respectively 2-MCPD concentration in the derivatization preparation of the samples

The concentration of the sample is stated in µg as absolute amount of 3-MCPD respectively 2-MCPD in the derivatization solution.

$$m_{3MCPD} = \frac{(R_{\text{Probe}} - b)}{a}$$

$m_{3\text{-}MCPD}$ = Absolute amount of 3-MCPD respectively 2-MCPD (µg) in the derivatization preparation of the standard
R_{Probe} = Response ratio of analyte / internal standard determined in the derivatization preparation of the standard
a = Slope of the regression line
b = Intercept of the regression line

15.1.3 Calculation of the 3-MCPD and 2-MCPD concentrations in the sample (mg/kg)

$$\omega = \frac{m_{3-MCPD}}{m}$$

ω = 3-MCPD respectively 2-MCPD concentration stated in mg/kg
m = Sample amount stated in g

The concentration should be given with an accuracy of one significant digit.

16 Selected Chromatograms

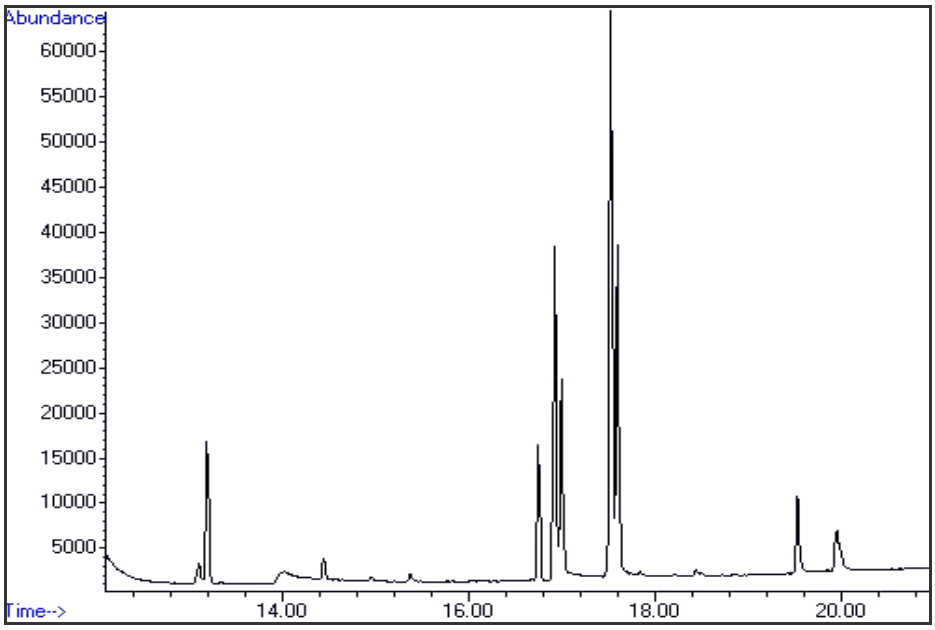


Figure 4: Scan chromatogram of a standard solution with 0.3 µg 3-MCPD / 0.3 µg d_5-3-MCPD as well as 0.3 µg 2-MCPD / 0.3 µg d_5-2-MCPD absolute in the derivatization preparation (3 mg/kg 3-MCPD /2-MCPD and 3 mg/kg d_5-3-MCPD / d_5-2-MCPD)

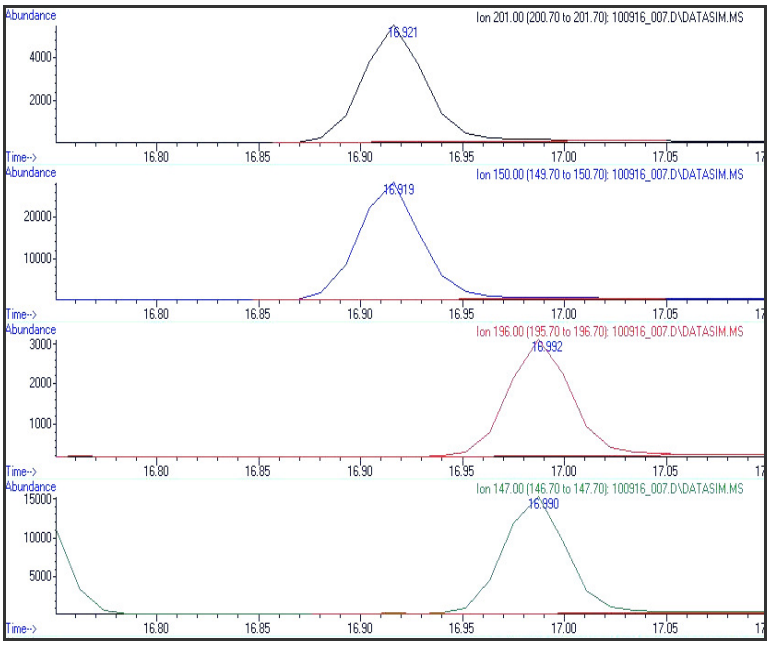


Figure 5: SIM ion chromatogram of a standard solution with 0.3 µg 3-MCPD and 0.3 µg d_5-3-MCPD absolute in the derivatization preparation (3 mg/kg 3-MCPD and 3 mg/kg d_5-3-MCPD)

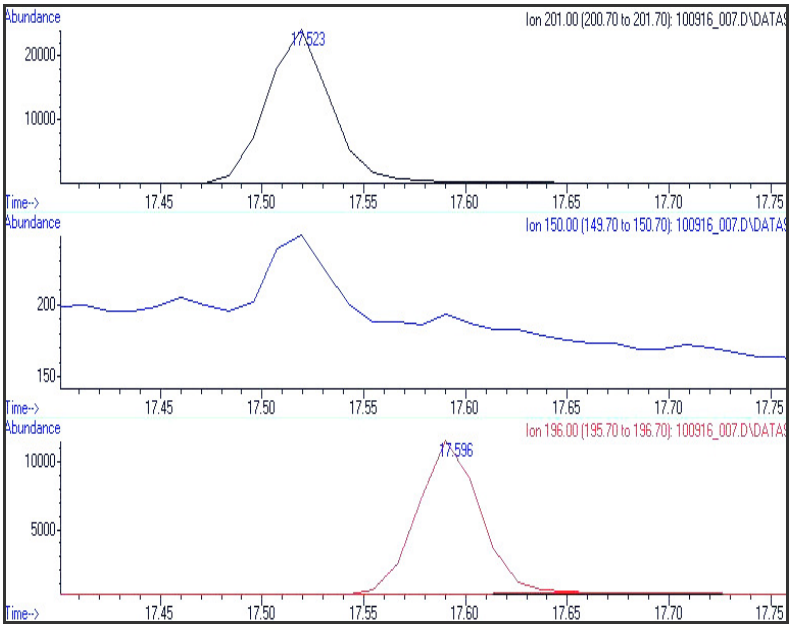


Figure 6: SIM ion chromatogram of a standard solution with 0.3 µg 2-MCPD und 0.3 µg d_5-2-MCPD absolute in the derivatization preparation (3 mg/kg 2-MCPD respectively. 3 mg/kg d_5-2-MCPD)

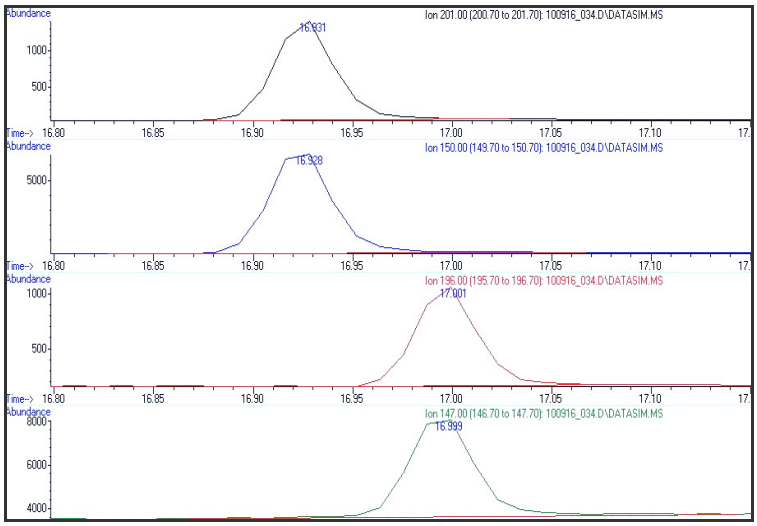


Figure 7: SIM ion chromatogram of a matrix sample (3 mg/kg 3-MCPD; spiked with 3 mg/kg d_5-3-MCPD)

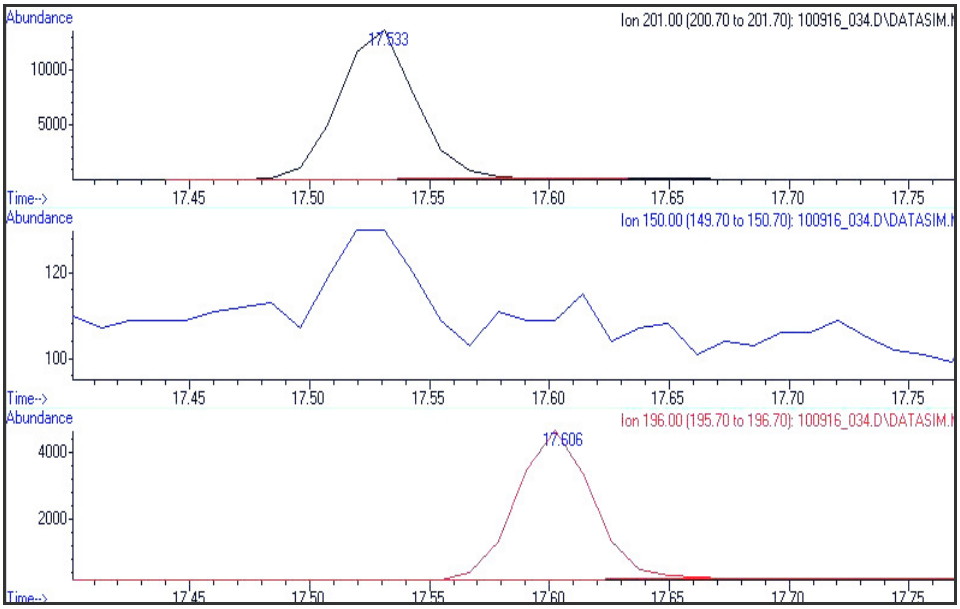


Figure 8: SIM ion chromatogram of a matrix sample (1.3 mg/kg 2-MCPD; spiked with 3 mg/kg d_5-2-MCPD)

17 Validation

17.1 Detection limit and quantification limit

Based on matrix calibration with **a)** spiking **before** fat extraction and **b)** spiking **after** fat extraction, the detection limit with an error probability of 5 % (α = 0.05) and the quantification limit with an uncertainty of measurement of 20 % (k = 2) were determined according to DIN 32645. For both analytes, evaluation was performed using the ion fragments m/z 196 (3-MCPD; 2-MCPD) and m/z 201 (d_5-3-MCPD; d_5-2-MCPD).

17.1.1 Matrix calibration with addition of analyte **before** accelerated solvent extraction (ASE)

Characteristic data for both, detection limit and quantification limit were obtained by spiking a blank sample: Analyte-free samples were spiked with calibration solutions of a 3-MCPD ester (1,2-bis-palmitol-3-monochloropropane-1,2-diol) as well as with 3-MCPD and 2-MCPD which had concentrations in the lower range of the detection limit (0.1 - 0.5 mg/kg). Equidistant spiking intervals were maintained. D_5-3-MCPD ester (d_5-1,2-bis-palmitol-3-monochloropropane-1,2-diol) as well as d_5-3-MCPD and d_5-2-MCPD at a concentration of 0.4 mg/kg were added as internal standard. Subsequently, the samples were subject to ASE (see Annex 1). 100 mg aliquots of the fat extracts were prepared as described in Annex 2.

The detection limit and quantification limit as well as the characteristic data of the linear regression are shown in Table 3.

Table 3: Detection limit and quantification limit according to DIN 32 645

Characteristic values	3-MCPD ester	3-MCPD	2-MCPD
Slope	15.142	25.666	27.992
y-intercept	- 0.005	0.118	- 0.090
Coefficient of determination (r)	0.9983	0.9967	0.9977
Detection limit (mg/kg); (α=0,05)	**0.03**	**0.09**	**0.03**
Quantification limit (mg/kg); (k = 2)	**0.05**	**0.13**	**0.06**

Results:

The limits determined according to DIN 32645 and calculated by means of a matrix calibration function with addition of analyte **before** fat extraction were found to be 0.03 mg/kg (detection limit) and 0.05 mg/kg (quantification limit) for 3-MCPD ester and 0.09 mg/kg (detection limit) and 0.13 mg/kg (quantification limit) for 3-MCPD.

For the 2-MCPD isomer, the detection limit was 0.03 mg/kg and the quantification limit was 0.06 mg/kg.

17.1.2 Matrix calibration with addition of analyte **after** ASE

Characteristic data for both, detection limit and quantification limit, were obtained by spiking a fat extract of a blank sample: An analyte-free sample was subject to ASE (see Annex 1) and fat extract portioned into 100 mg aliquots were spiked with calibration solutions of a 3-MCPD ester (1,2-bis-palmitol-3-monochloropropane-1,2-diol) as well as with 3-MCPD and 2-MCPD which had concentrations in the lower range of the detection limit (0.1 - 0.5 mg/kg). Equidistant spiking intervals were maintained. D_5-3-MCPD ester (d_5-1,2-bis-palmitol-3-monochloropropane-1,2-diol) as well as d_5-3-MCPD and d_5-2-MCPD at a concentration of 0.4 mg/kg were added as internal standard.

The detection limit and quantification limit as well as the characteristic data of the linear regression are shown in Table 4.

Table 4: Detection limit and quantification limit according to DIN 32 645

Characteristic values	3-MCPD ester	3-MCPD	2-MCPD
Slope	13.136	30.355	17.451
y-intercept	0.0533	0.3297	0.0120
Coefficient of determination (r)	0.9968	0.9977	0.9986
Detection limit (mg/kg); (α=0,05)	**0.04**	**0.05**	**0.02**
Quantification limit (mg/kg); (k = 2)	**0.07**	**0.10**	**0.05**

Results:

The limits determined according to DIN 32645 and calculated by means of a matrix calibration function with addition of analyte **after** fat extraction were found to be 0.04 mg/kg (detection limit) and 0.07 mg/kg (quantification limit) for 3-MCPD ester and 0.05 mg/kg (detection limit) and 0.10 mg/kg (quantification limit) for 3-MCPD.

For the 2-MCPD isomer, the detection limit was 0.02 mg/kg and the quantification limit was 0.05 mg/kg.

17.2 Determination of the recovery rate

In order to determine the recovery rate, an analyte-free sample was spiked with a 3-MCPD ester (1,2-bis-palmitol-3-monochloropropane-1,2-diol) at a concentration of 5.4 mg/kg fat before fat extraction. Assuming complete ester cleavage, this corresponds to 1 mg of free 3-MCPD/kg fat.

For the purpose of comparison, an analyte-free sample was spiked with 3-MCPD at a concentration of 1 mg/kg fat before fat extraction.

Since 2-MCPD ester was not available as a standard substance, an analyte-free sample was spiked with 2-MCPD at a concentration of 1 mg/kg fat before fat extraction.

100 mg aliquots of fat extract were prepared as shown in Annex 2.

The respective internal standards were added **a) before** fat extraction and **b) after** fat extraction**.**

Tables 5 to 10 show the mean values of recovery obtained for the respective concentrations. The recovery rate was determined (in %) as the ratio between the reference concentration and the concentration actually found.

Table 5: Recovery rate for 3-MCPD ester (samples were spiked before fat extraction)

Added 3-MCPD concentration	3-MCPD concentration	Mean value of recovery	Coefficient of variation (%)	Number of samples
5.40 mg/kg 3-MCPD ester	1.0 mg/kg	**114.1 %**	3.4	5

Table 6: Recovery rate for 3-MCPD ester (samples were spiked after fat extraction)

Added 3-MCPD concentration	3-MCPD concentration	Mean value of recovery	Coefficient of variation (%)	Number of samples
5.40 mg/kg 3-MCPD ester	1.0 mg/kg	**109.4 %**	4.8	5

Table 7: Recovery rate for 3-MCPD (samples were spiked before fat extraction)

Added 3-MCPD concentration	Mean value of recovery	Coefficient of variation (%)	Number of samples
1.00 mg/kg	**110.6 %**	2.3	5

Table 8: Recovery rate for 3-MCPD (samples were spiked after fat extraction)

Added 3-MCPD concentration	Mean value of recovery	Coefficient of variation (%)	Number of samples
1.00 mg/kg	**22.4 %**	4.8	5

Table 9: Recovery rate for 2-MCPD (samples were spiked before fat extraction)

Added 2-MCPD concentration	Mean value of recovery	Coefficient of variation (%)	Number of samples
1.00 mg/kg	**98.6 %**	2.5	6

Table 10: Recovery rate for 2-MCPD (samples were spiked after fat extraction)

Added 2-MCPD concentration	Mean value of recovery	Coefficient of variation (%)	Number of samples
1.00 mg/kg	**26.1 %**	6.0	6

Results:

A significant difference in their fat extraction behaviour was noticeable between free 3-MCPD respectively 2-MCPD and 3-MCPD ester in its bound form.

As to 3-MCPD ester, fat extraction entails a recovery loss of < 5 %, i.e., when applying ASE, 3-MCPD ester is completely extracted. Likewise could be confirmed that 3-MCPD ester is completely transesterified into free 3-MCPD during the process of alkaline saponification.

For MCPD in its free form, by contrast, the recovery loss following fat extraction with ASE is > 70 %. The extraction method does not seem to be appropriate for free MCPD. If d_5-3-MCPD or d_5-2-MCPD is used as internal standard, they may be added to the samples **after** fat extraction only.

Assuming that 2-MCPD esters show a similar behaviour as 3-MCPD esters, likewise a recovery rate of > 90 % should be obtained. Precise data cannot be provided, because standard substances for 2-MCPD esters have not been available commercially up to now.

17.3 Determination of the extraction yield with ASE

To determine the extraction efficiency, four different fat-containing foods were extracted repeatedly using ASE by the same technician (see Annex 1). The fat extracts were collected in vials, which had been previously calibrated; the solvent was evaporated at 40°C under a stream of nitrogen; the extraction yield was determined by weighing the vials again together with the extracts and by comparison with the fat content indicated on the labels of the corresponding food products.

Table 11: Extraction yield

					Mono-factorial analysis of variance, ANOVA (α = 0.05)		
Matrix	Numbers of determination	Mean value of the extraction yield (%)	Coefficient of variation (%)	Standard deviation	P-value	Test statistics (F)	crit. F-value
Strawberry spread	6	95.6	0.39	0.38	1.51	0.29	7.71
Chocolate spread	6	97.6	0.59	0.57	0.47	0.64	
Margarine	6	102.4	4.83	4.96	0.87	0.03	
Infant formula	6	96.3	0.26	0.25	0.36	1.05	

Results:

With respect to the parameter “extraction efficiency”, all samples were subject to mono-factorial analysis of variance. As a result, no significant differences were found (error probability of 5 %).

17.4 Laboratory precision under repeatability conditions and with change of technicians

In order to determine the method's precision, four fat-containing foods each with different 3-MCPD and 2-MCPD concentrations were subject to manifold analysis performed by two technicians.

The precision was determined first under repeatability conditions (same sample, same technician, and same laboratory apparatus); then the precision achieved with different technicians under repeatability conditions (same sample, same laboratory apparatus) was determined.

Tables 12 to 14 show the results of the analytical tests.

Table 12: Laboratory precision under repeatability conditions – and with change of technicians - using d_5-3-MCPD ester as internal standard

	Technician A				Technician B			
Matrix	Numbers of determination	Mean value of 3-MCPD (mg/kg)	Coefficient of variation (%)	Standard deviation (mg/kg)	Numbers of determination	Mean value of 3-MCPD (mg/kg)	Coefficient of variation (%)	Standard deviation (mg/kg)
Strawberry spread	6	**6.76**	2.7	0.18	6	**6.88**	2.2	0.15
Chocolate spread	6	**1.68**	3.3	0.06	5	**1.62**	3.9	0.06
Margarine	6	**1.59**	2.6	0.04	6	**1.39**	3.5	0.05
Infant formula	5	**0.68**	2.1	0.02	5	**0.65**	2.4	0.02

Table 13: Laboratory precision under repeatability conditions – and with change of technicians - using d_5-3-MCPD as internal standard

Matrix	Numbers of determination	Mean value of 3-MCPD (mg/kg)	Coefficient of variation (%)	Standard deviation (mg/kg)
Strawberry spread	5	**6.80**	2.0	0.12
Chocolate spread	5	**1.70**	3.8	0.03
Margarine	5	**1.27**	5.0	0.04
Infant formula	5	**0.69**	4.6	0.04

Table 14: Laboratory precision under repeatability conditions – and with change of technicians - using d_5-2-MCPD as internal standard

	Technician A				Technician B			
Matrix	Numbers of determination	Mean value of 2-MCPD (mg/kg)	Coefficient of variation (%)	Standard deviation (mg/kg)	Numbers of determination	Mean value of 2-MCPD (mg/kg)	Coefficient of variation (%)	Standard deviation (mg/kg)
Strawberry spread	6	**1.29**	2.8	0.04	6	**1.21**	2.4	0.03
Chocolate spread	5	**1.05**	3.6	0.04	5	**1.01**	1.3	0.01
Margarine	5	**0.50**	3.5	0.02	5	**0.46**	3.8	0.02
Infant formula	6	**0.28**	2.1	0.01	5	**0.26**	2.8	0.01

Results:

In the lower as well as in the upper concentration range, repeatability testing showed a satisfactory precision with a coefficient of variation of < 4 % for both 3-MCPD and 2-MCPD.

18 Annex

18.1 Annex 1

flow chart – fat extraction with ASE
annex 1 to Method_FC-022-01

Sample preparation

weighing 1,5-2,5 g sample in a beaker and homogenize with 4 g Isolute

load homogenized sample into a 22 ml ASE cell

weigh the empty ASE vial and record the weigh

Weighing according to fat content (**fc**):

2,5 g at **fc < 30 %**
2,0 g at **fc ~ 30-60 %**
1,5 g at **fc > 60 %**

Extraction parameter ASE

Extraction solvent	Petroleum ether / Isohexane / Acetone (2+2+1, v/v/v)
Extraction temperature	125 °C
Heat time	6 min
Static time	5 min
Flush volume	100 %
Flush time	60 sec
Cycles	2
Pressure	103,4 bar

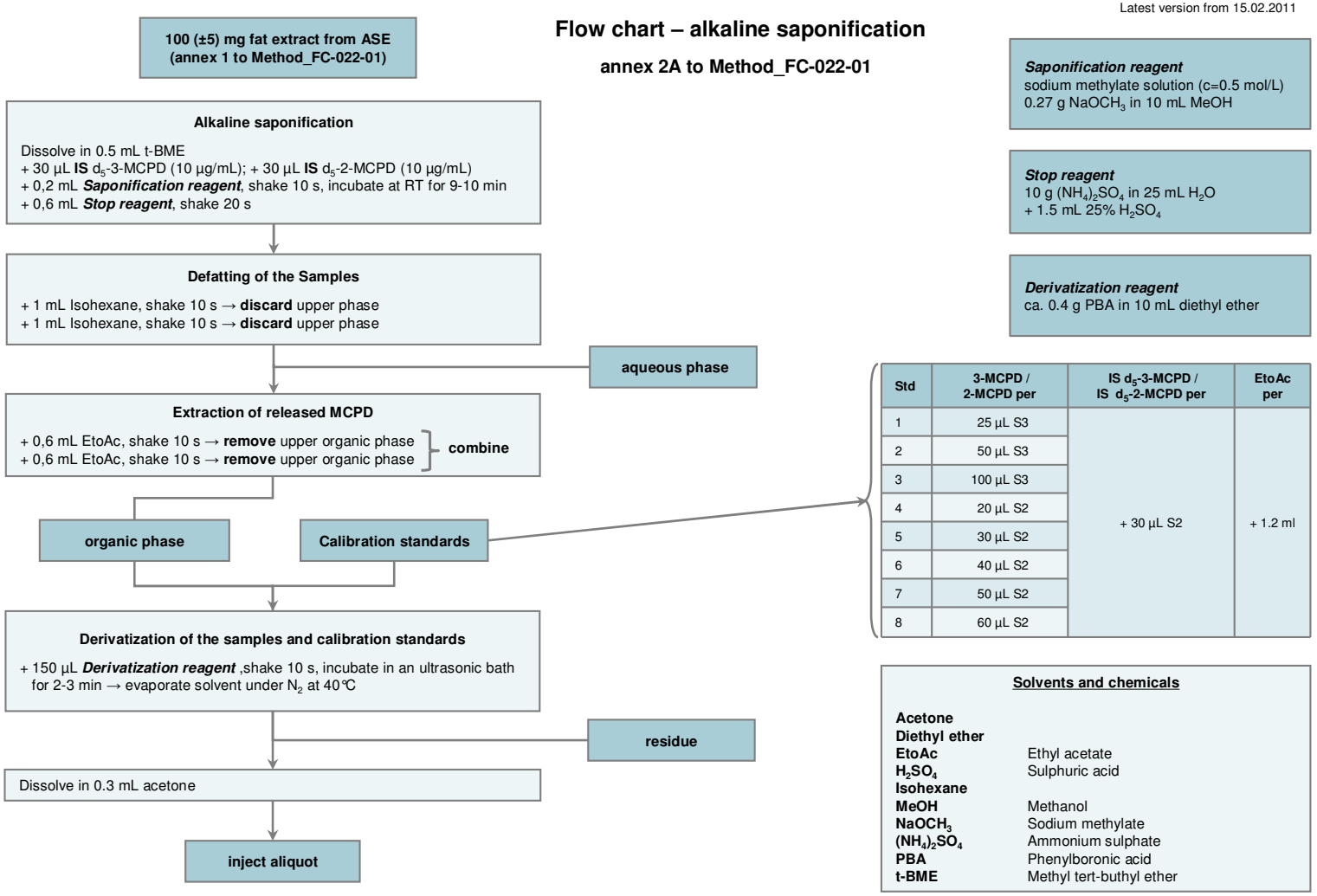
evaporate solvent under N_2 at 40°C

weigh again the ASE vials, record the weight and define the extraction yield for fat

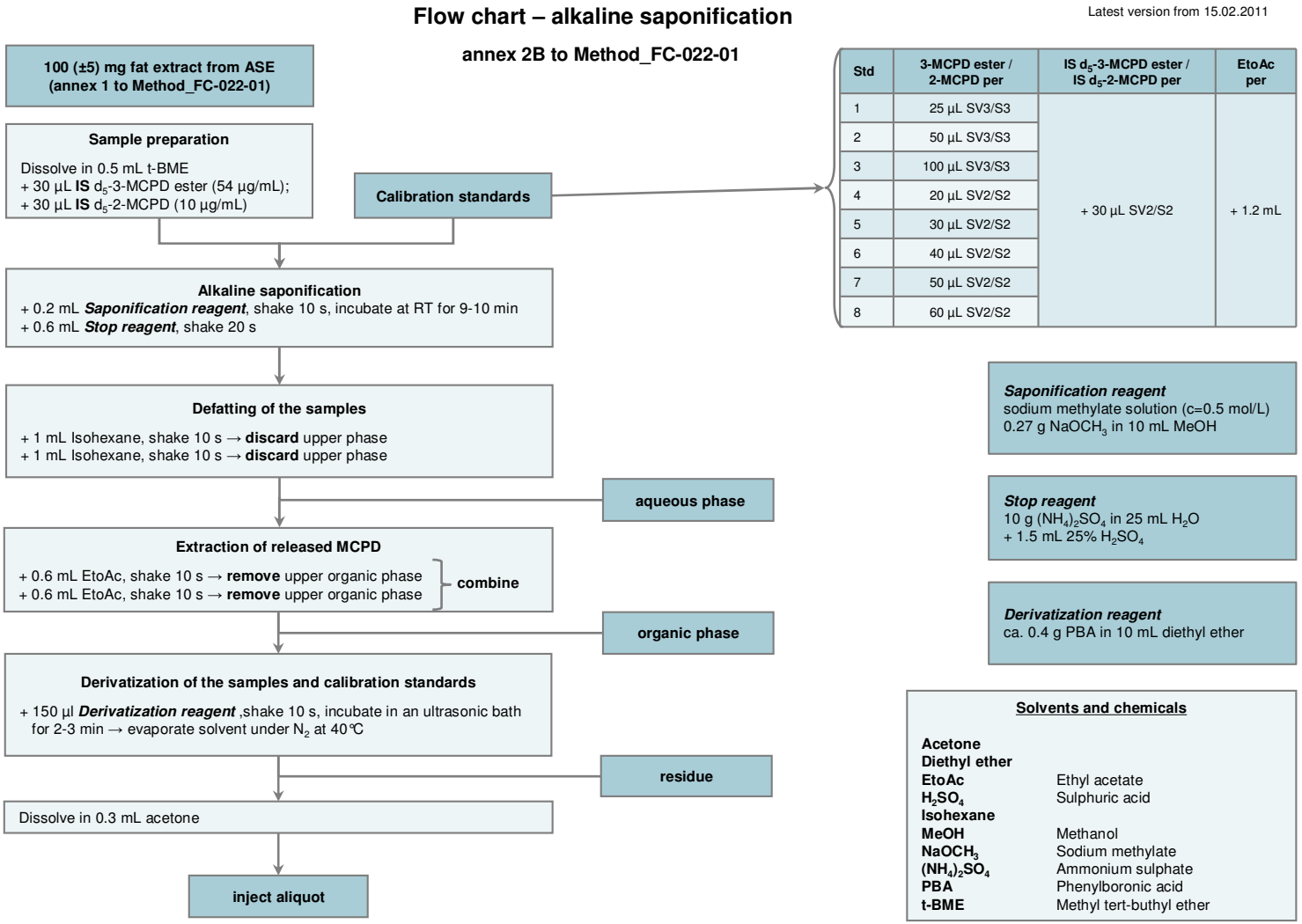
100 mg aliquot of the fat extract was analyzed to **annex 2A or 2B (Method_FC-022-01)**

Latest version from 15.02.2011

18.2 Annex 2A


Latest version from 15.02.2011
Flow chart – alkaline saponification
annex 2A to Method_FC-022-01
100 (±5) mg fat extract from ASE (annex 1 to Method_FC-022-01)
Alkaline saponification
Dissolve in 0.5 mL t-BME
+ 30 µL IS d5-3-MCPD (10 µg/mL); + 30 µL IS d5-2-MCPD (10 µg/mL)
+ 0,2 mL Saponification reagent, shake 10 s, incubate at RT for 9-10 min
+ 0,6 mL Stop reagent, shake 20 s
Defatting of the Samples
+ 1 mL Isohexane, shake 10 s → discard upper phase
+ 1 mL Isohexane, shake 10 s → discard upper phase
aqueous phase
Extraction of released MCPD
+ 0,6 mL EtoAc, shake 10 s → remove upper organic phase
+ 0,6 mL EtoAc, shake 10 s → remove upper organic phase
combine
organic phase
Calibration standards
Derivatization of the samples and calibration standards
+ 150 µL Derivatization reagent ,shake 10 s, incubate in an ultrasonic bath for 2-3 min → evaporate solvent under N2 at 40°C
residue
Dissolve in 0.3 mL acetone
inject aliquot
Saponification reagent
sodium methylate solution (c=0.5 mol/L)
0.27 g NaOCH3 in 10 mL MeOH
Stop reagent
10 g (NH4)2SO4 in 25 mL H2O
+ 1.5 mL 25% H2SO4
Derivatization reagent
ca. 0.4 g PBA in 10 mL diethyl ether
Std | 3-MCPD / 2-MCPD per | IS d5-3-MCPD / IS d5-2-MCPD per | EtoAc per
1 | 25 µL S3 | + 30 µL S2 | + 1.2 ml
2 | 50 µL S3
3 | 100 µL S3
4 | 20 µL S2
5 | 30 µL S2
6 | 40 µL S2
7 | 50 µL S2
8 | 60 µL S2
Solvents and chemicals
Acetone
Diethyl ether
EtoAc Ethyl acetate
H2SO4 Sulphuric acid
Isohexane
MeOH Methanol
NaOCH3 Sodium methylate
(NH4)2SO4 Ammonium sulphate
PBA Phenylboronic acid
t-BME Methyl tert-buthyl ether

18.3 Annex 2B


Flow chart – alkaline saponification
annex 2B to Method_FC-022-01
Latest version from 15.02.2011
100 (±5) mg fat extract from ASE (annex 1 to Method_FC-022-01)
Sample preparation
Dissolve in 0.5 mL t-BME
+ 30 µL IS d5-3-MCPD ester (54 µg/mL);
+ 30 µL IS d5-2-MCPD (10 µg/mL)
Calibration standards
Alkaline saponification
+ 0.2 mL Saponification reagent, shake 10 s, incubate at RT for 9-10 min
+ 0.6 mL Stop reagent, shake 20 s
Defatting of the samples
+ 1 mL Isohexane, shake 10 s → discard upper phase
+ 1 mL Isohexane, shake 10 s → discard upper phase
aqueous phase
Extraction of released MCPD
+ 0.6 mL EtoAc, shake 10 s → remove upper organic phase
+ 0.6 mL EtoAc, shake 10 s → remove upper organic phase
combine
organic phase
Derivatization of the samples and calibration standards
+ 150 µl Derivatization reagent ,shake 10 s, incubate in an ultrasonic bath for 2-3 min → evaporate solvent under N2 at 40°C
residue
Dissolve in 0.3 mL acetone
inject aliquot
Std
3-MCPD ester / 2-MCPD per
IS d5-3-MCPD ester / IS d5-2-MCPD per
EtoAc per
1
25 µL SV3/S3
2
50 µL SV3/S3
3
100 µL SV3/S3
4
20 µL SV2/S2
5
30 µL SV2/S2
6
40 µL SV2/S2
7
50 µL SV2/S2
8
60 µL SV2/S2
+ 30 µL SV2/S2
+ 1.2 mL
Saponification reagent
sodium methylate solution (c=0.5 mol/L)
0.27 g NaOCH3 in 10 mL MeOH
Stop reagent
10 g (NH4)2SO4 in 25 mL H2O
+ 1.5 mL 25% H2SO4
Derivatization reagent
ca. 0.4 g PBA in 10 mL diethyl ether
Solvents and chemicals
Acetone
Diethyl ether
EtoAc Ethyl acetate
H2SO4 Sulphuric acid
Isohexane
MeOH Methanol
NaOCH3 Sodium methylate
(NH4)2SO4 Ammonium sulphate
PBA Phenylboronic acid
t-BME Methyl tert-buthyl ether

BfR Method-23

Determination of 3-MCPD- and 2-MCPD-Fatty Acid Esters in fat-containing foods with GC/MS

Fat extraction with **ASE**
An indirect Determination by Detection of free 3-MCPD and 2-MCPD released from MCPD-esters by **Acidic Saponification**
Derivatization by **Phenylboronic Acid**

NOTE: Potential modifications of this method will be published on the homepage of the Federal Institute of Risk Assessment (BfR).

The number of participants in the method validation study using this method was not sufficient to comply with the requirement. Therefore, the status of this method remained as “test-method” (in-house validated method).

1 Scope of Application

This method describes the determination of ester-bound 3-chloropropane-1,2-diol (3-MCPD-fatty acid esters) and 2-chloropropane-1,3-diol (2-MCPD-fatty acid esters) in fat containing food by means of gas chromatography-mass spectrometry.

2 Principle of Method

To analyses MCPD-fatty acid esters in fat containing foods in a first step the samples must be extracted. Therefore accelerated solvent extraction (ASE) with a solvent mixture of petroleum ether / isohexane / acetone (2/2/1; v/v) in two extraction cycles and a temperature of 125°C is used. After extraction solvent of the ASE extract is dried under a stream of nitrogen, and the remaining fat residue is weighed to consistency and the extraction yield determined.

An appropriate amount of fat extract (100 - 200 mg) is weighed out and dissolved in t-BME and an internal standard (d_5-labeled 3-MCPD or d_5-labeled 3-MCPD ester, d_5-labeled 2-MCPD) is added. Cleavage of the ester bond is performed by acidic hydrolysis with a solution of sulphuric acid and methanol; as a result fatty acid methyl esters and free 3-MCPD respectively 2-MCPD are formed. The reaction is stopped with a solution of sodium hydrogen carbonate. The sample is defatted with isohexane, derivatized with phenylboronic acid. Subsequently the MCPD-derivative is extracted with cyclohexane and the extract evaporated to complete dryness. The residue is dissolved in isooctane and an aliquot is taken for analysis by GC-MS.

Warning and Safety Precautions

- When handling acids, bases, organic solvents and standard substances (pure substances and solutions) gloves must be used. Use solvents in places provided with a fume hood.
- Attention is drawn to the information contained in the Safety Data Sheet (SDS) and the regulations which specify the handling of reagents and solvents.
- All crucial steps in this method are marked by ***Note.***

3 Reagents and Products

Note: *The names of* manufacturers *have been mentioned for information purposes only.*

3.1 Reference Substances

3.1.1	3-MCPD	3-chloropropane-1,2-diol (e.g. FLUKA)
3.1.2	d_5-3-MCPD	fivefold deuterated 3-chloropropane-1,2-diol (e.g. CIL)
3.1.3	2-MCPD	2-chloropropane-1,3-diol (e.g. Toronto Research Chemicals)
3.1.4	d_5-2-MCPD	fivefold deuterated 2-chloropropane-1,3-diol (e.g. TRC)
3.1.5	3-MCPD ester	1,2-bis-palmitoyl-3-chloropropane-1,2-diol (e.g. TRC)
3.1.6	d_5-3-MCPD ester	fivefold deuterated 1,2-bis-palmitoyl-3-chloropropane-1,2-diol (e.g. TRC)

All 3-MCPD and 2-MCPD stock solutions are prepared in methanol; 3-MCPD-ester stock solutions are prepared in ethyl acetate and stored at +6 (±4) °C in the dark.

The working solutions of standard substances are prepared by diluting the stock solutions with methanol respectively ethyl acetate and are stored in the same way.

The working solutions of internal standards are prepared by diluting the stock solutions with methanol respectively ethyl acetate and are stored in the same way.

3.1.7 Stock Solutions:

3.1.7.1 3-MCPD stock solution (S0-solution):

Weigh 10 (±0.1) mg standard substance (3.1.1) into a 10 mL volumetric flask (4.8) and fill up to the mark by adding methanol (3.2.11) (c = 1 mg/mL).

3.1.7.2 d_5-3-MCPD stock solution (S0-solution):

Weigh 100 (±0.1) mg standard substance (3.1.2) into a 100 mL volumetric flask (4.8) and fill up to the mark by adding methanol (3.2.11) (c = 1 mg/mL).

3.1.7.3 2-MCPD stock solution (S0-solution):

Weigh 25 (±0.1) mg standard substance (3.1.3) into a 25 mL volumetric flask (4.8) and fill up to the mark by adding methanol (3.2.11) (c = 1 mg/mL).

3.1.7.4 d_5-2-MCPD stock solution (S0-solution):

Weigh 100 (±0.1) mg standard substance (3.1.4) into a 100 mL volumetric flask (4.8) and fill up to the mark by adding methanol (3.2.11) (c = 1 mg/mL).

3.1.7.5 3-MCPD ester stock solution (SV0-solution)

Weigh 10.8 (±0.1) mg standard substance (3.1.5) into a 2 mL volumetric flask (4.8) and fill up to the mark by adding ethyl acetate (3.2.7) (c = 5.4 mg/mL).

3.1.7.6 d_5-3-MCPD ester stock solution (SV0-solution)

Weigh 10.8 (±0.1) mg standard substance (3.1.6) into a 2 mL volumetric flask (4.8) and fill up to the mark by adding ethyl acetate (3.2.7) (c = 5.4 mg/mL)

3.1.8 3-MCPD standard solution for the calibration function

3.1.8.1 S2 standard solution:

Pipet 100 µL stock solution S0 (3.1.7.1) into a 10 mL volumetric flask (4.8) and fill up to the mark by adding methanol (3.2.11) (c = 10 µg/mL).

3.1.8.2 S3 standard solution:

Pipet 1 mL S2-standard solution (3.1.8.1) into a 10 mL volumetric flask (4.8) and fill up to the mark by adding methanol (3.2.11) (c = 1 µg/mL).

3.1.9 2-MCPD standard solution for the calibration function

3.1.9.1 S2 standard solution:

Pipet 100 µL stock solution S0 (3.1.7.3) into a 10 mL volumetric flask (4.8) and fill up to the mark by adding methanol (3.2.11) (c = 10 µg/mL).

3.1.9.2 S3 standard solution:

Pipet 1 mL S2-standard solution (3.1.9.1) into a 10 mL volumetric flask (4.8) and fill up to the mark by adding methanol (3.2.11) (c = 1 µg/mL).

3.1.10 3-MCPD ester standard solution for the calibration function

3.1.10.1 SV2 standard solution:

Pipet 100 µL stock solution SV0 (3.1.7.5) into a 10 mL volumetric flask (4.8) and fill up to the mark by adding ethyl acetate (3.2.7) (c = 54 µg/mL).

3.1.10.2 SV3 standard solution:

Pipet 1 mL SV2-standard solution (3.1.10.1) into a 10 mL volumetric flask (4.8) and fill up to the mark by adding ethyl acetate (3.2.7) (c = 5.4 µg/mL).

3.1.11 d_5-3-MCPD working solution as internal standard solution

3.1.11.1 S2 working solution d5-3-MCPD:

Pipet 1 mL stock solution S0 (3.1.7.2) into a 100 mL volumetric flask (4.8) and fill up to the mark by adding methanol (3.2.11) (c = 10 µg/mL).

3.1.12 d_5-2-MCPD working solution as internal standard solution

3.1.12.1 S2 working solution d5-2-MCPD:

Pipet 1 mL stock solution S0 (3.1.7.4) into a 100 mL volumetric flask (4.8) and fill up to the mark by adding methanol (3.2.11) (c = 10 µg/mL).

3.1.13 d_5-3-MCPD ester working solution as internal standard solution

3.1.13.1 SV2 working solution d5-3-MCPD ester:

Pipet 100 µL stock solution SV0 (3.1.7.6) into a 10 mL volumetric flask (4.8) and fill up to the mark by adding ethyl acetate (3.2.7) (c = 54 µg/mL).

3.2 Reagents

If not otherwise specified, all reagents shall have at least p.a. quality.

3.2.1 Acetone, for residue analysis (e.g. Merck 100012)

3.2.2 Ammonium sulphate (e.g. Merck 101217)

3.2.3 Cyclohexane, for GC (e.g. Merck 102817)

3.2.4 Distilled water

3.2.5 Ethanol absolute (e.g. Merck 100983)

3.2.6 Ethyl acetate (e.g. Promochem SO1191)

3.2.7 Isohexane, for residue analysis (e.g. Promochem SO1251)

3.2.8 Isolute HM-N (e.g. I.S.T. 9800-1000)

3.2.9 Isooctane, for GC (e.g. Merck 115440)

3.2.10 Methanol (e.g. Merck 106035)

3.2.11 Methyl tert-buthyl ether (t-BME), for GC (e.g. Merck 101995)

3.2.12 Petroleum ether (e.g. Promochem)

3.2.13 Phenylboronic acid > 95 %, for residue analysis (e.g. Fluka 78181)

3.2.14 Sodium hydrogen carbonate (e.g. Merck 106329)

3.2.15 Sulphuric acid 98 % (e.g. Merck 112080)

3.3 Solutions

3.3.1 Extracting reagent:

Prepare a mixture of 2:2:1 (v/v) petroleum ether (3.2.12), isohexane (3.2.7) und acetone (3.2.1) in a pressure resistant bottle.

3.3.2 Saponification reagent (0.3 M):

Dilute 1.8 mL sulphuric acid (3.2.15) in 100 mL methanol (3.2.9).

3.3.3 Stop reagent

Dissolve ca. 4.8 g sodium hydrogen carbonate (3.2.14) in 50 mL distilled water (3.2.4).

3.3.4 Derivatization reagent:

Dissolve 2.5 (±0.1) g phenylboronic acid (3.2.13) in a mixture of 19 mL acetone (3.2.1) and 1 mL distilled water (3.2.4).

3.3.5 Ammonium sulphate solution:

Dissolve 20 (±1) g ammonium sulphate (3.2.2) in 50 mL distilled water (3.2.4).

Note: *For the* preparation *of the solution 3.3.3 to 3.3.5 the use of an ultrasonic bath (4.21) is recommend to facilitate dissolution.*

3.4 Gases

3.4.1 Helium (5.0) (e.g. Air Liquide)

3.4.2 Nitrogen (5.0) (e.g. Air Liquide)

4 Apparatus

Note: *The names of* manufacturers *have been mentioned for information purposes only.*

4.1 11 mm crimp caps with PTFE/silicone/PTFE septa (e.g. Agilent 5181-1211)

4.2 2 ml crimp vials, clear glass (e.g. Agilent 5181-3375) with glass inserts (0.1 ml)

4.3 Accelerated Solvent Extractor ASE 200 with solvent reservoir and compressor modell 6-4 (e.g. Dionex)

4.4 Analytical balance

4.5 ASE 22 mL extraction cell with cell caps (e.g. Dionex)

4.6 ASE vials 60 mL and screw cap with septa (e.g. I-Chem)

4.7 Beaker

4.8 Calibrated volumetric flasks in various volume range

4.9 Cellulose filters for ASE cells d=1.983 cm (e.g. Dionex)

4.10 Centrifuge with cooler (e.g. Thermo Scientific RT7 and Rotor RTH 750)

4.11 Displacement or single channel pipettes

4.12 Drying oven

4.13 Evaporator (e.g. Barkey)

4.14 Manual crimper for 11 mm crimps caps (e.g. Chromacol CR-11)

4.15 Micro tips

4.16 Overhead shaker (e.g. Heidolph, Reax-2) or

4.17 Test tubs shaker (e.g. Heidolph, Multi Reax)

4.18 Pasteur pipettes

4.19 Polypropylene centrifuge test tubes 15 mL with screw cap (e.g. VWR)

4.20 Thick-walled test tubes ca. 5 mL (e.g. Hecht Assistent 75x12 mm, No.2775/6)

4.21 Ultrasonic bath

4.22 Vortex test tube shaker (e.g. Scientific Industries)

5 GC-MS System

5.1 Automatic Sampler

5.2 Capillary column (e.g. Agilent DB-5MS, 30 m x 0.25 mm, 0.25 µm)

5.3 Capillary gas chromatograph with an integrated programmable column oven providing a temperature up to at least 300°C (e.g. Agilent – 6890 / 7890A)

5.4 Glass liner, single taper, 4 mm ID, QW (e.g. Agilent 19251-60540)

5.5 Mass selective detector (e.g. Agilent MSD 5973 or 5975C) with ion source for electron-impact ionization

5.6 Pre-column (e.g. Phenomenex, Fused Silica, deaktiviert, 5 m x 0.32 mm)

5.7 Split/split less injector (e.g. Agilent)

6 Sample and Sampling

6.1 Lab samples

6.1.1 Sufficient sample amount should be provided to allow for triple determination at least.

6.1.2 Unequivocal identification of samples must be ensured throughout the process of sampling and sample packaging.

6.1.3 Please provide for proper packaging, preservation and transport of samples in order to ensure the good condition of the samples so that the analytical results are not affected. Store the samples at +6 (±4) °C in the dark.

6.1.4 Sufficient sample amount must be provided in order to ensure homogeneity.

6.2 Test samples

6.2.1 Blank sample

6.2.2 Reference sample

7 Procedure

7.1 Sample preparation

7.1.1 According to the fat content weigh (fc) 2.5 g (fc<30%), 2 g (fc 30-60%) or 1.5 g (fc>60%) sample by means of an analytical balance (10.4) into a beaker (10.7).

7.1.2 Add 4 g dispersing agent such as Isolute (3.2.8) to the sample and homogenize.

Note: *If problems occur during the fat extraction of infant formula additionally add 0.4 mL water (3.2.4) per* gram *sample to dispersing agent before homogenization.*

Therefore weigh 4 g Isolute into a beaker and mix with water. Subsequently add the infant formula and homogenize with the dispersing agent.

7.1.3 A disposable filter (4.9) is installed in the cell before the sample is loaded. The filter prevents blockage of the stainless steel frit in the bottom cap.

7.1.4 Unscrew the top cap from the cell body and place the filter in the cell at a slight angle. Position the insertion tool over the filter and slowly push the insertion tool into the cell. Make sure the filter is in full contact with the cell!

Note: *Always* hand-*tighten the bottom cell cap onto the cell body before installing the filter.*

Do not *place the filter in the bottom cap before installing the cap.*

7.1.5 Load the homogenized sample into a 22 mL cell (4.5).

Note: *Being careful to keep the threads clean.*

Take up fat residues *from the beaker with a tissue and put it inside the cell for extraction.*

If desired, fill any void volume in the cell with an inert material such as sea sand. This reduces the amount of solvent needed during the extraction.

7.1.6 Screw the top cap onto the cell body and **hand-tighten**.

Note: ***Do not use*** *a* wrench *or other tool to tighten the cap! This can damage the cell.*

7.1.7 Before starting the fat extraction weigh the empty ASE vials (4.6) and **record the weight**.

7.2 Fat extraction with ASE

7.2.1 Turn on the ASE extractor (4.3).

Note: *Additionally the* nitrogen *gas cylinder (3.4.2) must be open and turn on the compressor.*

7.2.2 Loading filled cells (4.5) into the upper tray slot in numerical order. Hang the cell vertically in the tray slots from their top caps.

7.2.3 Place the 60 mL vials (4.6) into the lower tray slot in numerical order.

7.2.4 Extraction parameter for ASE:

Solvent	petroleum ether (3.2.12) / isohexane (3.2.7) / acetone (3.2.1) (2:2:1, v/v)
Temperature [°C]	125
Pressure [bar]	103.4
Heat time [min]	6
Static time [min]	5
Flush volume [%]	100
Purge time [sec]	60
Cycles	2

Note: *Pressure was defined automatic by the Extractor.*

7.2.5 After the sequence, solvent of the extract is completely dried under a stream of nitrogen at 40°C.

7.2.6 Weigh again the ASE vials, **record the weight** and calculate the extraction yield for fat.

Note: *If only a non-heatable evaporator is available, evaporation of the solvent can take place at room temperature.*

The fat extracts *can dried additionally in a drying oven (4.12) at 70°C-100°C.*

7.2.7 After use, empty the cells and rinse the cell caps and cell bodies with water or organic solvent (for example ethanol or methanol). Place the pre-cleaned cell caps and cell bodies in a beaker witch is filled with an organic solvent and sonicate (4.21) for 15 min.

Note: *At regular* intervals *remove the snap ring from the cell cap, remove the cap inserts and clean separately.*

Also replacing *the cell PEEK Seal at regular intervals!*

7.3 Reprocessing the fat extracts

7.3.1 Prior to weighing, bring the fat extracts to room temperature.

7.3.2 Weighing the samples:

Weigh 100 (± 5) mg sample by means of an analytical balance (4.4) into a test tube (4.19) and **record the weight**.

Note: *The sample amount may be raised up to 200 mg, if the 3-MCPD respectively 2-MCPD* concentrations *are expected to be low (< 0.5 mg/kg). Higher sample amounts are not tested.*

7.3.3 Dissolution of samples:

Dissolve the samples in 0.5 mL t-BME (3.2.11). The use of a Vortex (4.22) for 20 s proved to be suitable for this purpose.

Note: *In case the sample has already solidified by then, dip the test tube into a hot water bath* for *a moment and re-dissolve. The water temperature required depends on the melting point of the solid fat.*

7.3.4 Add 30 µL of the internal standard **d_5-3-MCPD** work solution (3.1.11.1) **(Analysis according to annex 2A) or** add 30 µL of the internal standard **d_5-3-MCPD ester** work solution (3.1.13.1) **(Analysis according to annex 2B)**.

Note: *If d_5-3-*MCPD *ester is used as internal standard it is necessary to prepare the calibration standards at the same way as the samples (7.3.3).*

7.3.5 Add 30 µL of the internal standard **d_5-2-MCPD** work solution (3.1.12.1).

Note: *Up to now no 2-MCPD fatty acid esters or deuterated 2-MCPD fatty acid esters are available.*

7.4 3-MCPD ester and 2-MCPD calibration standards (Analysis according to annex 2B)

7.4.1 Preparation of the calibration standards in test tubes (4.19)

In succession pipet 30 µL, respectively, of d_5-3-MCPD ester working solution (3.1.13.1) d_5-2-MCPD working solution (3.1.12.1) into a test tube and add in the same way the respective volumes of the 3-MCPD ester standard solution SV2 (3.1.10.1), SV3 (3.1.10.2) and the 2-MCPD-standard solution S2 (3.1.9.1), S3 (3.1.9.2) (see Table 1).

Then add 0.5 mL each of t-BME (3.2.11) to all the standards.

Table 1: Pipetting scheme for calibration standards for acidic saponification

Standard	IS d_5-3-MCPD-ester (3.1.13.1) and d_5-2-MCPD (3.1.12.1)	3-MCPD-Ester (3.1.10.1)/(3.1.10.2) and 2-MCPD (3.1.9.1)/(3.1.9.2)	t-BME (3.2.11)	IS d_5-3-MCPD and d_5-2-MCPD	3-MCPD/2-MCPD absolute [µg] in the derivatization preparation	3-MCPD/2-MCPD [mg/kg] per 100 mg sample amount
1	30 µL SV2/S2	25 µL SV3/S3	0.5 mL	0.3 µg abs. in the derivatization preparation or 3 mg/kg sample	0.025	0.25
2		50 µL SV3/S3			0.05	0.5
3		100 µL SV3/S3			0.1	1.0
4		20 µL SV2/S2			0.2	2.0
5		30 µL SV2/S2			0.3	3.0
6		40 µL SV2/S2			0.4	4.0
7		50 µL SV2/S2			0.5	5.0
8		60 µL SV2/S2			0.6	6.0

7.5 Acidic saponification and subsequent defatting

The ester cleavage is achieved with a solution of 0.3 M sulphuric acid in methanol.

After adding **1.8 mL** saponification reagent (3.3.2), shake the sample and calibration standards (7.4) for 10 s (Vortex (4.22)).

Then cap the test tubes and shake the samples in a test tube shaker (4.16 or 4.17) for **2 hours**.

Note: *Since the two phases segregate immediately, this step is necessary in order to avoid under-rating results.*

After this, incubate the sample **at 40°C** for at least **16 hours** in the drying oven (4.12) (possible up to 20 h). Close the centrifuge tubes tightly (by using glass or plastic plugs for example).

Note: *Attention, formation of foam; add slowly with the necessary caution.*

After the incubation period, stop the reaction by adding **0.5 mL** stop reagent (3.3.3) and by shaking carefully at low rotation speed for 20 s (Vortex 4.22).

Samples are defatted by adding 1 ml isohexane (3.2.7) and shaking (10 s Vortex (4.22)). The **upper phase** is discarded.

Note: Subsequent *defatting is **not applicable** by the standards (7.4).*

n-hexane may *be used instead of isohexane for defatting.*

If necessary centrifuge the samples at 207 x g for 2 min at room temperature (10.10) to improve the phase separation.

Repeat defatting of the aqueous phase by adding 1 mL isohexane (3.2.7) and shaking (10 s Vortex (4.22)). The **upper phase** is discarded.

Note: *If necessary centrifuge the samples at 207 x g for 2 min at room temperature (4.10) to* improve *the phase separation.*

7.6 3-MCPD and 2-MCPD calibration standards (Analysis according to annex 2A)

7.6.1 Preparation of the calibration standards in test tubes (4.20):

In succession pipet 30 µL, respectively, of d_5-3-MCPD working solution (3.1.11.1) and of d_5-2-MCPD working solution (3.1.12.1) into a test tube and add in the same way the respective volumes of the 3-MCPD-standard solution S2 (3.1.8.1), S3 (3.1.8.2) and the 2-MCPD-standard solution S2 (3.1.9.1), S3 (3.1.9.2) (see Table 2).

Then add 1.8 mL each of ammonium sulphate solution (3.3.5) to all the standards.

Table 2: Pipetting scheme for calibration standards for acidic saponification

Standard	IS d_5-3-MCPD (3.1.11.1) and d_5-2-MCPD (3.1.12.1)	3-MCPD (3.1.8.1)/(3.1.8.2) and 2-MCPD (3.1.9.1)/(3.1.9.2)	$(NH_4)_2SO_4$ solution (3.3.5)	IS d_5-3-MCPD and d_5-2-MCPD	3-MCPD/2-MCPD absolute [µg] in the derivatization preparation	3-MCPD/2-MCPD [mg/kg] per 100 mg sample amount
1	30 µL	25 µL S3	1.8 mL	0.3 µg abs. in the derivatization preparation or 3 mg/kg sample	0.025	0.25
2		50 µL S3			0.05	0.5
3		100 µL S3			0.1	1.0
4		20 µL S2			0.2	2.0
5		30 µL S2			0.3	3.0
6		40 µL S2			0.4	4.0
7		50 µL S2			0.5	5.0
8		60 µL S2			0.6	6.0

7.7 Derivatization

Note: *The* derivatization *of the pre-prepared standards (7.4 respectively 7.6) and the samples (7.3) has to be performed simultaneously*.

Add 250 µL each of the derivatization reagent (3.3.4) to all the samples and standards.

7.7.1 The derivatization reaction takes place in the ultrasonic bath (4.21) at room temperature for 2-3 min.

7.8 Extraction of the phenylboronic acid derivatives of the standards (Analysis according to Annex 2A)

The phenylboronic acid derivatives of the calibration standards are extracted with 300 µL isooctane (3.2.9) by shaking for 20 s (Vortex 4.22). Transfer an aliquot of the residue into a GC crimp vial with a glass insert (4.2) and close with a crimp cap (4.1).

Note: *If* necessary *centrifuge the samples at 207 x g for 2 min at room temperature (4.10) to improve the phase separation.*

If only a non-*refrigerated centrifuge is available, centrifugation can take place at room temperature.*

The extracts can be stored *at 4 °C for 3 days prior to GC/MS-analysis.*

7.9 Subsequent processing of the derivatized samples and standards (Analysis according to Annex 2B)

To remove matrix components that could interfere with GC-MS analysis, the phenylboronic acid derivatives of the samples are extracted with a nonpolar organic solvent which has to be carefully dried. Then the analytes are re-dissolved into a more polar solvent.

7.9.1 Extraction of the phenylboronic acid derivatives:

Note: *Prior extraction of the derivatives evaporate solvent of the standards at 40 °C under a stream of nitrogen to a minimum volume of 1.5 mL.*

Pipet 1 mL cyclohexane (3.2.3) to the sample and standards, shake the mixture (10 s Vortex (4.22)) and subsequently centrifuge at **207 x g for 2 min at 10 °C** (4.10) to improve the phase separation.

Transfer the **upper organic phase** to a test tube (4.20) and repeat this extraction of the lower phase two times.

Note: *During the extraction with cyclohexane take care that no water passed into the organic extracts. To* avoid *this, it is better to transfer the upper organic phase incompletely; in this case an additional extraction with ethyl acetate may be performed.*

If only a non refrigerated *centrifuge is available, centrifugation can take place at room temperature.*

7.9.2 Drying the merged extracts:

Evaporate the merged extracts at 40 °C under a stream of nitrogen to completely dryness. During the drying process a white precipitate forms at the rim of the glass tube.

Note: *If only a non-*heatable *evaporator is available, evaporation of the solvent can take place at room temperature.*

7.9.3 Dissolution of the residue:

Dissolve the residue in 300 µL isooctane (3.2.9) under shaking (10 s Vortex (4.22)) and subsequently centrifuge at **207 x g for 2 min at 10°C** (4.10).

Note that the white precipitate at the rim of the glass tube remains there.

Transfer an aliquot of the residue into a GC crimp vial with a glass insert (4.2) and close with a crimp cap (4.1).

Note: Cyclohexane *or acetone may be used instead of isooctane for the dissolution.*
If only *a non-refrigerated centrifuge is available, centrifugation can take place at room temperature.*

The extracts can be stored at 4 °C for 3 days prior to GC/MS-analysis.

8 GC/MS-Analysis

The following specifications are given by way of example.

The GC-MS analysis is based on electron-impact ionization operated in SIM mode with the following parameters:

8.1 Injector conditions

Split/split less injector	
Mode	Pulsed split less
Injector temperature	180 °C
Insert liner	Liner, single taper with glass wool (5.4)

8.2 GC and MS conditions

GC column	DB-5MS (5.2)
Pre-column	Fused Silica, deactivated (5.6)
Flow	1.2 ml/min (constant)
Carrier gas	Helium (3.4.1)
GC oven programme	60 °C (kept constant for 1 min); 6 °C/min up to 190 °C; 30 °C/min up to 280 °C (kept constant for 10 min)
Temperature transfer line	280 °C
Temperature ion source	230 °C
Temperature quadrupole	150 °C

8.2.1 Selected fragment ions of the PBA derivative for SIM method

3-MCPD derivative	Ions (m/z)	Dwell (ms)
	196	80
	147	80
d_5-3-MCPD derivative	Ions (m/z)	Dwell (ms)
	201	80
	150	80
2-MCPD derivative	Ions (m/z)	Dwell (ms)
	196	80
d_5-2-MCPD derivative	Ions (m/z)	Dwell (ms)
	201	80

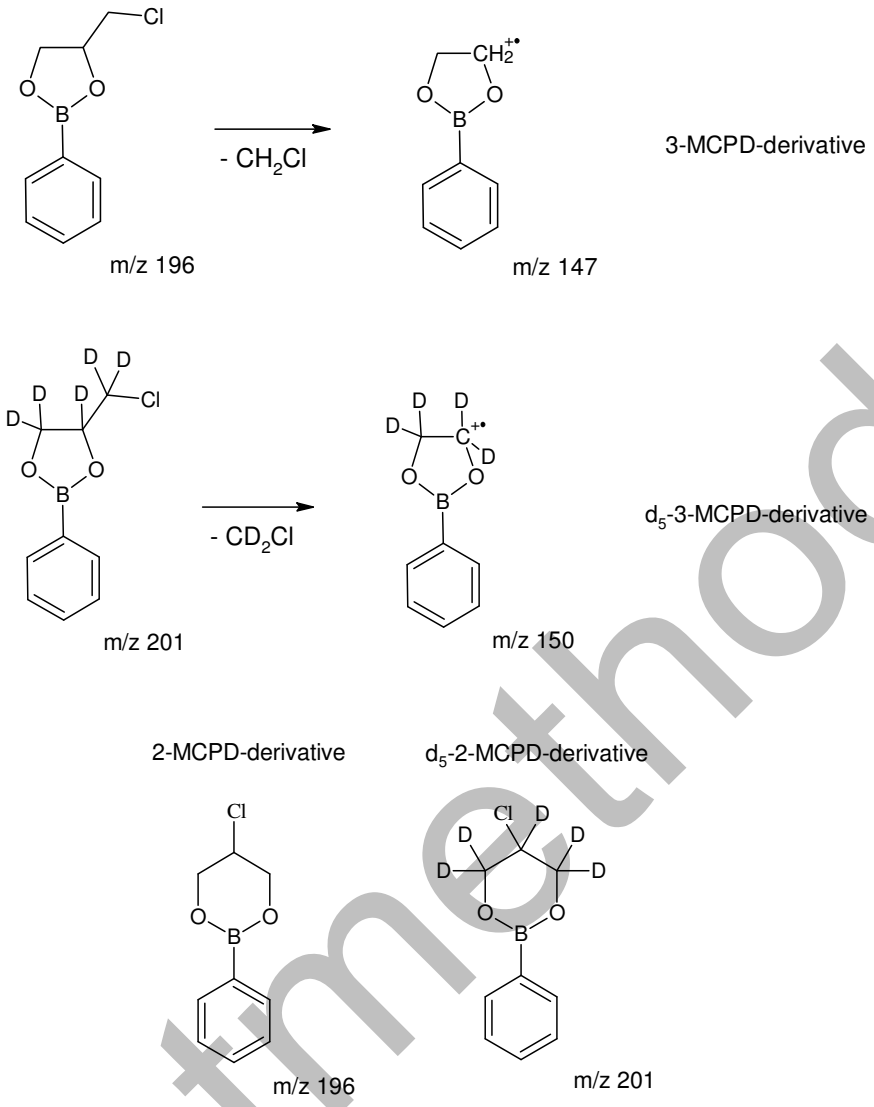


Figure 1: Selected fragment ions for the SIM method

9 Evaluation

9.1 GC/MS Evaluation

9.1.1 Response ratios and calibration function

Ion m/z 196 and ion m/z 147 shall be used as quantifying ions for 3-MCPD and ions m/z 201, 150 for the internal standard d_5-3-MCPD. For the isomer 2-MCPD shall be used ion m/z 196 and ion m/z 201 for the internal standard d_5-2-MCPD.

Based on the calibration standards, determine the areas of the quantifying ions of the phenylboronic acid derivatives of the 3-MCPD respectively 2-MCPD as well as d_5-3-MCPD respectively d_5-2-MCPD internal standard, and form the response ratios of analyte/internal standard.

$$R = \frac{A \quad (m/z \quad 196)}{A \quad (m/z \quad 201)} \qquad \text{respectively} \qquad R = \frac{A \quad (m/z \quad 147)}{A \quad (m/z \quad 150)}$$

R = Response ratio of standard/internal standard
A = Response area

To set up the calibration function, plot the response ratio of the 3-MCPD standard and the d_5-3-MCPD internal standard against the concentration of the 3-MCPD standard (µg). Calculate the calibration function by means of linear regression. Proceed in the same manner for the concentration of the 2-MCPD standard (µg).

$$R = a * m_{3-MCPD} + b$$

R = Response ratio of standard/internal standard
a = Slope of the regression line
b = Intercept of the regression line
$m_{3\text{-}MCPD}$ = Absolute amount of 3-MCPD respectively 2-MCPD (µg) in the derivatization preparation of the standard

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* Interference due to matrix and/or GC-MS properties (such as conditions of the column or of the ion source, altered by age or other) may cause faulty ion traces of the 3-MCPD phenylboronic acid derivatives. Therefore, it is recommended to determine the response ratio of the ion traces 147 and 196. The response ratios should range between 4.5 and 5.8. Any outlying ratio suggests a disturbance.

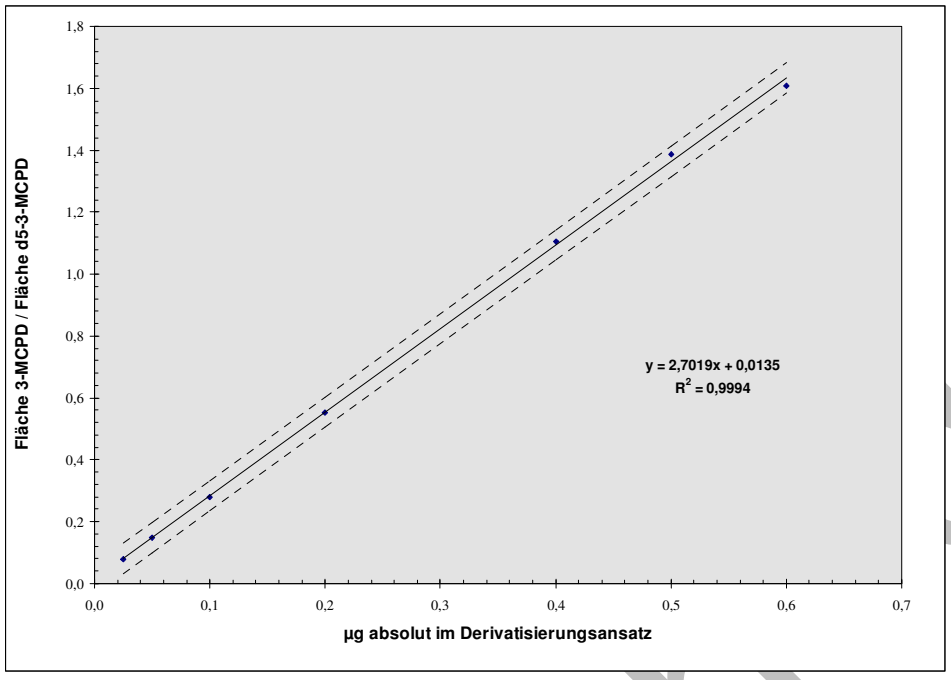


Figure 2 **Model for a 3-MCPD calibration line**

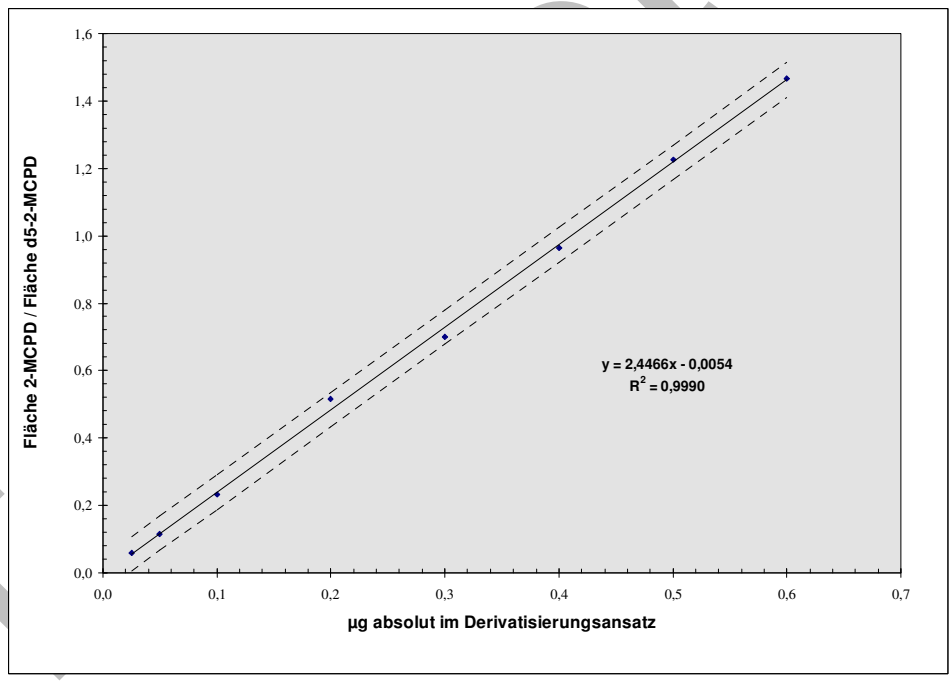


Figure 3: Model for a 2-MCPD calibration line

9.1.2 Calculation of the released 3-MCPD respectively 2-MCPD concentration in the derivatization preparation of the samples

The concentration of the sample is stated in µg as absolute amount of 3-MCPD respectively 2-MCPD in the derivatization solution.

$$m_{3MCPD} = \frac{(R_{\text{Probe}} - b)}{a}$$

$m_{3\text{-}MCPD}$ = Absolute amount of 3-MCPD respectively 2-MCPD (µg) in the derivatization preparation of the standard
R_{Probe} = Response ratio of analyte / internal standard determined in the derivatization preparation of the standard
a = Slope of the regression line
b = Intercept of the regression line

9.1.3 Calculation of the 3-MCPD and 2-MCPD concentrations in the sample (mg/kg)

$$\omega = \frac{m_{3-MCPD}}{m}$$

ω = 3-MCPD respectively 2-MCPD concentration stated in mg/kg
m = Sample amount stated in g

The concentration should be given with an accuracy of one significant digit.

10 Selected Chromatograms

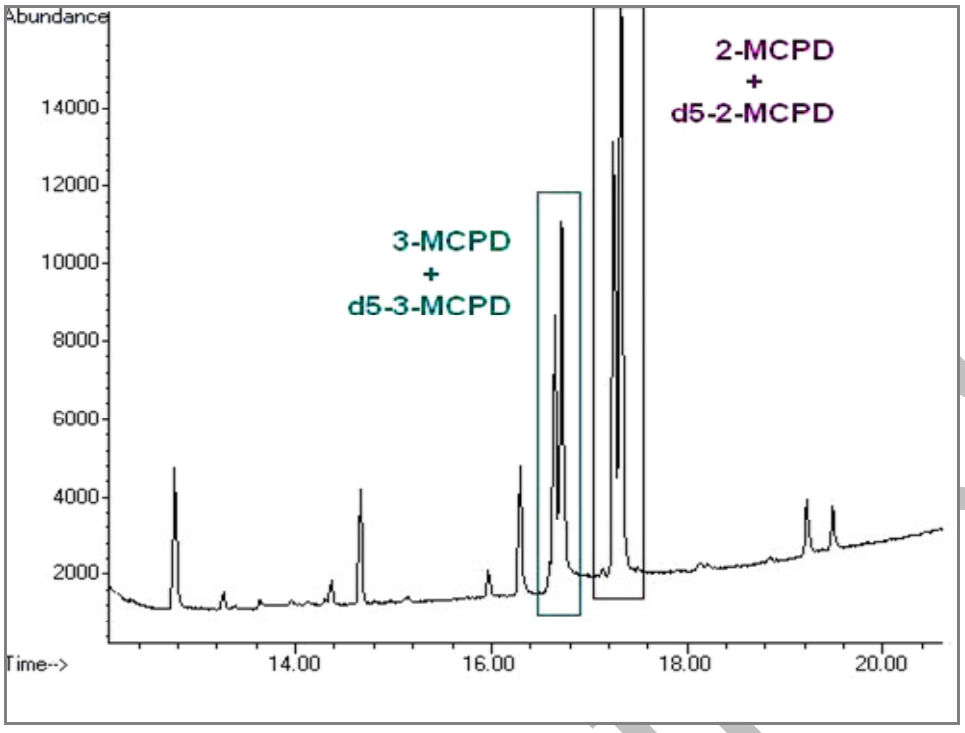


Figure 4: Scan chromatogram of a standard solution with 0.3 µg 3-MCPD / 0.3 µg d5-3-MCPD as well as 0.3 µg 2-MCPD / 0.3 µg d5-2-MCPD absolute in the derivatization preparation (3 mg/kg 3-MCPD /2-MCPD and 3 mg/kg d5-3-MCPD / d5-2-MCPD)

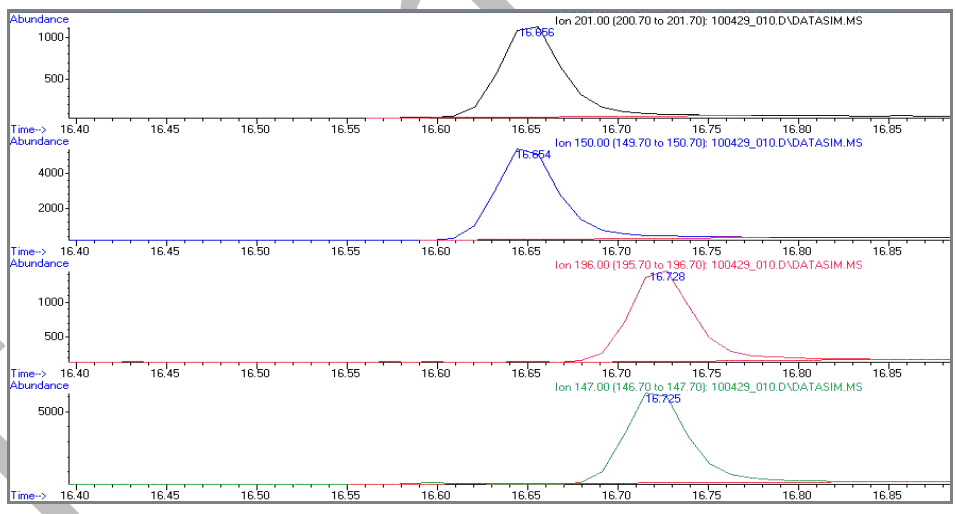


Figure 5: SIM ion chromatogram of a standard solution with 0.3 µg 3-MCPD and 0.3 µg d5-3-MCPD absolute in the derivatization preparation (3 mg/kg 3-MCPD and 3 mg/kg d5-3-MCPD)

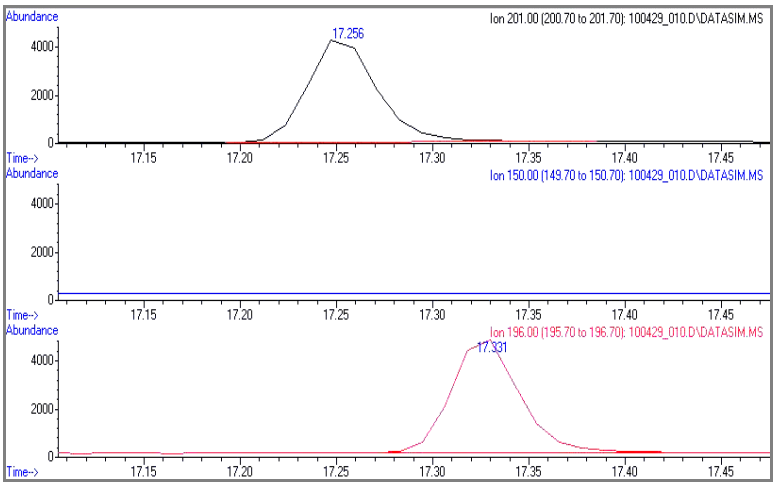


Figure 6: SIM ion chromatogram of a standard solution with 0.3 µg 2-MCPD und 0.3 µg d5-2-MCPD absolute in the derivatization preparation (3 mg/kg 2-MCPD respectively. 3 mg/kg d5-2-MCPD)

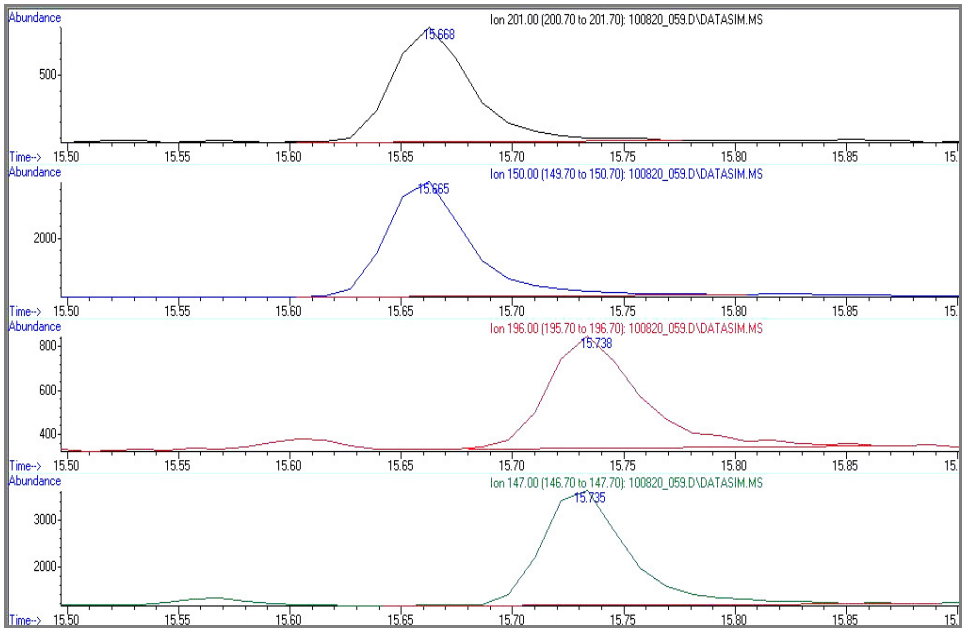


Figure 7: SIM ion chromatogram of a matrix sample (3 mg/kg 3-MCPD; spiked with 3 mg/kg d5-3-MCPD)

11 Validation

11.1 Determination of the recovery rate

In order to determine the recovery rate, an analyte-free sample was spiked with a 3-MCPD ester (1,2-bis-palmitol-3-monochloropropane-1,2-diol) at a concentration of 5.4 mg/kg fat before fat extraction. Assuming complete ester cleavage, this corresponds to 1 mg of free 3-MCPD/kg fat.

For the purpose of comparison, an analyte-free sample was spiked with 3-MCPD at a concentration of 1 mg/kg fat before fat extraction.

Since 2-MCPD ester was not available as a standard substance, an analyte-free sample was spiked with 2-MCPD at a concentration of 1 mg/kg fat before fat extraction.
100 mg aliquots of fat extract were prepared as shown in Annex 2.

The respective internal standards were added **a) before** fat extraction and **b) after** fat extraction**.**

Tables 3 to 8 show the mean values of recovery obtained for the respective concentrations. The recovery rate was determined (in %) as the ratio between the reference concentration and the concentration actually found.

Table 3: Recovery rate for 3-MCPD ester (samples were spiked before fat extraction)

Added 3-MCPD concentration	3-MCPD concentration	Mean value of recovery	Coefficient of variation (%)	Number of samples
5.40 mg/kg 3-MCPD ester	1.0 mg/kg	**100.5**	4.7	5

Table 4: Recovery rate for 3-MCPD ester (samples were spiked after fat extraction)

Added 3-MCPD concentration	3-MCPD concentration	Mean value of recovery	Coefficient of variation (%)	Number of samples
5.40 mg/kg 3-MCPD ester	1.0 mg/kg	**98.5**	1.4	5

Table 5: Recovery rate for 3-MCPD (samples were spiked before fat extraction)

Added 3-MCPD concentration	Mean value of recovery	Coefficient of variation (%)	Number of samples
1.00 mg/kg	**101.6**	4.1	6

Table 6: Recovery rate for 3-MCPD (samples were spiked after fat extraction)

Added 3-MCPD concentration	Mean value of recovery	Coefficient of variation (%)	Number of samples
1.00 mg/kg	**24.2**	2.7	5

Table 7 Recovery rate for 2-MCPD (samples were spiked before fat extraction)

Added 2-MCPD concentration	Mean value of recovery	Coefficient of variation (%)	Number of samples
1.00 mg/kg	**110.9**	5.2	5

Table 8 Recovery rate for 2-MCPD (samples were spiked after fat extraction)

Added 2-MCPD concentration	Mean value of recovery	Coefficient of variation (%)	Number of samples
1.00 mg/kg	**51.2**	26.1	5

Results:

A significant difference in their fat extraction behaviour was noticeable between free 2-MCPD and 3-MCPD ester in its bound form.

As to 3-MCPD ester, fat extraction entails a recovery loss of < 2 %, i.e., when applying ASE, 3-MCPD ester is completely extracted. Likewise could be confirmed that 3-MCPD ester is completely transesterified into free 3-MCPD during the process of acidic saponification.

For MCPD in its free form, by contrast, the recovery loss following fat extraction with ASE is > 60 %. The extraction method does not seem to be appropriate for free MCPD. If d_5-3-MCPD or d_5-2-MCPD is used as internal standard, they may be added to the samples **after** fat extraction only.

Assuming that 2-MCPD esters show a similar behaviour as 3-MCPD esters, likewise a recovery rate of > 90 % should be obtained. Precise data cannot be provided, because standard substances for 2-MCPD esters have not been available commercially up to now.

11.2 Determination of the extraction yield with ASE

To determine the extraction efficiency, four different fat-containing foods were extracted repeatedly using ASE by the same technician (see Annex 1). The fat extracts were collected in vials, which had been previously calibrated; the solvent was evaporated at 40°C under a stream of nitrogen; the extraction yield was determined by weighing the vials again together with the extracts and by comparison with the fat content indicated on the labels of the corresponding food products.

Table 9: Extraction yield

					Mono-factorial analysis of variance, ANOVA (α = 0.05)		
Matrix	Numbers of determination	Mean value of the extraction yield (%)	Coefficient of variation (%)	Standard deviation	P-value	Test statistics (F)	crit. F-value
Strawberry spread	6	95.6	0.39	0.38	1.51	0.29	7.71
Chocolate spread	6	97.6	0.59	0.57	0.47	0.64	
Margarine	6	102.4	4.83	4.96	0.87	0.03	
Infant formula	6	96.3	0.26	0.25	0.36	1.05	

Results:

With respect to the parameter “extraction efficiency”, all samples were subject to mono-factorial analysis of variance. As a result, no significant differences were found (error probability of 5 %).

11.3 Laboratory precision under repeatability conditions and with change of technicians

In order to determine the method’s precision, four fat-containing foods each with different 3-MCPD and 2-MCPD concentrations were subject to manifold analysis performed by two technicians.

The precision was determined first under repeatability conditions (same sample, same technician, and same laboratory apparatus); then the precision achieved with different technicians under repeatability conditions (same sample, same laboratory apparatus) was determined.

Tables 10 to 12 show the results of the analytical tests.

Table 10: Laboratory precision under repeatability conditions – and with change of technicians - using d5-3-MCPD ester as internal standard

	Technician A				Technician B			
Matrix	Numbers of determination	Mean value of 3-MCPD (mg/kg)	Coefficient of variation (%)	Standard deviation (mg/kg)	Numbers of determination	Mean value of 3-MCPD (mg/kg)	Coefficient of variation (%)	Standard deviation (mg/kg)
Strawberry spread	6	6.08	2.7	0.17	6	6.14	3.9	0.24
Chocolate spread	6	1.55	3.7	0.06	6	1.68	1.7	0.03
Margarine	6	1.62	4.7	0.08	5	1.62	1.9	0.03
Infant formula	6	0.53	4.7	0.03	5	0.61	4.0	0.02

Table 11: Laboratory precision under repeatability conditions – and with change of technicians - using d5-3-MCPD as internal standard

	Technician A				Technician B			
Matrix	Numbers of determination	Mean value of 3-MCPD (mg/kg)	Coefficient of variation (%)	Standard deviation (mg/kg)	Numbers of determination	Mean value of 3-MCPD (mg/kg)	Coefficient of variation (%)	Standard deviation (mg/kg)
Strawberry spread	5	6.05	3.5	0.14	6	6.25	2.6	0.12
Chocolate spread	6	1.54	2.0	0.03	6	1.62	3.9	0.06
Margarine	5	1.50	2.0	0.03	5	1.49	4.8	0.07
Infant formula	6	0.57	2.8	0.01	5	0.63	3.4	0.04

Table 12: Laboratory precision under repeatability conditions – and with change of technicians - using d5-2-MCPD as internal standard

	Technician A				Technician B			
Matrix	Numbers of determination	Mean value of 2-MCPD (mg/kg)	Coefficient of variation (%)	Standard deviation (mg/kg)	Numbers of determination	Mean value of 2-MCPD (mg/kg)	Coefficient of variation (%)	Standard deviation (mg/kg)
Strawberry spread	6	1.51	2.9	0.04	6	1.46	2.0	0.03
Chocolate spread	6	0.73	2.1	0.02	6	0.72	4.8	0.03
Margarine	6	0.77	3.4	0.03	5	0.81	3.9	0.03
Infant formula	6	0.39	2.8	0.01	5	0.40	4.8	0.02

Results:

In the lower as well as in the upper concentration range, repeatability testing showed a satisfactory precision with a coefficient of variation of < 5 % for both 3-MCPD and 2-MCPD.

12 Anhang

12.1 Anhang 1

flow chart – fat extraction with ASE
annex 1 to Method_FC-023-01

Sample preparation

weighing 1,5-2,5 g sample in a beaker and homogenize with 4 g Isolute

load homogenized sample into a 22 ml ASE cell

weigh the empty ASE vial and record the weigh

Weighing according to fat content (**fc**):

2,5 g at **fc < 30 %**
2,0 g at **fc ~ 30-60 %**
1,5 g at **fc > 60 %**

Extraction parameter ASE

Extraction solvent	Petroleum ether / Isohexane / Acetone (2+2+1, v/v/v)
Extraction temperature	125 °C
Heat time	6 min
Static time	5 min
Flush volume	100 %
Flush time	60 sec
Cycles	2
Pressure	103,4 bar

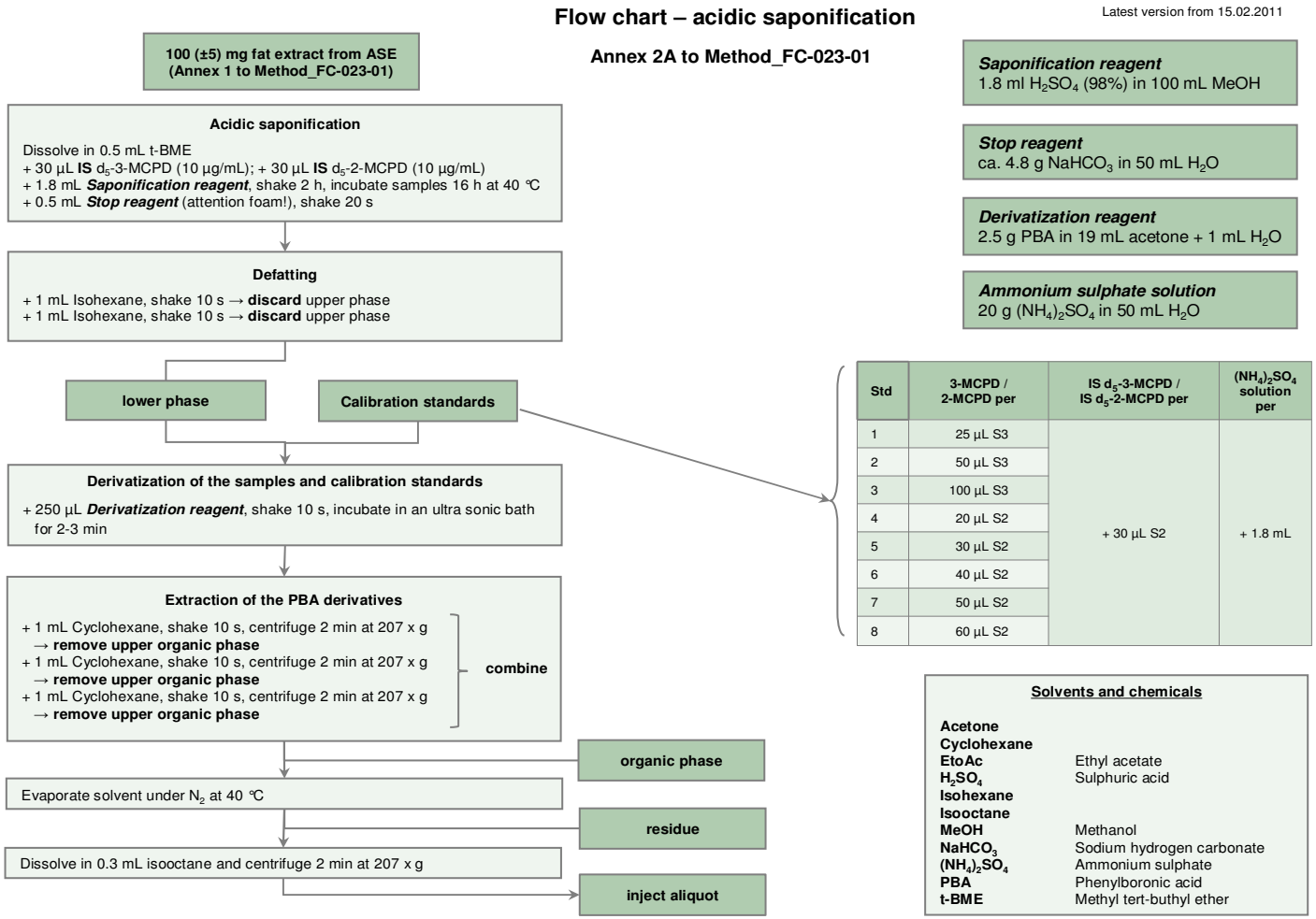
evaporate solvent under N_2 at 40°C

weigh again the ASE vials, record the weight and define the extraction yield for fat

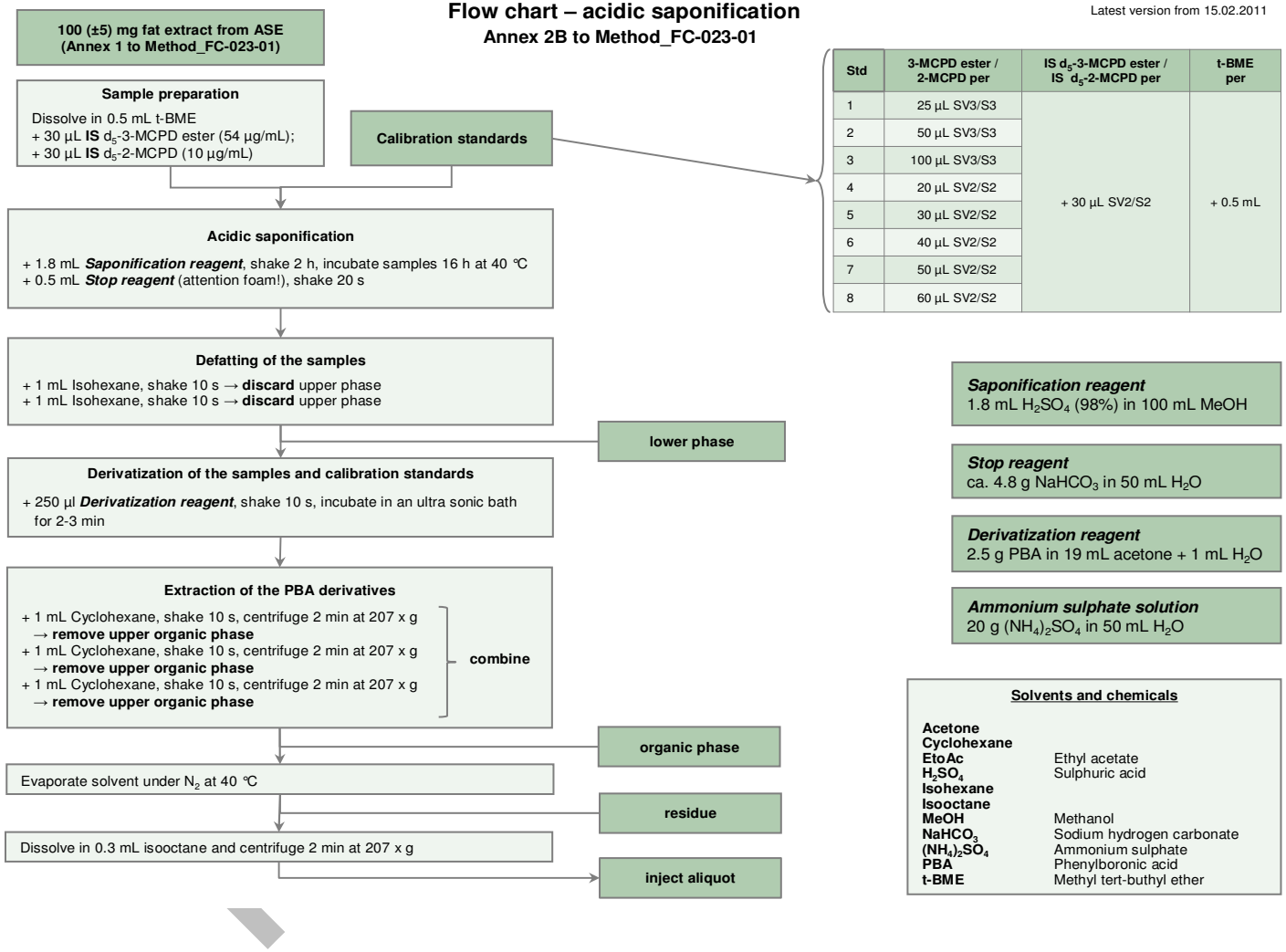
100 mg aliquot of the fat extract was analyzed to **annex 2A or 2B (Method_FC-023-01)**

Latest version from 15.02.2011

12.2 Anhang 2A



12.3 Anhang 2B



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