

**Ester-bound 3-chloropropane-1,2-diol (3-MCPD esters) and glycidol (glycidyl esters)**

Determination in fats and oils by GC-MS

**1 Scope of application**

1.1 The method describes a procedure for the separate determination of ester-bound 3-chloro-propane-1,2-diol (3-MCPD esters) in fats and oils by means of gas chromatography/mass spectrometry after cleavage of esters with methanol and sodium methoxide. Provided that with the exception of glycidol no other compounds are present, which are forming 3-MCPD esters under the conditions of the analysis, the method also enables the determination of glycidol (glycidyl esters).

1.2 This method is applicable to solid and liquid fats present as such or obtained by cautious extraction from processed fat and oil-containing foodstuffs.

*Note: To extract fat from fat-containing foodstuffs, neither acidic nor alkaline cleavage procedures shall be used. Digestion procedures according to Weibull-Stoldt or similar shall not be used, because significant amounts of 3-MCPD esters are formed in this way. Suitable solvents for fat extraction are t-butyl methyl ether or mixtures of t-butyl methyl ether with hexane or petroleum ether. Under certain circumstances it may turn out that 3-MCPD monoesters cannot be extracted completely with hexane or petroleum ether.*

**2 Definition**

**Ester-bound 3-chloropropane-1,2-diol (3-MCPD esters)** is the sum of all monoesters and diesters of 3-MCPD with different fatty acids,

determined according to this method.

*Note: The initial content of free 3-MCPD in fats and oils usually is so low that it can be ignored.*

The content, calculated as a mass fraction of free 3-MCPD, is stated in milligram per kilogram. Regarding fat-containing foods, the content generally refers to the fat content in the food.

**Ester-bound glycidol (glycidyl esters)** is defined as the sum of all esters of glycidol with different fatty acids, determined according to this method.

*Note: The initial content of free glycidol in fats and oils usually is so low that it can be ignored.*

The content, calculated as a mass fraction of free glycidol, is stated in milligram per kilogram. Regarding fat-containing foods, the content generally refers to the fat content in the food.

### 3 Principle of method

The sample is dissolved in butyl methyl ether and d<sub>5</sub>-labeled 3-MCPD is added as an internal standard. 3-MCPD is released from ester bonds by transesterification with methanolic NaOCH<sub>3</sub>, fatty acids are transformed into fatty acid methyl esters. The reaction is stopped with acetic acid. Fatty acid methyl esters and non-saponifiable compounds are removed with hexane. The released 3-MCPD is transformed into a cyclic boronic acid ester by adding phenylboronic acid dissolved in NaCl solution. In this step, glycidol is transformed nearly quantitatively into the phenylboronic acid ester of 3-MCPD. The phenylboronic acid derivatives of 3-MCPD and of the internal standard are extracted with hexane and analysed by GC-MS.

At first the total content of ester-bound 3-MCPD and glycidol is determined in the sample as described above. The content is expressed as 3-MCPD (Concentration A).

In a second trial the sample is treated with a mixture of propanol/sulphuric acid under mild conditions. The epoxide ring of the glycidyl ester is opened and glycidol is quantitatively removed forming different reaction products.

After this treatment the content of ester-bound 3-MCPD in the sample is determined again (Concentration B). Concentration B corresponds to the original content of ester-bound 3-MCPD in the sample.

Provided that the difference of the two determinations (concentrations B minus A) is most exclusively due to the occurrence of glycidol, the difference is used to calculate the content of ester-bound glycidol using a stoichiometric conversion factor.

#### 4 Reagents

**WARNING:** Attention is drawn to the regulations which specify the handling of hazardous substances. Technical, organisational and personal safety measures must be followed.

If not otherwise specified,

- Reagents of analytical grade must be used,
- Water must be double-distilled or of equivalent purity.

- 4.1 Mixture of propanol and sulphuric acid: Dissolve 0,5 mL of conc. sulphuric acid (free of chloride) in 100 mL of n-propanol suitable for residue analysis or of comparable quality. The solution shall be freshly prepared every day.
- 4.2 Hexane or isohexane, suitable for residue analysis or of comparable quality;

- 4.3 Sodium methylate ( $\text{NaOCH}_3$ ),  $c = 0,5 \text{ mol/L}$  in methanol;
- 4.4 Solvent mixture A, composed of t-butyl methyl ether (tBME) and ethyl acetate, suitable for residue analysis or of comparable quality:  
Mix 8 mL tBME with 2 mL ethyl acetate (volume fraction tBME  $\varphi = 8 \text{ mL}/10 \text{ mL}$ , volume fraction ethyl acetate  $\varphi = 2 \text{ mL}/10 \text{ mL}$ );
- 4.5 Acetic acid, at least  $w = 99 \%$ ;
- 4.6 Sodium chloride ( $\text{NaCl}$ ) solution,  $\rho = 200 \text{ g/L}$ ; dissolve 200 g  $\text{NaCl}$  in 1 litre water;
- 4.7 Solvent mixture B, composed of acetic acid and  $\text{NaCl}$ -solution:  
Dissolve 1 mL acetic acid (4.5) in 30 mL  $\text{NaCl}$  solution (4.6), (volume fraction acetic acid  $\varphi = 3,3 \text{ mL}/100 \text{ mL}$ , volume fraction  $\text{NaCl}$  solution  $\varphi = 96,7 \text{ mL}/100 \text{ mL}$ ). Prepare fresh solutions daily;
- 4.8 Derivatisation reagent:  
Phenylboronic acid  $\rho \sim 2,5 \text{ g}/20 \text{ mL}$ ; dissolve approx. 2,5 g phenylboronic acid in 19 mL acetone and 1 mL water;
- 4.9 Internal standard solution:
- 4.9.1 Stock solution 3-MCPD- $d_5$  ( $\rho \sim 100 \text{ mg}/50 \text{ mL}$ ):  
Dissolve ca. 100 mg 3-MCPD- $d_5^*$  in 50 mL of absolute ethanol. The solution can be stored at  $(0 - 6) \text{ }^\circ\text{C}$  for at least one year.
- 4.9.2 Working solution 3-MCPD- $d_5$ , ca.  $20 \text{ } \mu\text{g}/\text{mL}$  in t-butyl methyl ether:  
Place 1 mL stock solution in a volumetric flask and complete to 100 mL by adding t-butyl methyl ether. The solution can be stored at  $0 \text{ }^\circ\text{C}$  to  $6 \text{ }^\circ\text{C}$  for at least three months.
- 4.10 3-MCPD-calibration solutions ( $\rho \sim 100 \text{ } \mu\text{g}/\text{mL}$ ): Weigh approx. 25 mg 3-chloropropane-1,2-diol (3-MCPD) into a 250 mL volumetric flask and make up to the mark by adding  $\text{NaCl}$  solution (4.6).

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\* Supplier: e.g. Promochem, Wesel

The stock solution can be stored at 0 °C to 6 °C for at least three months.

Prepare fresh calibration solutions from the stock solutions daily by diluting with NaCl solution.

4.11 Helium, suitable for GC-MS.

## 5 Apparatus

5.1 Test tubes with screw caps, volume approx. 10 mL;

5.2 Heatable ultrasonic bath, with temperature control

5.3 Heating block or water bath with temperature control, suitable for test tubes (5.2);

5.4 Various glass pipettes and/or piston-stroke pipettes;

5.5 GC-MS system, equipped with a gas chromatograph, column oven provided with a thermo regulator, a split/splitless injector, a mass spectrometer, and an evaluation system.

*Note: The use of an injector provided with a programmed-temperature vaporiser (e. g. PTV injector), a non-polar pre-column and a back flush unit, is recommended, but is not indispensable.*

5.6 Chromatographic column: Fused-silica-capillary, coated with methyl silicon (95 %) and phenyl silicon (5 %), for example Rtx-5MS (0,25 mm i.d., 30 m length, 0,25 µm film thickness).

5.7 Measuring flask (class A); 50 mL, 100 mL and 250 mL content.

## 6 Sample

### 6.1 Sampling:

Sampling is not part of the method described. A recommended sampling method is given in the DGF Standard methods C-I 1 to 5.

## 6.2 Preparation of test sample:

Heat solid and semi-solid fats above their melting point, and homogenise carefully without overheating. Filter off visible impurities after mixing; in the presence of water, use a hydrophobised filter.

## 7 Procedure

### 7.1 Option A: Determination of the sum of ester-bound 3-MCPD and glycidol

#### 7.1.1 Ester Cleavage:

Weigh approx. 100 mg fat into a screw capped test tube, and dissolve in 0,5 mL of solvent mixture A (4.4).

After the addition of 100  $\mu$ L of internal standard solution (working solution 4.9.2) and 1 mL of NaOCH<sub>3</sub>-solution (4.3) - in this order - close the test tube tightly, and allow it to stand for 5 minutes to maximum 10 minutes at room temperature.

After this, add in immediate succession 3 mL hexane and 3 mL solvent mixture B (4.7).

Remove the organic phase (upper phase) with a pipette as completely as possible and discard it.

Add further 3 mL hexane and shake carefully. After phase separation, remove the hexane phase (upper phase) with a pipette as completely as possible and discard it. Continue treating the aqueous phase as described in clause 7.3 (derivatisation).

### 7.2 Option B: Determination of ester-bound 3-MCPD after removal of glycidol with propanol/sulphuric acid:

### 7.2.1 Removal of glycidol

Weigh approx. 100 mg of the fat into a screw capped tube and dissolve in 0,5 mL of solvent mixture A (4.4).

Add 0,5 mL n-propanol/sulphuric acid (4.1), mix and put the closed tube for 15 minutes into an ultrasonic bath at 45 °C (starting temperature).

### 7.2.2 Ester cleavage:

After the addition of 100 µl of the internal standard solution (4.9.2) and 1 mL of NaOCH<sub>3</sub> solution (4.3) (in this order) the tube is closed tightly and left for 5 minutes to 10 minutes at room temperature.

Then add 3 mL of hexane und 3 mL of solvent mixture (4.7).

By means of a pipette withdraw and discard the organic (upper) phase as completely as possible.

After the addition of further 3 mL of hexane the sample is shaken carefully. After the separation of the phases the upper hexane phase is completely withdrwan with a pipette and discarded. Continue treating the aqueous phase as described in clause 7.3 (derivatisation).

### 7.3 Derivatisation:

Add 500 µL of the derivatisation reagent (4.8) to the aqueous phase (7.1.1 or 7.2.2), close the tube tightly and heat at 80 °C for 20 minutes.

To perform the derivatisation of a calibration solution, place 100 µL of internal standard solution in a test tube and remove the major part of solvent cautiously. Then complete to a final volume of approx. 3 mL by adding the respective calibration solution (3-MCPD in NaCl-solution in 4.10) and additional NaCl-solution (4.6). After addition of 500 µL

derivatisation reagent, close the tube tightly and heat at 80 °C for 20 minutes.

After cooling to room temperature, extract the 3-MCPD derivative by shaking it with 3 mL hexane.

Draw off ca. 1-2 mL of the hexane phase and transfer it to a GC-vial.

To protect the GC-MS system and to achieve higher sensitivity, the hexane phase can be carefully evaporated and the residue can be re-dissolved in heptane or octane.

#### 7.4 Gas Chromatography-Mass Spectrometry:

The following conditions have been found to be suitable:

Injection volume:	2 µL
Program for PTV injector:	50 °C, 10 °C/sec to 180 °C (5 min),
Splitless time:	1,50 min
Split flow:	20 mL/min
Carrier gas:	helium, 1,2 mL/min, constant flow
Temperature program:	60 °C (1 min) with 6 °C/min to 190 °C with 20 °C/min to 280 C (keep 10 min to 30 min)
Mass Spectrometry:	EI+, SIM mode
Internal standard:	m/z = 201; optional 150
3-MCPD:	m/z = 196 (quantifier); 147 (qualifier)

### 7.5 Evaluation:

For evaluation use the SIM masses 196 and 147 for 3-MCPD, and 201 for the internal standard 3-MCPD-d<sub>5</sub>, mass trace 147 is only used as a qualifier. Optional SIM masses 150 and 147 can be used for quantification.

The peak areas of the mass traces of 3-MCPD are related to the peak area of the internal standard:

$$Q = \frac{A(196)}{A(201)} \quad \text{and} \quad Q = \frac{A(147)}{A(201)}$$

where:

$Q$  is the ratio of peak areas;  
 $A$  is the peak area.

To produce the calibration line, derivatise appropriate standard solutions as described in 7.2. In many cases an amount of 0,05 µg to 1 µg of standard solution (absolute amount in the derivatisation mixture) is suitable.

Plot the 3-MCPD amounts (absolute amounts in microgram) against the calculated response ratios and determine the regression line:

$$Q = a \cdot m_{3-MCPD} + b \quad (\text{equation of the regression line})$$

where:

$Q$  is the ratio of peak areas;  
 $m_{(3-MCPD)}$  is the amount (µg) of 3-MCPD in the derivatisation mixture;  
 $a$  is the slope of the regression line;  
 $b$  is the intercept on the regression line.

## 8 Results of Determination

### 8.1 Calculation:

Based on the regression line obtained from the response ratio of the sample, the 3-MCPD amount =  $w_{3\text{-MCPD}}$  ( $\mu\text{g}$ ) in the sample solution is calculated according to the following equation:

$$w_{3\text{-MCPD}} = \frac{Q_{\text{sample}} - b}{a}$$

Calculate the content  $w_{3\text{-MCPD-Ester}}$  of the (ester-bound) 3-MCPD in the sample in milligrams per kilogram according to the following equation:

$$w_{3\text{-MCPD-Ester}} = \frac{w_{3\text{-MCPD}}}{m}$$

where

$Q_{\text{sample}}$  is the peak area ratio of the sample;

$w_{3\text{-MCPD-ester}}$  is the mass fraction of ester-bound 3-MCPD in microgram per kilogram;

$w_{3\text{-MCPD}}$  is the amount of 3-MCPD ( $\mu\text{g}$ ) in the sample solution;

$m$  is the mass of test sample in gram.

The result of the determination is calculated for both methods A and B:

$w_A = w_{3\text{-MCPD-Ester}}$  for option A (7.1 following).

$w_B = w_{3\text{-MCPD-Ester}}$  for option B (7.2 following).

### 8.2 Calculation of glycidol content

The content  $w_A$  corresponds to the sum of the content of ester-bound 3-MCPD and glycidol and is expressed as a mass fraction of 3-MCPD.

The content  $w_B$  corresponds to the content of ester-bound 3-MCPD and is expressed as a mass fraction of 3-MCPD.

The content of glycidol  $w_{\text{Glycidol}}$  is calculated as follows:

$$w_{Glycidol} = 0,67 * (w_A - w_B)$$

The results are expressed as a mass fraction in milligrams per kilogram as follows:

For concentration  $\leq 1$  mg/kg with two decimal places,

For concentration  $> 1$ mg/kg with one decimal place,

For concentration  $> 100$  mg/kg as a whole number.

### 8.3 Precision of method:

The precision of the method is the result of an interlaboratory study<sup>\*)</sup>. The study was organised in 2008 by the Federal Institute for Risk Assessment (BfR Berlin) on 10 samples . In this test the sum of ester-bound 3-MCPD and ester-bound glycidol was determined. The results, calculated as 3-MCPD in mg/kg, are given in tab. 1.

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<sup>\*)</sup> Evaluated according to DGF Standard Method A-II 1: *Execution and evaluation of ring tests* and ISO 5725:1994: *Accuracy (trueness and precision) of measurement methods and results – Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method*.

Tab. 1: Summary of statistical results – Sum of 3-MCPD and glycidol esters (Results in mg/kg)

Sample	Safflower seed oil		Fat		Rapeseed oil		Grape seed oil		Olive oil	
	Number of participating laboratories ( <i>N</i> )	19	19	19	20	8	8	20	19	18
Number of laboratories retained after eliminating outliers ( <i>n</i> )	17	15	17	17	8	7	17	16	16	15
Number of individual test results of all laboratories on each sample ( <i>z</i> )	67	60	68	68	31	26	67	64	64	60
<b>Mean value (<i>m</i>)</b>	<b>2,45</b>	<b>2,46</b>	<b>8,28</b>	<b>8,18</b>	<b>0,24</b>	<b>0,25</b>	<b>3,55</b>	<b>3,57</b>	<b>1,22</b>	<b>1,17</b>
Repeatability standard deviation ( <i>s<sub>r</sub></i> )	0,14	0,15	0,33	0,30	0,06	0,06	0,17	0,17	0,11	0,07
Repeatability coefficient of variation ( <i>RCV<sub>r</sub></i> ), %	5,6	6,2	3,9	3,7	24,1	25,2	4,8	4,8	8,9	5,6
<b>Repeatability limit <i>r</i> (<i>s<sub>r</sub></i> x 2,8)</b>	<b>0,38</b>	<b>0,42</b>	<b>0,91</b>	<b>0,84</b>	<b>0,16</b>	<b>0,17</b>	<b>0,48</b>	<b>0,48</b>	<b>0,3</b>	<b>0,18</b>
Reproducibility standard deviation ( <i>s<sub>R</sub></i> )	0,27	0,18	0,86	1,06	0,07	0,06	0,35	0,30	0,19	0,14
Reproducibility coefficient of variation ( <i>RCV<sub>R</sub></i> ), %	11,1	7,4	10,5	13,0	28,5	26,5	9,9	8,3	15,7	12,3
<b>Reproducibility limit <i>R</i> (<i>s<sub>R</sub></i> x 2,8)</b>	<b>0,78</b>	<b>0,51</b>	<b>2,42</b>	<b>2,97</b>	<b>0,19</b>	<b>0,18</b>	<b>0,99</b>	<b>0,83</b>	<b>0,53</b>	<b>0,40</b>
Horrat ( <i>Ho<sub>R</sub></i> )	0,8	0,5	0,8	1,1	1,4	1,3	0,8	0,6	0,9	1

#### 8.4 Repeatability limit ( $r$ ):

The repeatability limit ( $r$ ) is the value less than or equal to the absolute difference between two test results which can be expected with a probability of 95 %, under repeatability conditions.

Repeatability conditions are defined as conditions under which test results are obtained with the same method, on identical test material, in the same laboratory, by the same operator, using the same equipment and reagents, within a short interval of time.

#### 8.5 Reproducibility limit ( $R$ ):

The reproducibility limit ( $R$ ) is the value less than or equal to the absolute difference between two test results which can be expected with a probability of 95 %, under reproducibility conditions.

Reproducibility conditions are defined as conditions under which test results are obtained with the same method, on identical test material, in different laboratories, by different operators, using different equipment and reagents, within a short interval of time.

### 9 Analysis Report

Results of the determination must be stated with reference to the method here described. Furthermore, all characteristics identifying the sample, any non-standard treatment, where applicable, as well as any individual experimental steps not mentioned in this method should be included in the report, and, where applicable, any particular evaluation method should also be recorded.

## 10 References

R. Weisshaar, R. Perz: Fatty acid esters of glycidol in refined fats and oils. *European Journal of Lipid Science and Technology* **111**, (2009).

R. Weisshaar: Determination of total 3-chloropropane-1,2-diol in edible oils by GC-MS after ester cleavage with sodium methoxide. *European Journal of Lipid Science and Technology* **110**, 183-186 (2008).

Z. Zelinkova, B. Svejrovska, J. Velisek, M. Dolezal: Fatty acid esters of 3-chloropropane-1,2-diol in edible oils. *Food Additives and Contaminants* **23**, 1290-1298 (2006).