

# Determination of glyceroltriheptanoate (GTH) in processed animal by-products by gas chromatography

6<sup>th</sup> Edition

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# **Determination of glyceroltriheptanoate (GTH) in processed animal by-products by gas chromatography**

## **1. Scope**

Glyceroltriheptanoate (GTH) is a marker for animal by-products belonging to category 1 and 2, which are defined in the EU animal by-products Regulation (EC) No 1069/2009 [1]. The use of this specific marker is a requirement according to Commission Regulation (EC) No 142/2011 [2].

This analytical procedure specifies a gas chromatographic (GC) method for the determination of glyceroltriheptanoate (GTH) in dry meat and bone meal (MBM) as produced in animal by-product rendering plants and rendered fat. Mass spectrometry detection (MS) can be used to quantify GTH in target materials (i.e. Category 1 and 2 according to Regulation EC (No) 1069/2009) containing GTH around the target concentration (250mg GTH per kg fat), and to confirm the presence or absence of GTH in non-targeted materials (i.e. Category 3 according to [1]).

This method protocol has been successfully single-laboratory validated by the Institute for Reference Materials and Measurements of the European Commission's Joint Research Centre [3] and subjected to a full ring trial validation [4].

## **2. Definition**

Glyceroltriheptanoate (CAS 620-67-7 – C<sub>24</sub>H<sub>44</sub>O<sub>6</sub> – molecular weight 428.6) is a synthetic triglyceride derived from enanthic acid (n-heptanoic acid).

## **3. Principle**

The GTH is extracted with petroleum benzine applying the Soxhlet method. The extract is then purified on amino bonded solid phase cartridges (SPE). The solvent is removed by evaporation and the residue is dissolved in isooctane and diluted to the required concentration. The content of GTH is determined using a gas chromatography (GC) method. The detection is performed coupling the GC to a bench-top mass spectrometry detector (MS). For the quantification with GC an internal standard (5 $\alpha$  cholestane) is used.

## 4. Reagents

During the analysis, unless otherwise stated, use only reagents of recognised analytical grade.

### 4.1 Chemicals

4.1.1 *n*-hexane, for analysis

4.1.2 Isooctane, GC grade.

4.1.3 Diethylether, for analysis.

4.1.4 Petroleum benzine, boiling range 40-60°C for analysis

### 4.2 SPE eluent

Combine 850 ml *n*-hexane (4.1.1) with 150 ml Diethylether (4.1.3) in a 1 litre flask. Mix well.

### 4.3 Standards

4.3.1 Glyceroltriheptanoate (1,2,3 – Trienathoylglycerol), puriss.,  $\geq 99.0\%$  (GC), CAS 620-67-7 (Fluka 92855 or equivalent).

4.3.2 5 $\alpha$  cholestane, purum,  $\geq 96.0\%$  (GC), CAS 481-21-0 (Fluka C8003 or equivalent).

### 4.4 Standard solutions

4.4.1 GTH stock standard

The target concentration is ca. 1 mg ml<sup>-1</sup>. Weigh, to the nearest 0.1 mg, 50 mg of GTH (4.3.1) into a 50 ml volumetric flask. Dissolve in isooctane (4.1.2) and fill up to the mark. Store the GTH stock standard solution in a refrigerator.

4.4.2 GTH working standard

The target concentration is 0.01 mg ml<sup>-1</sup>. Take an aliquot of 0.5 ml of the GTH stock standard (4.4.1) and fill up to the mark of a 50 ml volumetric flask with isooctane (4.1.2).

4.4.3 Cholestane stock standard

The target concentration is ca. 1 mg ml<sup>-1</sup>. Weigh, to the nearest 0.1 mg, 50 mg of 5 $\alpha$  cholestane (4.3.2) into a 50 ml volumetric flask and fill up to the mark with isooctane (4.1.2).

#### 4.4.4 Cholestane working standard

The target concentration is 0.03 mg ml<sup>-1</sup>. Take an aliquot of 3 ml of the cholestane stock standard (4.4.3) and make up to 100 ml with isooctane in a volumetric flask.

*Warning 1: Work in a fume hood when handling the solvents and solutions. Wear safety glasses and protective clothing.*

*Note 1: The target concentration of the stock and working solution may be adjusted to the specific performance profile of the MS used.*

*Note 2: It is recommended to prepare the working solution on a daily basis.*

## 5. Apparatus

Usual laboratory apparatus and, in particular, the following.

### 5.1 GC equipped with a split/splitless or on-column injector and a MS detector

#### 5.1.1 GC capillary column:

Select a column allowing triglyceride separation. Select the column length (around 25-30 m), the internal diameter (between 0.25 mm and 0.10 µm) and the film thickness (0.25 µm or more) taking into account the laboratory experience and the injection system applied. In any case the selected column and the corresponding temperature programme (see Annex 1) shall produce a complete separation between the cholestane peak, the GTH one and other possible interfering peaks. Examples of suitable columns are:

- DB5, non polar, low bleed characteristics, length 30 m, film thickness 0.25 µm, internal diameter 0.25 mm, upper temperature limit 350°C.
- CP-TAP CB, medium-polarity stationary phase, special type for triglycerides, length 25 m film thickness 0.25 µm, internal diameter 0.10 µm, upper temperature limit 355/360°C.

### 5.2 Balances, one analytical, of 10 g capacity or greater with 0.1 mg readability, and one of 100 g capacity or greater with 0.01 g readability

### 5.3 Volumetric flasks

### 5.4 Rotary evaporator (rotavapor)

### 5.5 Soxhlet apparatus

Use a Soxhlet extraction apparatus with an extraction chamber of 100 ml capacity, reflux condenser and a 250 ml round bottom flask. The flask needs to fit to the rotavapor (5.4). The reflux rate should be adjusted at 10 cycles per hour.

5.5.1 *Extraction thimbles, free of soluble matter in petroleum benzine (4.1.4), (Whatman, Schleicher & Schuell, part no. 10 350 242, or equivalent).*

5.5.2 *Glass wool, fat free*

5.5.3 *Glass beads*

**5.6 Heating system, suitable for heating the flask of the Soxhlet (5.4)**

**5.7 Drying oven, air-oven set at 100°C ± 3 °C**

**5.8 Desiccators**

**5.9 SPE manifold**

5.9.1 *SPE cartridges, amino bonded solid phase, 500 mg, 3ml*

*Note 3: Amino bonded SPE cartridges are considered suitable if they are capable of retaining the free fatty acids in the extracted fat fraction ensuring that the measured GTH concentration expressed in terms of percentage recovery rate is above 80% as described by von Holst et al. [3].*

**5.10 Nitrogen evaporator unit**

**5.11 Glass test tubes, 5 ml, with stopper**

**5.12 Sample vials, suitable for the injection system that is used**

**5.13 Vortex mixer**

**5.14 Sieve with 1mm apertures**

## **6. Procedure**

In the case of MBM analysis the full procedure has to be applied. In the case of rendered fat samples the procedure starts from step 6.3.

### **6.1 Preparation of the sample**

Grind the MBM sample so that it passes through a sieve with 1mm apertures.

### **6.2 Sample extraction**

Weigh, to the nearest 0.1 g, 10 g MBM sample in clean extraction thimbles (5.5.1), cover with glass wool (5.5.2) and place it in the extraction tube of the Soxhlet apparatus (5.5). Add glass beads (5.5.3) to the Soxhlet flask (5.5) in order to promote smooth boiling. Weigh, to the nearest 0.1g, and register the tare of the flask

(glass beads included). Add 150 ml of petroleum benzine to the flask (4.1.4). Connect the apparatus and the condenser to a cold water source under the fume hood. Start up the system and set the heating system (5.6) in order to fulfil the requirements specified in 5.5. Let the Soxhlet (5.5) extract continuously for six hours. After six hours, distil off the solvent with the rotavapor (5.4). Dry the residue by placing the flask for one and a half hours in the drying oven (5.7). Leave to cool in a desiccator (5.8) and weigh. Dry again for 30 minutes to ensure that the weight of the fat remains constant (loss in weight between two successive weightings must be less than 1 mg).

*Note 4: Measuring the difference of the flask with and without the fat fraction allows for the determination of the percentage fat content of the MBM. However, this value is not required for the purpose of this analysis, since the GTH concentration is related to fat and not to the original matrix [2].*

*Note 5: Other extraction methods can be applied provided that extraction efficiency for fat and GTH, obtained with Soxhlet and the alternative method, is equivalent.*

### **6.3 Sample purification**

Melt in an oven rendered fat or fat extracted from the MBM according to the procedure described in 6.2 and weigh, to the nearest 0.01g, approximately 0.5g of the fat into 5 ml volumetric flask. Add n-hexane (4.1.1) and fill up to the mark. Mix well (5.13).

Prepare the glass test tubes (5.11) adding 100 µl of cholestane working standard (4.4.4) in each of them. Place the amino bonded cartridges (5.9.1) in the SPE manifold (5.9). Please note that the use of a vacuum pump is not recommended. Condition each cartridge with 7.5 ml hexane ensuring that a thin layer of hexane remains on top of the solid phase. Place the test tube prepared under each cartridge; pipette an aliquot of 0.2 ml of the diluted fat (from the 5ml volumetric flask) to the top of the cartridge. Ensure that the aliquot permeates the solid phase completely. Elute with 3 ml of SPE eluent allowing the extract to be collected completely in the corresponding glass test tube by gravity (4.2).

Evaporate the cleaned extract to dryness in the nitrogen evaporating unit under a gentle nitrogen stream (please note that it is advisable to kindly warm up the residue during the evaporation process). Dissolve the residue in 5ml of isooctane (4.1.2), cap the tubes, and vortex each tube for 30 seconds ensuring that the complete dissolution of the fat happens. Transfer the final extract to a sample vial and proceed injecting the sample according to the analysis procedure described in 6.5.2.



*Warning 2: Wash all glassware very carefully to avoid GTH blind values, for instance by rinsing the glassware with acetone prior to the washing procedure in the dishwasher.*

## 6.4 Calibration curve

Prepare a series of appropriate calibration solutions, expressed in micrograms per millilitres, by diluting the GTH working standard (4.4.2) with isooctane (examples are given in Table 1).

*Table 1: Example of calibration standard solutions*

Correspondent GTH content in the extracted fat	GTH concentration in the calibration solution	GTH working std (4.4.2)	Cholestane working std	Volumetric flask
mg kg <sup>-1</sup>	µg ml <sup>-1</sup>	ml	ml	ml
0	0	0	0.1	5
75	0.3	0.15	0.1	5
125	0.5	0.25	0.1	5
250	1	0.5	0.1	5
375	1.5	0.75	0.1	5
500	2	1	0.1	5
625	2.5	1.25	0.1	5

## 6.5 Sample analysis

### 6.5.1 GC conditions

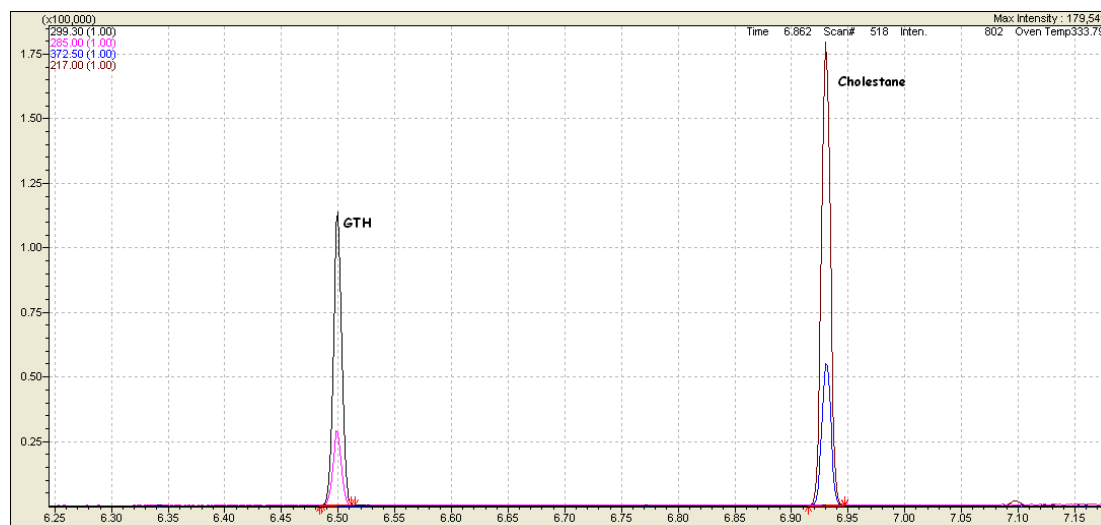
Different GC methods could be applied provided that they allow for a gas chromatographic separation of GTH, the internal standard and other possible interfering compounds.

The selected mass ions for GTH are 299.3 (quantifier ion) and 285.0 (confirmatory ion); the selected mass ions for cholestane are 217.0 (quantifier ion) and 372.5 (confirmatory ion). Please note that these conditions are given for guidance; other conditions may be used provided that equivalent results are obtained.

*Note 6: Select appropriate GC conditions to ensure that less volatile components of the sample have passed the GC column prior to the next injection (e.g. keeping the column at maximum temperature achievable for at least 15 minutes).*

Typical chromatogram obtained using DB5 column and split injection is shown in Figure 1.

Figure 1. Chromatogram at 250 mg GTH kg<sup>-1</sup> fat. SIM mode.



### 6.5.2 Analysis

The sequence of analysis should be as follows: one set of calibration standards, a series of samples, and a second set of calibration standards.

Calculate the response factor ratio (GTH peak area divided by the cholestane peak area) of the calibration samples and the unknown samples. Plot the response factor ratios of the calibration standard against the respective concentrations of GTH. To draw the curve use all the values pooled from the first set and from the second set of calibration standards. Determine the GTH concentration of the extracts of the unknown samples in micrograms per millilitre by reference of their response factor ratios to the pooled calibration curve.

*Note 7: It is advisable to condition the system injecting 4 times the highest point of the calibration curve before proceeding with the sequence of analysis.*

## 7. Expression of results

### 7.1 Calculation

Calculate the GTH content in the test sample by the equation

$$w = \frac{c \times V_1 \times V_3}{m \times V_2}$$

where

- w is the numerical value of the GTH content of the test sample in milligrams per kilograms fat.
- c is the average of two numerical values of the GTH concentration obtained from two injections of the same purified extract, in micrograms per millilitres
- m is the weight of the fat sample as determined in 6.3.

- $V_1$  is the volume of the fat solution, in millilitres (5 ml according to 6.3).
- $V_2$  is the volume of the extract aliquot placed on the top of the cartridge, in millilitres (0.2 ml according to 6.3).
- $V_3$  is the volume in which the dry extract is redissolved, in millilitres (5 ml according to 6.3).

## 8. Quality control samples

Each batch of analyses contains at least one experiment in which an aliquot of a known GTH solution, corresponding to a sample having a GTH concentration around  $250 \text{ mg kg}^{-1}$  on fat basis, is analysed. Prepare the Quality control sample solution transferring  $250 \mu\text{l}$  of the GTH stock standard solution (4.4.1) in a 10 ml volumetric flask, evaporate the transferred solution to dryness and fill up to the mark with n-hexane (4.1.1). Place  $200 \mu\text{l}$  on the SPE cartridge and follow the procedure as described in chapter 6.3. The system is considered under control, when the measured GTH concentration expressed in terms of percentage recovery rate is above 80%.

## 9. Performance profile of the method

### 9.1 Trueness and precision

The method has been ring trial validated [4] and it has delivered the method performance characteristics that are summarised in Table 2.

*Table 2: Results from the statistical evaluation:  $S_r$ : standard deviation of repeatability;  $RSD_r$ : relative standard deviation of repeatability;  $S_R$ : standard deviation of reproducibility;  $RSD_R$ : relative standard deviation of reproducibility. The number in brackets indicates the number of laboratories identified as outliers due to deviation from the method or by statistical tests. All concentrations are related to the fat content. The target concentrations have been established during the homogeneity study.*

Processed product	Mat 6	Mat 7	Mat 8	Mat 10	Mat 11	Mat 12
	MBM	MBM	MBM	FAT	FAT	FAT
No. of laboratories	19 (4)	19 (4)	19 (4)	19 (4)	19 (4)	19 (5)
Target concentration ( $\text{mg kg}^{-1}$ )	191	256	455	186	301	61
Mean ( $\text{mg kg}^{-1}$ )	188	273	467	181	306	58
$S_r$ ( $\text{mg kg}^{-1}$ )	10.8	13.6	16.1	8.5	13.9	4.6
$RSD_r$ (%)	5.7	5	3.4	4.7	4.5	7.8
$S_R$ ( $\text{mg kg}^{-1}$ )	22.1	24.5	47.1	22.8	41.4	9.6
$RSD_R$ (%)	11.8	9	10.1	12.6	13.5	16.5
HORRAT value	1.6	1.3	1.6	1.7	2	1.9

These results demonstrated that the precision and trueness of the method is fit for the intended purpose and can be used for official control purposes to determine GTH in processed animal by-products from Category 1 and Category 2 [2].

## 9.2 Determination of limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ of the method have been determined by fortifying a blank MBM sample with GTH at  $1 \text{ mg kg}^{-1}$  (corresponding to  $8.5 \text{ mg kg}^{-1}$  on fat basis). This concentration was expected to be close to the background noise. Based on the chromatogram of the analysis of this sample the LOD and LOQ were set at the signal that corresponds to 3 times and 10 times of the background noise, respectively. The concentration of GTH is expressed as  $\text{mg kg}^{-1}$  fat:

*Table 3: Sensitivity of the method (calculated on fat basis)*

<b>LOD</b> $\text{mg kg}^{-1}$	4.8
<b>LOQ</b> $\text{mg kg}^{-1}$	16.0

## 10. References

- [1] European Communities. 2009. REGULATION (EC) No 1069/2009 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 21 October 2009 laying down health rules as regards animal by-products and derived products not intended for human consumption and repealing Regulation (EC) No 1774/2002 (Animal by-products Regulation). Official Journal of the European Union, L 300/1-33
- [2] European Union. 2011. COMMISSION REGULATION (EU) No 142/2011 of 25 February 2011 implementing Regulation (EC) No 1069/2009 of the European Parliament and of the Council laying down health rules as regards animal by-products and derived products not intended for human consumption and implementing Council Directive 97/78/EC as regards certain samples and items exempt from veterinary checks at the border under that Directive. Official Journal of the European Union, L 54/1-254
- [3] von Holst C, Boix A, Bellorini S, Serano F, Androni S, Verkuylen B, Margry R. 2009. Use of glyceroltriheptanoate as marker for processed animal by-products: Development and validation of an analytical method. Food Additives and Contaminants 26: 466-474
- [4] Boix A, Bellorini S, von Holst C. 2010. Validation of an analytical method for the determination of glyceroltriheptanoate (GTH) in processed animal by-products: Results of a collaborative study. Food Additives and Contaminants 27: 793-800

## 11. Annex 1

Example of applicable GC method fit for the purpose:

<b>Method</b>	
<i>Detector</i>	MS
<i>Column*</i>	DB5
<i>Injection</i>	Split (1:50)
<i>Flow (ml/min)</i>	2.1
<i>Carrier gas</i>	Helium
<i>Temperature programme</i>	70°C for 1'
	rate 45°C
	345°C for 15'
<i>Injector T</i>	250°C
<i>Detector T</i>	260°C (325°C interface)
<i>Volume injected (µl)</i>	1

Please note that these conditions are given for guidance; other conditions may be used provided that equivalent results are obtained.

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**Abstract**

This analytical procedure specifies a gas chromatographic (GC) method for the determination of glyceroltriheptanoate (GTH) in dry meat and bone meal (MBM) as produced in animal by-product rendering plants and rendered fat. Mass spectrometry detection (MS) is used to quantify GTH in target materials (i.e. Category 1 and 2 according to Regulation EC (No) 1774/2004) containing GTH around the target concentration (250mg GTH per kg fat) and to confirm the presence or absence of GTH in non-targeted materials (i.e. Category 3). This method protocol has been successfully single-laboratory and ring trial validated by the Institute for Reference Materials and Measurements of the European Commission's Joint Research Centre.

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