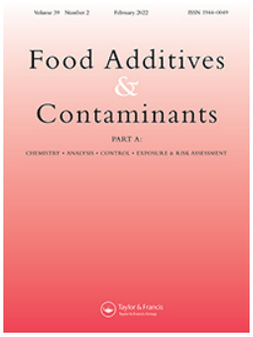

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Food Additives & Contaminants: Part A

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/tfac20

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To cite this article: Sabina Pederiva, Rosa Avolio, Sara Morello, Maria Cesarina Abete & Daniela Marchis (2022): Glyceroltriheptanoate (GTH) occurrence in animal by-products: a monitoring study to minimise safety-related risk of misuse, Food Additives & Contaminants: Part A, DOI: 10.1080/19440049.2022.2059109

To link to this article: https://doi.org/10.1080/19440049.2022.2059109

Published online: 05 Apr 2022.

FOOD ADDITIVES & CONTAMINANTS: PART A
https://doi.org/10.1080/19440049.2022.2059109

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Glyceroltriheptanoate (GTH) occurrence in animal by-products: a monitoring study to minimise safety-related risk of misuse

Sabina Pederiva[a,b], Rosa Avolio[a,b], Sara Morello[a,b], Maria Cesarina Abete[a] and Daniela Marchis[a,b]

[a]Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, Turin, Italy; [b]Italian National Reference Laboratory of Animal Proteins in Feed, Turin, Italy

ABSTRACT

Early in this century, the crisis connected to the spread of bovine spongiform encephalopathy caused a great concern related to the use of animal by-products (ABPs). According to the Commission Regulation (EU) No 1069/2009, these materials are classified in three categories according to their related risk. In 2011 Commission Regulation (EU) No 142/2011 established that meat and bone meal (MBM) and fat deriving from ABPs not intended for human consumption (category 1 and 2) are required to be permanently marked with glyceroltriheptanoate (GTH), at a minimum concentration of 250 mg kg^{-1} of fat, while category 3 processed animal proteins (PAPs) must not contain this compound. PAPs are bio resources, which could be used in a renewable and regenerative way in a circular economy model for a conscious usage of raw materials. The aim of this study was to provide information on GTH occurrence in MBM and, if any, in PAPs. Samples were collected from 2017 to 2021 and analysed by GC-MS. Detected non-compliant samples were exclusively of MBM category 1 and 2, probably due to the addition of an inadequate amount of GTH during the manufacturing processes. These results highlighted the importance of National Monitoring Programs as a useful tool to minimise safety related risk due to the misuse of GTH. Thus, investigating the critical points in feed supply-chain and sharing the information on its occurrence may help to improve animal and human wellness and safety.

ARTICLE HISTORY
Received 7 February 2022
Accepted 17 March 2022

KEYWORDS
Glyceroltriheptanoate (GTH); GC-MS; animal by-products; processed animal proteins; meat and bone meal

Introduction

The misuse of certain animal by-products (ABPs) has led in the past to the spread of bovine spongiform encephalopathy (BSE), which resulted in a concrete risk for human and animal health (EFSA Panel on Biological Hazards (BIOHAZ) 2018).

ABPs are mostly entire bodies or parts of animals, and in the EU they are classified into three categories (Commission Regulation (EU) No 1069/2009), according to their related risk. Category 1 APBs include high risk by-products (cadavers, products impounded for health reasons and specific risk materials) intended exclusively for incineration; category 2 ABPs are classified as moderate risk by-products, which can be used at technical purpose or as fertiliser, biogas and compost. Finally, category 3 ABPs include low risk by-products, which can be used in pet food manufacturing or, after specific rendering processes, for processed animal proteins (PAPs) production. PAPs could be used as ingredient in feed for some species of food producing animals, in pet food (Commission Regulation (EU) 1372/2021 2021) or as organic fertiliser and soil improvers or other derived products (Commission Regulation (EU) 1069/2009 2009).

In order to prevent and minimise the related risk due to the use of these materials, but most of all to protect the safety of the food and feed chain, Commission Regulation (EU) 142/2011 (2011) established that fat and MBM produced from ABPs not indented for human consumption (category 1 and 2) must be marked permanently with glyceroltriheptanoate (GTH) at a minimum concentration of 250 mg kg^{-1} of fat, to clearly differentiate them from category 3 PAPs, which must not contain GTH.

GTH, which is a synthetic fat with three *n*-heptanoic acids esterified with glycerol, has

CONTACT Sabina Pederiva sabina.pederiva@izsto.it Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, Turin, Italy

physicochemical characteristics that make it the most appropriate marker for fat and meat and bone meal (MBM). After its addition, GTH cannot be removed, allowing discrimination between categories and avoiding the misuse of these by-products. The Joint Research Centre's Institute for Reference Materials and Measurements (JRC-IRMM, Belgium) in 2010 developed and validated an analytical method for the detection of GTH in processed ABPs by gas chromatographic analysis (von Holst et al. 2009; Boix et al. 2010).

In 2013, the Italian National Reference Laboratory of Animal Proteins in feed (NRL-AP) validated a method for detection and quantification of GTH analysis in category 1 and 2 fat and MBM (Marchis et al. 2013), lately revised and extended to category 3 PAPs. Nowadays, this is the sole laboratory in Italy to carry out these specific analyses.

Since 2015, Italian Competent Authorities (CA) have implemented the National Animal Feed Plan to control and detect GTH in category 1 and 2 fat and MBM, sampled in rendering industries approved, according to Regulation (EU) 1069/2009. From 2018, category 3 PAPs were added to this specific official control.

The aim of this monitoring study was to provide and share information on GTH occurrence in MBM and PAPs, in order to improve controls and to minimise safety related risk of a possible misuse of these products.

Indeed, a further strengthening for efficient and effective controls on these matrices is recommendable, as the re-use of such by-products represents a key approach to the development of circular pathways for a more conscious usage of resources. In fact, the growing concern on this topic has a global impact, as it is expected that the demand for resources will double by 2050 (Geng 2019).

Learning how to benefit the use of sustainable, reusable and safety materials is the first step to act in a purposeful and mindful way and save sources for the planet.

Material and methods

Data collection and samples selection

Data collection and samples selection were carried out by Italian CA, according to Commission Regulation (EU) 691/2013 (2013), as part of the official feeding stuffs controls between 2017 and 2021 in Italy.

As reported in Table 1, 167 samples were collected and processed: 24 in 2017, 35 in 2018, 42 in 2019, 38 in 2020 and 28 in 2021. Of these, 29% were category 1 MBM, 25% category 2 MBM, 43% were category 3 PAPs and 3% were category 3 fat.

A blank category 3 animal fat was selected as negative control, after checking the absence of GTH by GC-MS analysis applying the developed method, described below.

Standards and reagents

Solvents used were *n*-hexane for analysis (VWR, Milan, Italy), isooctane GC grade (Merck, Darmstadt, Germany), petroleum ether for analysis (boiling range 40 °C–60 °C, Honeywell, Charlotte, NC, USA), and diethyl ether for analysis (Merck, Darmstadt, Germany). Solid phase extraction (SPE) cartridges, amino bonded solid phase, 500 mg, 3 mL were supplied by Phenomenex (Torrance, CA, USA). GTH standard, purity ≥99%, (CAS 620-67-7) and 5-α-cholestane, purity ≥96%, (CAS 481-21-0), used as internal standard (ISTD) to verify injection step, were purchased from Sigma-Aldrich (Milan, Italy).

GTH standard stock solution (1000 µg mL^{-1}), GTH working standard solution (10 µg mL^{-1}), ISTD stock solution (1000 µg mL^{-1}) and ISTD working standard solution (30 µg mL^{-1}) were prepared in isooctane. All solutions were stored at 4 °C in the dark for no longer than 6 months.

Method optimization

The method was developed and validated in 2013, following the JRC-IRMM method (Boix

Table 1. Samples description: number and percentage divided per year and sample type.

	Sampling year						Percentage
Sample type	2017	2018	2019	2020	2021	Total	(%)
Category 1 MBM	16	14	13	1	4	48	29
Category 2 MBM	5	6	7	18	6	42	25
Category 3 PAP	3	14	21	17	17	72	43
Category 3 Fat	0	1	1	2	1	5	3
Total per year	24	35	42	38	28	167	100

et al. 2010; Marchis et al. 2013), for routine analysis of GTH in MBM and fat. The extraction was carried out in a Soxhlet apparatus of 100 mL capacity and a 250 mL round-bottom flask. The weighed sample (10 g) was extracted with 150 mL of petroleum ether for 6 h at reflux rate of 10 cycles per hour. Then, the extract was evaporated with a rotary evaporator (Sample Concentration Hei-Vap Precision, Heidolph Instruments, Schwabach, Germany).

In 2019, the extraction step was modified as described in the next paragraph, and the method was validated to extend the scope to PAPs of category 3.

Extraction and clean-up procedure

An amount of 10 g (MBM category 1 and 2) or 15 g (category 3 PAP) was added to 100 mL of petroleum ether and shaken for 30 min with an overhead shaker (Heidolph Reax 2, Schwabach, Germany). Supernatant was filtered using a paper filter and collected in a 500 mL round-bottom flask. This procedure was repeated twice. Solvent was then evaporated with a rotary evaporator (50 °C ± 5 °C; 300–150 mBar; Sample Concentration Hei-Vap Precision, Heidolph Instruments, Schwabach, Germany) and 0.5 g of the residual fat was dissolved in 5 mL of *n*-hexane. Category 3 fat samples are weighted without a previous extraction with petroleum ether. After that, 0.2 mL of diluted extract was loaded on SPE cartridge, which was previously conditioned with 10 mL of *n*-hexane. The fraction containing the GTH was eluted through the cartridge with 8 mL of a mixture of *n*-hexane/diethyl ether (85:15, v/v) by gravity, and collected in a tube containing 0.100 mL ISTD (0.3 mg mL^{-1} in isooctane). The eluate was finally evaporated to dryness under a gentle stream of nitrogen (50 °C ± 5 °C) and the dry residue was dissolved in 5 mL of isooctane by vortexing.

Quality controls were carried out dissolving 0.5 g of blank fat sample in 5 mL of *n*-hexane. Two negative control samples were spiked with 1000 µg mL^{-1} GTH solution in isooctane at concentration of 250 mg kg^{-1} as positive control for category 1 and 2 MBM; for category 3, positive controls samples were spiked at 50 mg kg^{-1}. All control samples were then purified on SPE cartridge and processed as previously described for the in-field collected samples.

Aliquots of the purified extracts were transferred into GC vials and GC-MS analyses were performed (Thermo Electron Corporation, Austin, TX, USA).

GC-MS analytical method

Chromatographic and mass spectrometer conditions

The GC-MS chromatographic conditions were optimised to ensure the most appropriate separation of GTH and ISTD from other possible interfering compounds (Marchis et al. 2013).

The GC-MS analysis was carried out on a GC-MS Thermo Scientific instruments: DSQ™ single quadrupole mass spectrometer, FOCUS™ GC gas Chromatograph and the AS 3000 autosampler (Thermo Electron Corporation, Austin, TX, USA).

Chromatographic separation was performed on a DB-5MS 30 m capillary column (I.D. 0.25 mm; film thickness: 0.25 µm, Supelco SLB™ 5 ms). The GC oven program was set as follows: 75 °C (hold 1 min), to 290 °C at 45 °C min^{-1} (hold 1 min), then to 345 °C at 15 °C min^{-1} (hold 5 min). Other GC parameters were carrier gas (helium) flow mode at 1 mL min^{-1}, splitless injection 1 mL at 250 °C with purge valve on at 2 min.

The MS operated in electron impact, single ion monitoring mode, as follows: transfer line at 270 °C, ion source at 250 °C and solvent delay set to 7.5 min.

Quantification ions (m/z) were 113 for GTH and 217 for ISTD while qualification ions (m/z) were 285, 299 for GTH and 357, 372 for ISTD.

Quantification of GTH

For quantification, standard solutions of GTH were prepared in isooctane respectively at concentration levels 0.3, 0.5, 1.0, 1.5, 2.0 and 2.5 mg mL^{-1} for category 1 and 2 MBM, and at concentration levels 0.1, 0.2, 0.3, 0.4 and 0.5 mg mL^{-1} for category 3 PAPs and fat. ISTD concentration was 0.3 mg mL^{-1} in all standard solutions. Ratio of the GTH peak area to the

ISTD peak area against GTH concentration was used to plot the calibration curve.

Quality controls

GTH analyses were carried out applying an accredited in-house method, according to UNI CEI EN ISO/IEC 17025. The fixed limits of Quantitation (LOQ) were 125 mg kg^{-1} for category 1 and 2 MBM and 50 mg kg^{-1} for category 3 PAPs and fat.

Each analytic session had to comply with the following requirements to be acceptable: no signals blank samples; ISTD to be present in each sample at least 30% of the average calculated on the points of calibration curve; correlation coefficient (R^2) of calibration curve greater than 0.99; finally, recovery calculated on positive samples had to fulfill the requirements of the control chart (Shewart chart), routinely used to check the maintenance of repeatability and accuracy conditions.

Results

Validation results

Validation results highlighted that the method is suitable for both routine analyses of official controls in our laboratory, and for in-house monitoring processes in rendering industries, as it showed a good linearity, repeatability (RSD%), recovery and acceptable uncertainty results for all the categories analysed (Table 2; Marchis et al. 2013).

Table 2. Validation results sorted by category.

	Category 3 PAP and fat		Category 1 and 2 MBM	
Linearity	25–125 mg kg^{-1}		75–625 mg kg^{-1}	
LOQ	50 mg kg^{-1}		125 mg kg^{-1}	
LOD	16.6 mg kg^{-1}		41.6 mg kg^{-1}	
Ruggedness	Yes		Yes	
Specificity	Yes		Yes	
Working range	50–125 mg kg^{-1}		125–500 mg kg^{-1}	
	Concentration levels			
	50 mg kg^{-1}	125 mg kg^{-1}	250 mg kg^{-1}	500 mg kg^{-1}
Repeatability (RSD%)	7.43	3.36	5.09	7.87
Recovery (%)	111	88.0	89.0	85.0
Repeatability limit (r)	13.5	52.9	46.2	143
Uncertainty	25%	–	14%	–

Table 3. Non-compliant samples: number and percentage divided per year and sample type.

	Sampling Year				
	2017	2018	2019	2020	2021
Sample type					
MBM category 1	1	2	0	0	1
MBM category 2	4	1	2	5	0
PAPs category 3	0	0	0	0	0
Fat category 3	0	0	0	0	0
Total non-compliant samples	5	3	2	5	1
Total sample analysed	24	35	42	38	28
Ratio of non-compliant and analysed (%)	21	9	5	13	4

Table 4. Non-compliant sample concentration.

Year	MBM category	Concentration (mg kg^{-1})
2017	2	<125
	2	<125
	2	<125
	2	171
	1	<125
2018	1	<125
	1	<125
	2	<125
2019	2	129
	2	175
2020	2	<125
	2	<125
	2	<125
	2	<125
	2	170
2021	1	<125

Retrospective study results

As reported in Regulation (EU) 142/2011 the target content of GTH should be at least 250 mg kg^{-1} of category 1 and 2 fats. As GTH is a marker to discriminate category 1 and category 2 from category 3 materials and it should not be present in category 3 fats and PAPs.

Out of 167 samples analysed in the five-year monitoring study, 16 MBM samples (category 1 and 2) were declared non-compliant because GTH concentration was quantified as lower than 250 mg kg^{-1}. However, all category 3 PAPs samples were in accordance with the requirements as they were below the LOQ method (50 mg kg^{-1}). Non-compliant samples percentages were as follows: 21% in 2017 (5 samples out of 24 samples collected), 9% in 2018 (3 out of 35), 5% in 2019 (2 out of 42), 13% (5 out of 38) in 2020 and 4% in 2021 (1 out of 28) (Table 3).

As reported in Table 4, category 1 MBM were found at a concentration lower than the LOQ (125 mg kg^{-1}) once in 2017 and 2021, and twice in 2018. All the other non-compliant samples were category 2 MBM. Most of MBM samples were found lower than the LOQ, while in 2017

and 2020 one sample per year was quantified, at 171 mg kg^{-1} and 170 mg kg^{-1}, respectively. In 2019, the two non-compliant samples were quantified at 129 and 175 mg kg^{-1}, both category 2 MBM.

According to the EU legislation, all reported results were corrected adding the expanded uncertainty percentage to provide the more advantageous result for the customer.

Discussion and conclusions

This retrospective study highlights that category 2 MBM intended to be used as fertiliser is the most common non-compliant sample type, while all category 3 PAPs fulfill the requirement. In order to ensure its homogeneous distribution, GTH has to be added to category 1 and 2 MBM or fat during the rendering process, at core temperature of at least 80 °C. The addition of a not uniformly distributed, or an inadequate amount of GTH during the manufacturing processes may explain this trend. This retrospective study highlighted a decreasing number of non-compliant samples over the years. Thanks to these encouraging results we can assume that surveillance positively contribute to the reduction of GTH misuse related risk. However, continuous efforts must be carried out in order to ensure reliable monitoring.

Feed business operators are encouraged to apply quality management schemes, such as Hazard Analysis Critical Control Points (HACCP), rules and Good Manufacturing Practices, in order to enhance the correct use of GTH as marker in MBM production procedures. In particular, HACCP plans must be based on the critical parameters such as temperature, pressure, time and microbiological criteria as provided by Regulation (EU) 142/2011.

In 2018, EFSA BIOHAZ Panel highlighted the lack of information on GTH monitoring programs in the European Union. In fact, the only validated method available was provided by an inter-laboratory study (Boix et al. 2010) and few European laboratories monitor their performances (Marchis et al. 2013). The great concern is related to both the misuse of GTH and the incorrect labelling of ABPs, which means a wrong classification of products, often documented by audit reports from the Food and Veterinary Office (EFSA Panel on Biological Hazards (BIOHAZ) 2018).

In 2019, in order to improve and harmonise monitoring programs in all the member states, DG SANTE commissioned the European Union Reference Laboratory for Animal Protein (EURL-AP) to verify the analytical capabilities of the available methods by an inter-laboratory study (Veys and Fumière 2020). Nineteen laboratories in different countries took part to the study (Austria, Belgium, Finland, France, Germany, Greece, Hungary, Italy, the Netherlands, Poland, Romania, Slovakia, Slovenia and Switzerland). It has been demonstrated that procedures currently in use are able to support the implementation of Regulation (EU) 142/2011 granting a total traceability of category 1 and 2 MBM in order to avoid the re-entering of such products in the feed and food chain.

The growing demand of resources led the EU to enact the European Green Deal in 2019, which aims to develop a new model of circular economy based on the reduction of waste and the use of sustainable matrices. Thanks to their high-quality proteins content, low cost and slaughter waste origin, PAPs represent both an advantageous raw material and an alternative resource which may contribute to environmental pollution reduction (Alao et al. 2017), with 10% of the greenhouse gas emissions of soya meal (EFPRA 2021). Therefore, it is crucial to guarantee a safe matrix, coming from fully traceable supply lines.

In conclusion, sharing knowledge on GTH occurrence in MBM and PAPs could not only support the investigation of critical points in feed production, but could also contribute to the evolution of a civic consciousness for a better reuse of raw materials, in order to grant animal and human wellness and safety.

Acknowledgments

The Authors thank the Italian veterinary service for the precious contribution in sample collection.

Disclosure statement

No potential competing interest was reported by the author(s).

ORCID

Sabina Pederiva http://orcid.org/0000-0003-3704-9329
Daniela Marchis http://orcid.org/0000-0003-2575-0337

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