Interlaboratory study on the detection of glycerol triheptanoate (GTH) in fats by GC-MS

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Introduction

Among the measures related to the feed ban in the European Union, Commission Regulation (EU) 142/2011 [1] provides for a compulsory permanent marking of derived products from Category 1 and 2 materials with glyceroltriheptanoate (GTH) at a minimum mass fraction of at least 250 mg per kg of fat. This end point marking is intended to ensure a total traceability and to prevent such derived products of re-entering the feed and food chain. Controls for GTH detection is based on the use of gas chromatography coupled to mass spectrometry (GC-MS) [2, 3].

In 2019, DG SANTE mandated the EURL-AP to organise an inter-laboratory study on the implementation of the method for the determination of GTH in processed animal by-products. This request was initiated in a broader framework of questions as to whether the current method would be robust enough to be applied to by-products used as organic fertilisers, would be sufficiently sensitive to be applied on compound feeds possibly adulterated with Category 1 and 2 materials and for which the detection level of the method would not be low enough due to the dilution originating by the matrix. In addition the request for inter-laboratory study was also supported by questions raised by Member States on reported difficulties in the implementation of the method. After a first expert meeting where several scenarios were proposed and after joined discussion with the JRC Geel on the design of the study, a simple sample set was designed. It was based on industrial fats adulterated or not with GTH at different mass fraction levels.

Participating laboratories were designated for that purpose by the competent authorities of the Member States, based on their experience in GTH detection by using or not the official method. The objective of the study was thus to assess the method at EU level and to foresee if its revision would be required at a later stage. In addition, the applicability of the method was evaluated by assessing the laboratories' network capability to correctly determine the content of GTH in the test samples.

The project was substantially supported by Ursula Vincent and Christoph von Holst from the European Commission's Joint Research Centre Geel, Belgium, especially when setting up the design of the study and for the statistical evaluation of the analytical results submitted by the laboratories.

Study organisation

Laboratories to which the invitation was sent to join the interlaboratory study were selected by the Member States of the European Union based on an inquiry form distributed on behalf of DG SANTE to the NRLs of the EURL-AP network [ANNEX I] in January 2019. The inquiry form was intended to gather information on the expertise level in GTH detection by GC-MS of each potential participating laboratory. In June 2019, nineteen laboratories were thus invited to participate to the study [ANNEX II]. Their participation was free of any charge. Participating laboratories are listed in ANNEX III. Each participating lab received a randomly assigned code ranging from 1 to 20 (#18 was not assigned).

A protected file containing detailed instructions, a deadline for results delivery (set on the 6th of September) and a report form [ANNEX IV] was sent to the participants. Among the instructions, a responsibility agreement had to be signed to commit to following strictly the instructions. Aside the analytical result on the GTH content of each of the sample, a set of additional data related to the method implementation and parameters had to be filled. It concerned information on the reagents, the type of SPE cartridge, the type of GC column, the temperature program, information on the injection, the type of mass spectrometer analyser, and finally information on the mass ions selected for both quantification and confirmation. Participants were also invited to send the chromatograms by copying them and inserting them into a worksheet of the report form. The report form included the generation of an automated report summary which had to be sent to the organiser as a signed PDF document.

Acceptance criteria for participants' results were (1) the respect of the result return date and (2) the completeness of the results according to the instructions. No participant had to be rejected.

Materials and method

Materials

Two fats were used as matrices for the sample set; both of them were industrially produced and were commercially available. The first fat was a category 3 material from poultry origin used to produce the GTH-containing samples by spiking GTH. The second fat was a category 2 material from mixed animal origin industrially marked with GTH at an unknown mass fraction level. Both fats were collected in 10 L buckets packaging.

To ensure better homogeneity within the 10 L buckets, the fats were melted at 60°C and filtered to remove insoluble impurities. The filtrated fats were then stirred to allow homogenisation for 4-5 h and finally cooled down to solid state. The two fats were stored at 4°C before sample preparation.

The GTH utilized for spiking was of an industrial grade, also named "Special oil 107" (Oleochemicals Sasol, Germany GmbH) used in a former study [4]. Its purity was measured at 91.1%.

Sample preparation

Category 3 poultry fat without any addition of GTH was prepared first as a negative control sample. Category 2 mixed fat with an unknown GTH mass fraction was kept as such as a real industrial sample.

Other samples of Category 3 poultry fats fortified at several mass fraction levels of GTH were prepared as follows:

Initial weights of industrial GTH (ca 1 mg) were transferred into a 1 L evaporating flask. Serial fats quantities were added to the evaporating flask, in total in 8 steps, to achieve the desired mass fraction levels. Weights of fats were measured at 1 mg for the 6 first steps, and at 0.1 g for the 2 last steps. In between each step the vials containing the fats were rinsed twice with n-hexane and poured into the evaporating flask to ensure recovery of the whole fat content contained in each vial. n-Hexane was evaporated at each step by mixing under rotavapor until condensation stopped, then mixing was continued for another 3 min.

From each sample, aliquots of 25 g were taken and transferred into hermetically closed vials composing the sample set. Sample sets were produced in appropriate quantities to enable the homogeneity study, a stability study and to constitute a reserve of sets in case of damages during their transport to the participants.

All distributed units were blind for the participants; the only information was the unit numbers mentioned on the vials. Each number was unique and coded.

Sample set, assigned values and corresponding uncertainties

Each participant received a sample set composed of 6 distributed units (Table 1).

Table 1: Composition of the sample set, target assigned values and uncertainties on the assigned values.

	Industrial GTH content [mg kg^{-1}]	Target assigned value x_{pt} [mg kg^{-1}]	$u(x_{pt})$ (k=1) [mg kg^{-1}]	σ_{pt}	$u(x_{pt})/\sigma_{pt}$ (>0.3?)	score to use
Blank Cat 3	0	/	/	/	/	/
GTH25	25	22.77	1.29	4.55	0.28	z
GTH50	50	45.55	2.57	6.83	0.38	z'
GTH100	100	91.10	5.14	13.66	0.38	z'
GTH250	250	227.70	12.85	34.15	0.38	z'
Cat 2 fat	unknown	737.46*	140.56	110.61	1.27	z'

$u(x_{pt})$ is the measurement uncertainty of the assigned value
σ_{pt} is the standard deviation for the proficiency assessment
* estimated from robust mean calculated from the results of the laboratories that participated in this study

Based on the formulation or the amount of industrial GTH added, four nominal values (or target assigned values after correction for purity) were obtained from 23 mg kg^{-1} fat to 228 mg kg^{-1} fat.

Details on the assigned values and associated uncertainties of the prepared samples were calculated according to ISO 13528 [5] and are also presented in Table 1. The values of standard deviation for proficiency assessment (σ_{pt}) were estimated based on the values of relative reproducibility standard deviations (RSD_R) obtained from the validation study of the method [3]: 20% for the lowest GTH mass fraction (GTH25) and 15% for all other GTH containing samples.

For Cat 2 fat sample, the mass fraction of industrially added GTH was unknown as well as the purity grade of the used marker. Therefore the assigned value was estimated by the robust mean as a consensus of the participants results (Table 5). This choice was justified since the frequency of distribution of the values was symmetrical and unimodal as demonstrated by the kernel density estimate [ANNEX VI]. A robust standard deviation of 19 % was found. However for consistency and comparison issues with the other samples, the same σ_{pt} value of 15 % from the validation study was selected.

Homogeneity study

Prior to the distribution of the sample set, according to ISO 13528 [5] and the IUPAC guidelines [6] a random selection of 10 units of each sample was performed and analysed in duplicate by GC-MS under repeatability conditions by an expert laboratory to assess the homogeneity. The homogeneity

was estimated by comparing the sampling standard deviation (s_{sam}) calculated using a single-factor ANOVA to the standard deviation for proficiency assessment (σ_{pt}). The values of σ_{pt} were, as explained in a previous section, of 20% for the lowest GTH mass fraction (GTH25) and 15% for all other GTH containing samples, including the unknown Cat 2 fat. Homogeneity study results are summarised on Table 2.

Table 2: Homogeneity of the sample set (all values are expressed in mg kg^{-1}).

	Blank Cat 3	GTH25	GTH50	GTH100	GTH250	Cat 2 fat
mean	< 10*	21.75	41.45	77.40	204.60	662.70
s_{sam}	/	0.33	0.00	3.03	5.80	9.69
σ_{pt} (%)	/	20	15			
σ_{pt}	/	4.35	6.22	11.61	30.69	99.41
s_{sam} / σ_{pt}	/	0.08	/	0.26	0.19	0.10
result	passed	passed	passed	passed	passed	passed

* all negative results were reported as <10 by the expert laboratory

s_{sam} is the between-sample standard deviation

σ_{pt} is the standard deviation for proficiency assessment

test item are adequately homogeneous if $s_{sam} < 0.3\ \sigma_{pt}$

The homogeneity study showed the base material (Blank Cat 3) as effectively free from GTH. All other samples containing GTH were assessed as adequately homogeneous for the analyte at the sample intake of 200 µl.

Stability study

The stability results are summarised in Table 3.

Table 3: Stability of GTH50 from homogeneity study to results reporting (all values are expressed in mg kg^{-1}).

	Control *t1*-RT	Experiment *t1*-40°C
replicate 1	43.00	45.00
	42.00	42.00
replicate 2	42.00	41.00
	45.00	42.00
mean (y_1)	43.00	42.50
mean hom. *t0* (y_2)	41.45	
$\lvert y_1-y_2 \rvert$	1.55	1.05
0.3 σ_{pt}	1.87	
$\lvert y_1-y_2 \rvert \leq 0.3\ \sigma_{pt}$	passed	passed

Four units of the GTH50 (45.55 mg kg^{-1}) were randomly chosen before the sending of the samples for the homogeneity study and the sending of the sample sets to the participants. Two were stored at room temperature (control) and the two others were stored at 40 °C (experiment). After the return

of the results by the participants, these four samples were sent for analyses in duplicate to the same expert laboratory having performed the homogeneity study. The stability was thus assessed on a long-term since it covered the whole study period from the homogeneity study (t0) to the results reporting (t1). Analyses were randomly performed under repeatability conditions. Comparison of means was performed according to ISO 13528 [5].

The sample was considered as adequately stable during the course of the study.

Evaluation of results

The participants results were assessed by z scores or by z' scores when, according to ISO 13528 [5], the measurement of the uncertainty around the assigned value was not negligible i.e. accounted for more than 30% of the standard deviation for the proficiency assessment (as to see from Table 1).

Interpretations of z and z' scores were as follows: $|value| \leq 2$ were satisfactory, $2 < |value| < 3$ were questionable and $|value| \geq 3$ were unsatisfactory.

Results and discussion

Laboratory results

Original results of the participants are presented in ANNEX V.

The first sample, Blank Cat 3 fat, served as a negative control for the study. Only two labs (L5, L16) reported it as positive although at very low mass fraction levels of respectively 4.8 and 6.6 mg kg^{-1} of GTH. All other labs reported the blank as truly negative. This represents a specificity score of 89%.

Graphical representations of the participants' results sorted by ascending values as well as kernel density plots for estimating the probability density function were performed for all GTH containing samples and presented in ANNEX VI. Figure 1 (next page), related to GTH100 sample, is commented hereunder, it is illustrating the overall situation of noticed through the sample set.

Figure 1 shows a distribution of the results within the acceptance range for a large majority of participants. In the depicted case, only one result (L17) was obviously downwards deviating. The frequency of distribution is unimodal and symmetrical around the assigned value and the outlying value appears as a distinct peak as to see from the kernel distribution pattern. Same conclusions could be drawn from the other sample results distribution [ANNEX VI]: only a few apparent outlying results per result set were noted and a same associated kernel distribution pattern.

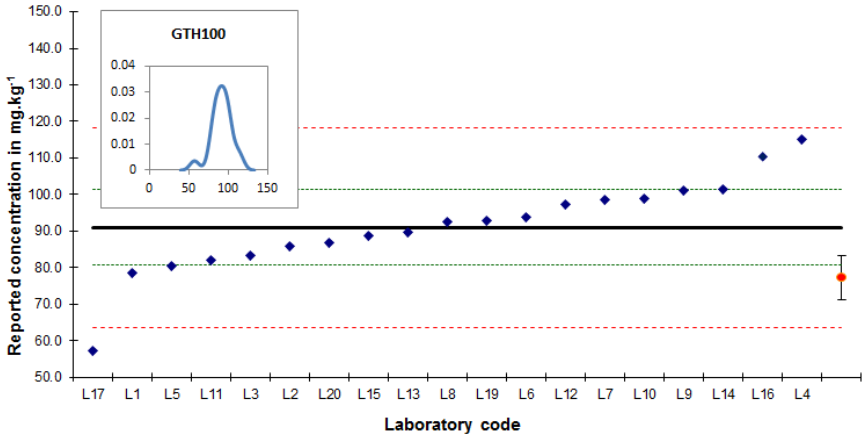

Fig.1: Sorted result range for sample GTH100 and corresponding kernel density plot. Assigned value (x_{pt}) : black line; Assigned range ($x_{pt} \pm u(x_{pt})$ (k=2)): green dotted lines; Acceptance range ($x_{pt} \pm 2\sigma_{pt}$): red dotted lines; Mean value from the homogeneity study (mean $_{hom} \pm 2u_{hom}$): red bullet.

Table 4: z and z' scores (assigned values are expressed in mg kg^{-1}).

	GTH 25	GTH 50	GTH 100	GTH 250	Cat 2 Fat
Assigned value value :	**22.77**	**45.55**	**91.10**	**227.70**	**737.46**
	z score	**z' score**			
LAB CODE					
L1	0.7	-0.3	-0.9	-0.8	-0.6
L2	0.3	-0.2	-0.4	0.2	0.1
L3	1.1	0.5	-0.6	-0.7	-0.5
L4	**10.0**	**7.8**	1.8	**-2.1**	0.7
L5	0.3	-0.1	-0.8	-1.0	-0.2
L6	0.2	1.3	0.2	0.0	-0.1
L7	**7.6**	**4.1**	0.6	1.8	2.0
L8	0.3	0.6	0.1	-0.1	0.2
L9	1.3	0.9	0.7	1.1	0.2
L10	**3.1**	-1.1	0.6	**2.8**	2.0
L11	1.4	0.5	-0.7	-0.3	-0.2
L12	1.5	**-2.3**	0.4	**2.1**	-0.3
L13	-1.3	-1.8	-0.1	**5.0**	-0.7
L14	**2.4**	1.1	0.8	0.4	0.2
L15	1.1	0.2	-0.2	0.7	0.2
L16	**3.1**	**2.7**	1.4	1.4	1.7
L17	**2.7**	-1.8	**-2.5**	-1.9	-1.3
L19	0.3	1.5	0.1	-0.7	-0.4
L20	0.4	-1.4	-0.3	-0.1	-1.6
Nb of lab	19	19	19	19	19
Satisfactory	13	15	18	15	19
Questionable	2	2	1	3	0
Unsatisfactory	4	2	0	1	0
% satisfactory z or z'	**68%**	**79%**	**95%**	**79%**	**100%**

The portion of satisfactory z and z' scores (Table 4) ranged from 68 % (for GTH25) to 95 % (for GTH100) and 100 % (for Cat 2 industrial fat) of the participants. The percentage of unsatisfactory scores was very limited (0 % for GTH100 and Cat 2 industrial fat, 5 % for GTH250 and 11 % for GTH50) with the exception of the lowest level of mass fraction (21 % for GTH25). The percentage of questionable scores accounted for 8 % of the total results. Furthermore, the robust mean calculated from the results reported are statistically comparable to the corresponding assigned values of GTH present in the test items (Table 5). An additional aspect allowing to evidence the fitness-for-purpose of the method was comparing the robust means and their associated standard error (*i.e.* the reproducibility standard deviation divided by the square root of the number of participants) calculated from all reported results for all GTH fortified sample types (logically excluding the Cat 2 fat for which the assigned value was estimated by the robust mean). Figure 2 displays a graphical representation of these robust values versus the assigned values.

Therefore, it can be concluded that the study is globally satisfactory. Performance achieved tends to demonstrate a global good implementation of the GTH detection by GC-MS.

Table 5: Statistical comparison of the robust mean with the assigned value for GTH in each test sample

	x_{pt} [mg kg^{-1}]	σ_{pt} [mg kg^{-1}]	Robust mean [mg kg^{-1}]	Robust standard deviation [mg kg^{-1}]	RSD [%]	Standard Error
GTH 25	22.77	4.55	29.16	6.74	23.1	1.55
GTH 50	45.55	6.83	47.70	12.04	25.2	2.76
GTH 100	91.10	13.66	91.72	10.56	11.5	2.42
GTH 250	227.70	34.15	236.78	51.67	21.8	11.86
Cat 2 Fat	-	110.61	737.46	140.14	19.0	32.15

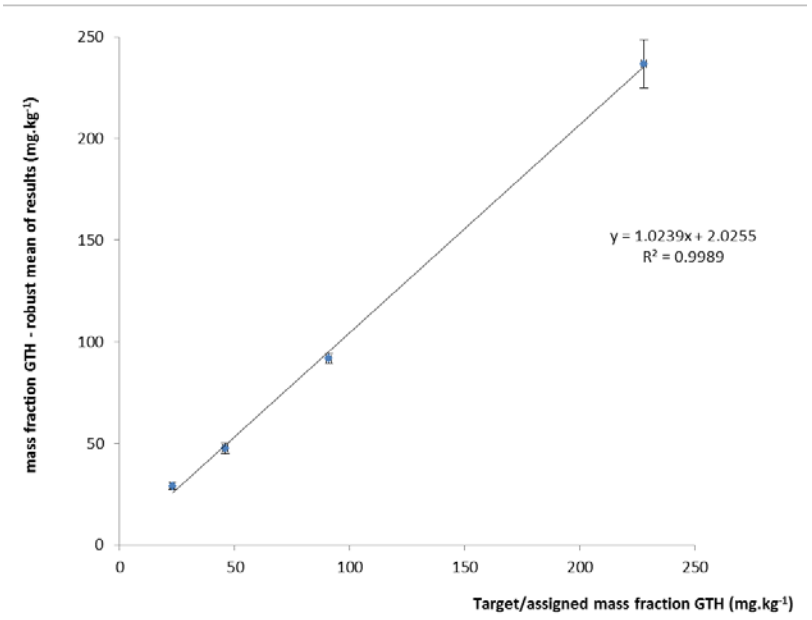

Fig.2: Robust means (± SEM) vs target values of GTH.

An analysis of the z and z' score values through the sample set nevertheless points to some laboratories collecting a high number (more than one on the series of 6 samples) of unsatisfactory scores: L4, L7 and L10. All of these unsatisfactory scores are due to overestimations of the GTH content. No unsatisfactory score for underestimation of the GTH content was noted in the present study. Questionable values of z and z' scores were evenly caused by underestimations (3 out of 8 questionable values) or by overestimations (5 out of 8 questionable values) of GTH content.

The high value of the coefficient of determination (R^2= 0.99) (Figure 2) evidences the respective agreement of the robust means with the assigned values of GTH demonstrating an absence of significant bias through the extended mass fraction range.

Complement of information from collected additional data

With the exception of L2, all participants returned their results with the result report form properly filled with complementary information as requested. These additional data are presented in ANNEX VII.

The collected complementary information intended to study the variation in the implementation of the GC-MS through a set of variable parameters. This set of parameters offers certain flexibility but no specific impact, individually considered or combined, on the overall method performance could be signalled out.

Conclusion

This inter-laboratory study was organised in 2019 by the EURL-AP mandated by DG SANTE to assess the implementation of the method for the determination of GTH in processed animal by-products in the EU Member States. Another objective of the study was also to assess the method at EU level and to foresee if its revision would be required at a later stage.

The overall performance of the participants was satisfactory; according to the z or z' score, 95 % of the laboratories perform correctly for the determination of GTH at 100 mg kg^{-1} in fat, 79 % at 50 mg kg^{-1} and 250 mg kg^{-1}, respectively and 68 % at 25 mg kg^{-1}. As for the Cat 2 industrial fat sample, 100 % of the participants reported satisfactory results. These results confirm their analytical capabilities to support the implementation of Commission Regulation (EU) 142/2011 on a compulsory permanent marking of derived products from Category 1 and 2 materials with glyceroltriheptanoate (GTH) at a minimum mass fraction of at least 250 mg per kg of fat, as part of the measures related to the feed ban in the European Union and intended to ensure a total traceability and to prevent such derived products of re-entering the feed and food chain.

Lastly, the method having proven to be fit for the intended purpose, the study also allowed to conclude that within the current legal scope, namely the detection of GTH in processed animal by-products, no revision was deemed necessary. Considering the broader scope of questions referred into the introduction as to whether this method would be adapted for the detection of GTH in other matrices such as fertilisers or compound feed, this study establishes a first step for further modelling and investigations.

Acknowledgements

The authors are grateful to the participating laboratories who volunteered to this study and for their eagerness to deliver complete results as well as complementary information on request of the organiser. The expert panel for GTH detection, set up on behalf of DG SANTE and the EURL-AP played an important role in the setting up of this study. The authors are grateful to them: Giuseppina Amato and Daniela Marchis (IZSTO - Italy), Thorsten Buhrke (BfR - Germany), Harald Dahlmans (NutriControl – the Netherlands), Aleksandra Grelik and Ewelina Kowalczyk (PIWet - Poland), Gabrijela Tavčar-Kalcher (University of Ljubljana - Slovenia). A special thanks to Lisa Plasmans, Julien Maljean, Julie Hulin for their technical assistance in the sample preparation.

References

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ANNEX I

European Union Reference Laboratory for Animal Proteins in feedingstuffs

Walloon Agricultural Research Centre, Valorisation of Agricultural Products Department
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Chaussée de Namur 24, B – 5030 GEMBLOUX

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Invitation to participate in an inter-laboratory study on the implementation of the analytical procedure for the determination of glyceroltriheptanoate (GTH) in animal by-products

Gembloux, 31th January 2019

Commission Regulation (EU) 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 provides for a compulsory marking of derived products from Category 1 and 2 materials with glyceroltriheptanoate (GTH). GTH detection is based on the use of gas chromatography coupled to mass spectrometry (GC-MS). The method is available for download from https://ec.europa.eu/jrc/sites/jrcsh/files/JRC_68602_GTH_protocol_JRC_technical_note_6th_edition.pdf

DG SANTE mandated the EURL-AP to organise in 2019, an inter-laboratory study on the implementation of the methods for determination of GTH in processed animal by-products, by the laboratories designated for that purpose by the competent authorities of the Member States, with the objective to assess the method at EU level and to foresee if required its revision at a later stage.

As a first step, this study intends to collect information regarding the state of play of the implementation of the analytical procedure for the detection of GTH in each Member State. As Member of the National Reference Laboratory network, you are asked to provide the following information:

- List of laboratories carrying out analysis for the determination of GTH in your Member State;
- Method used:
  - does it strictly follow the analytical procedure as described in the JRC technical note? In case of negative answer, please explain the deviations to the JRC method.
  - is the method validated?
  - what are the critical points identified in the implementation of the method?
- Number of GTH analyses per year,
- Name and e-mail of contact person in each laboratory,
- Name of the laboratory, address, postcode, city, phone number that wishes to participate in the proficiency testing (at least one per Member State).

Please send this information by e-mail to the EURL-AP secretary by end of February 2019 at the latest.

The second step of the study will consist on implementation testing. Fat samples will be distributed for GTH determination, by end of June 2019. The participants' results should be communicated to the organiser by the beginning of September.

More information and a detailed timetable will be directly communicated to the participating laboratories once designated.The participation is free of charge for the participating laboratories whether they are public or private.

Any complementary information can be asked by e-mail at the EURL-AP secretary address.
Yours Sincerely,

Dr Gilbert Berben,
EURL-AP Director

Dr Pascal Veys
NRL network manager

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ANNEX II

European Union Reference Laboratory for Animal Proteins in feedingstuffs

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Information on the inter-laboratory study on the implementation of the analytical procedure for the determination of glyceroltriheptanoate (GTH) in animal by-products

Gembloux, 21th June 2019

Dear Colleagues,
Dear Participants,

By end of February, you were designated by your Member State as a participant to the implementation test on the detection of GTH in animal by-products by gas chromatography coupled to mass spectrometry (GC-MS). The study is organised by the EURL-AP on behalf of DG SANTE.

In order to prepare you to the study, we are sending you the present letter with first practical information:

1. **Objective of the study**: assessment of the method at EU level to foresee if revision of the GTH detection method is required at a later stage. This is thus *not a proficiency assessment of the participants*.

2. **Time table**: as announced in the invitation, the samples should be sent by end of June 2019 to the participants. At writing time the homogeneity study of the sample set is on-going. So depending on the results the samples may be sent a bit later (*but no later than mid July 2019*). Upon sending of the samples you will have 6 weeks to send the results back to the organiser. A fixed deadline will be communicated in the sample acknowledgement form that you have to return once the samples will be received. This deadline will also be mentioned into the instructions and results reporting form.

3. **Number and type of samples**: the sample set will be composed of 6 fat samples (marked or not with GTH). Each sample is identified by a unique number.

4. **Identification of participants**: each participant will be identified by a unique number which will be communicated on the sample acknowledgement form included in the shipment box. This identifier is only known to the organiser and the corresponding participant.

5. **Instructions and reporting**: simultaneously to the sending of the sample set, a protected excel file containing both the instructions and the report form will be sent by email to each participant individually.

For reminder your participation is totally free of charge.

Any other practical information shall be found into the instructions/reporting excel file. Nevertheless if you would need complementary information or explanation do not hesitate to contact the organisers directly by e-mail at the EURL-AP secretary address referred in the heading.

Yours Sincerely,

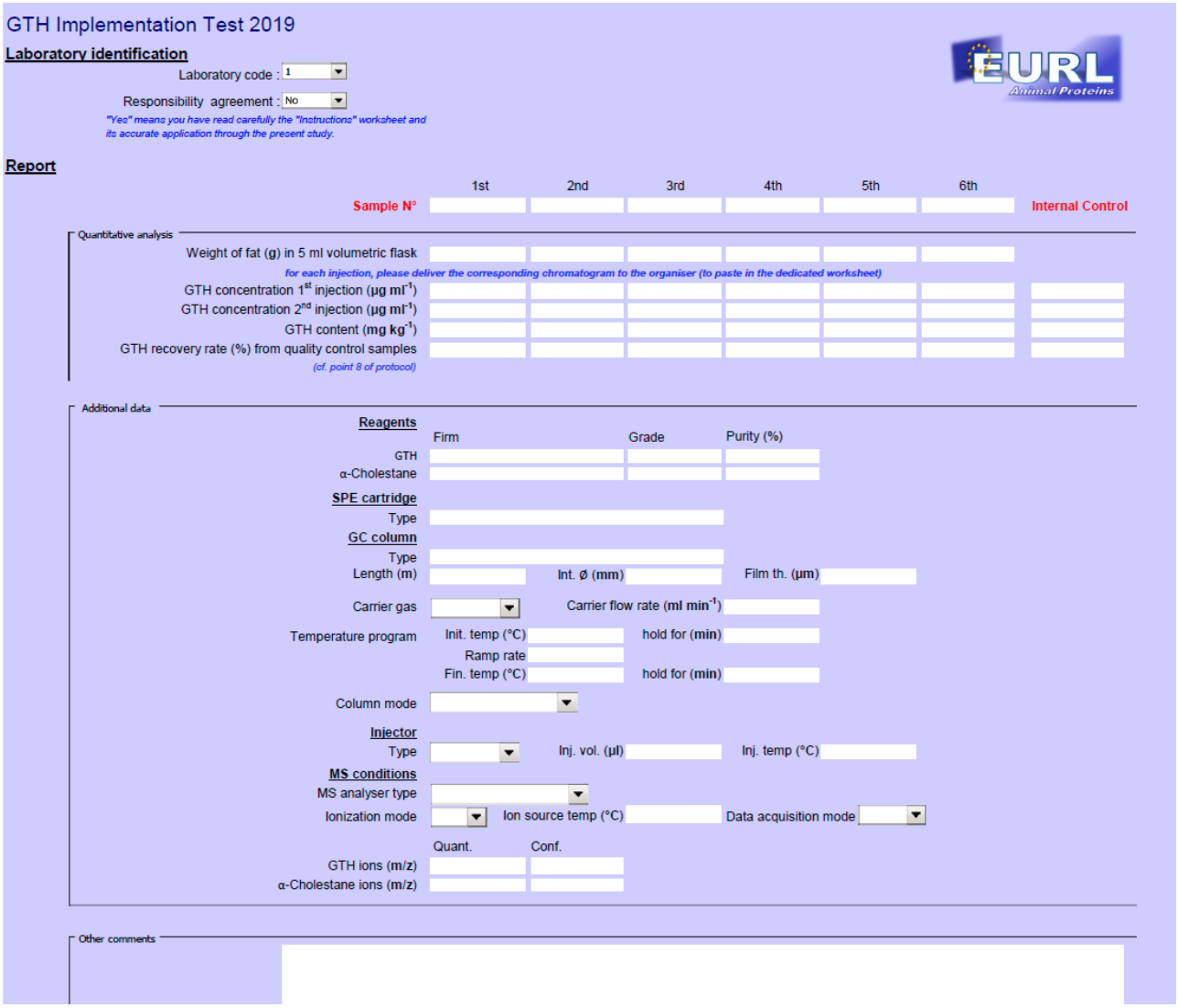
Dr Olivier Fumière,
o.fumiere@cra.wallonie.be

Dr Pascal Veys
p.veys@cra.wallonie.be

ANNEX III

Country	Laboratory name
Austria	AGES - Group for Contaminant and Special Analysis
Belgium	Servaco Food Control
Belgium	FLVVT
Finland	Finnish Food Authority - Chemistry Unit
France	Service Commun des laboratoires ; Etablissement de RENNES SCL L35
France	Inovalys
Germany	Chemisches und Veterinäruntersuchungsamt (CVUA) Sigmaringen
Germany	Chemisches und Veterinäruntersuchungsamt Westfalen (CVUA Westfalen), Anstalt des öffentlichen Rechts, Standort Arnsberg
Germany	Federal Institute for Risk Assessment (BfR), Standort Jungfernheide
Greece	General Chemical State Lab - A Chemical Service of Athens - section B
Hungary	Analytical National Reference Laboratory, NÉBIH - National Food Chain Safety Office
Italy	Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta
Netherlands	NutriControl
Poland	National Veterinary Research Institute - Department of Feedingstuffs Hygiene
Poland	Wojewódzki Inspektorat Weterynarii w Kielcach
Romania	Institutul de Igienã si Sãnãtate Publicã Veterinarã Institute for Hygiene and Veterinary Public Health
Slovakia	State veterinary and food institute Dolny Kubin
Slovenia	University of Ljubljana, Veterinary Faculty, National Veterinary Institute
Switzerland	Federal Department of Economic Affairs, Education and Research EAER, Agroscope, Division Method Development and Analytics

ANNEX IV



GTH Implementation Test 2019

Laboratory identification

Laboratory code : 1

Responsibility agreement : No

"Yes" means you have read carefully the "Instructions" worksheet and its accurate application through the present study.

Report

	1st	2nd	3rd	4th	5th	6th	
Sample N°							Internal Control

Quantitative analysis

Weight of fat (g) in 5 ml volumetric flask							
for each injection, please deliver the corresponding chromatogram to the organiser (to paste in the dedicated worksheet)							
GTH concentration 1st injection (µg ml^{-1})							
GTH concentration 2nd injection (µg ml^{-1})							
GTH content (mg kg^{-1})							
GTH recovery rate (%) from quality control samples *(cf. point 8 of protocol)*							

Additional data

Reagents

	Firm	Grade	Purity (%)
GTH			
α-Cholestane			

SPE cartridge

Type

GC column

Type

Length (m) | Int. Ø (mm) | Film th. (µm)

Carrier gas | Carrier flow rate (ml min^{-1})

Temperature program: Init. temp (°C) | hold for (min); Ramp rate; Fin. temp (°C) | hold for (min)

Column mode

Injector

Type | Inj. vol. (µl) | Inj. temp (°C)

MS conditions

MS analyser type

Ionization mode | Ion source temp (°C) | Data acquisition mode

	Quant.	Conf.
GTH ions (m/z)		
α-Cholestane ions (m/z)		

Other comments

Picklists to ensure correct reporting were available.

ANNEX V

Original results

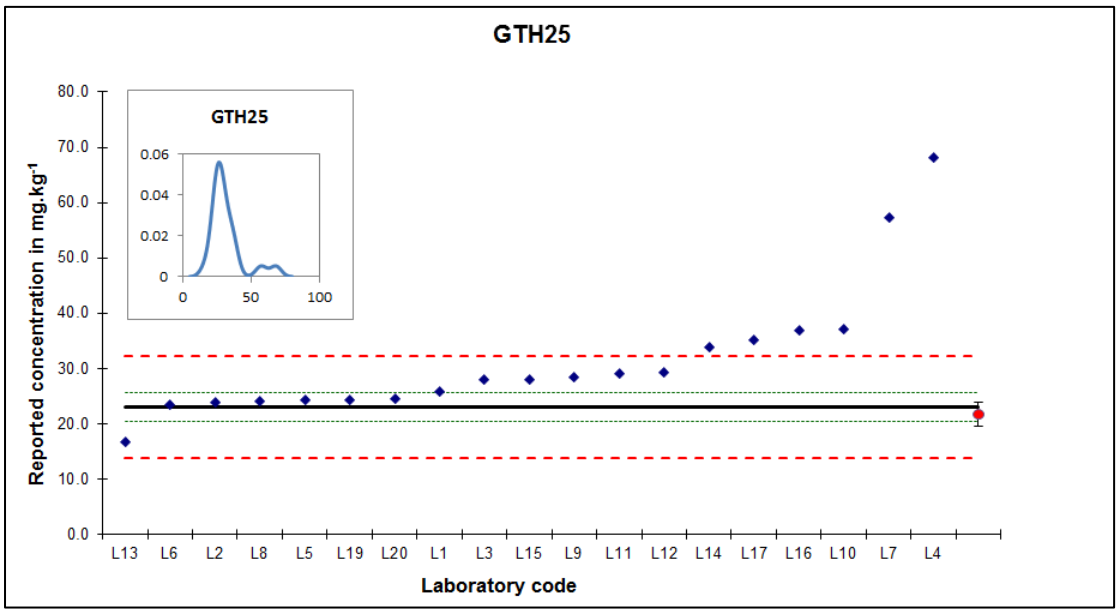
Target values :	Blank Cat 3 0	GTH 25 23	GTH 50 46	GTH 100 91	GTH 250 228	Cat 2 Fat 737
LAB CODE						
L1	0.0	25.9	43.3	78.5	201.7	636.8
L2	0.0	24.0	44.0	86.0	233.0	762.0
L3	0.0	28.0	48.8	83.3	202.7	649.6
L4	0.0	68.1	98.5	115.2	155.7	871.2
L5	4.8	24.3	44.6	80.4	194.1	709.9
L6	0.0	23.5	54.5	93.9	228.4	728.6
L7	0.0	57.3	73.8	98.7	287.5	1092.3
L8	0.0	24.2	49.8	92.6	225.4	767.9
L9	0.0	28.5	51.4	101.0	266.6	781.6
L10	0.0	37.1	38.1	99.0	323.3	1088.0
L11	0.0	29.0	49.0	82.0	219.0	702.0
L12	0.0	29.4	29.7	97.2	298.0	684.2
L13	0.0	16.7	33.3	89.8	397.0	611.0
L14	0.0	33.8	52.8	101.4	241.1	769.9
L15	0.0	28.0	46.6	88.7	250.0	772.0
L16	6.6	36.9	63.9	110.4	275.6	1038.0
L17	0.0	35.2	33.4	57.2	163.0	504.4
L19	0.0	24.3	56.0	92.9	204.0	673.6
L20	0.0	24.6	36.2	86.8	223.0	455.0

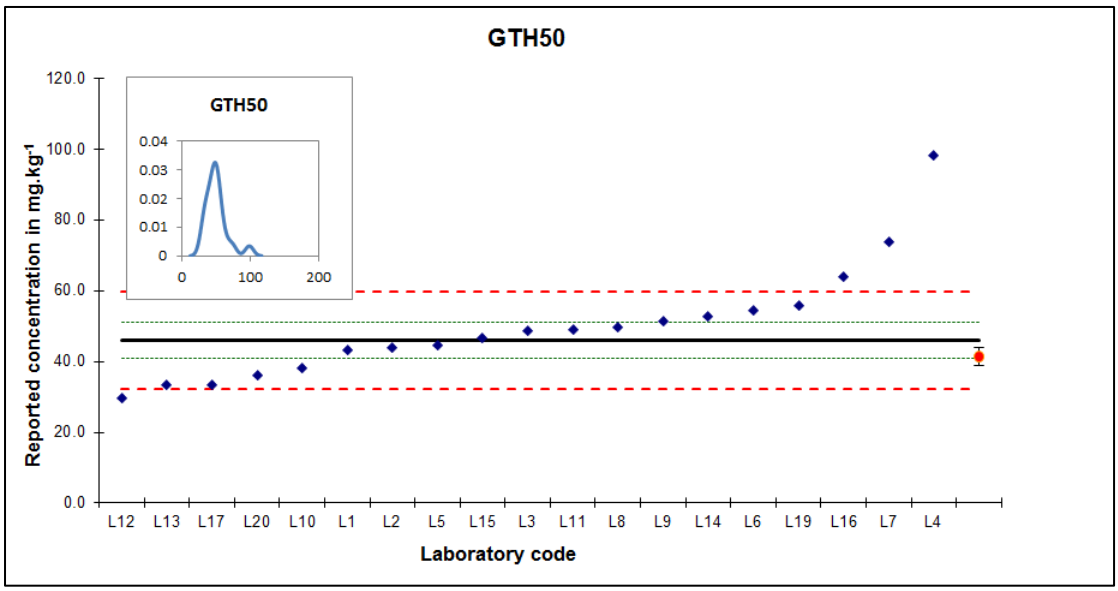
All data are in mg kg^{-1} of fat

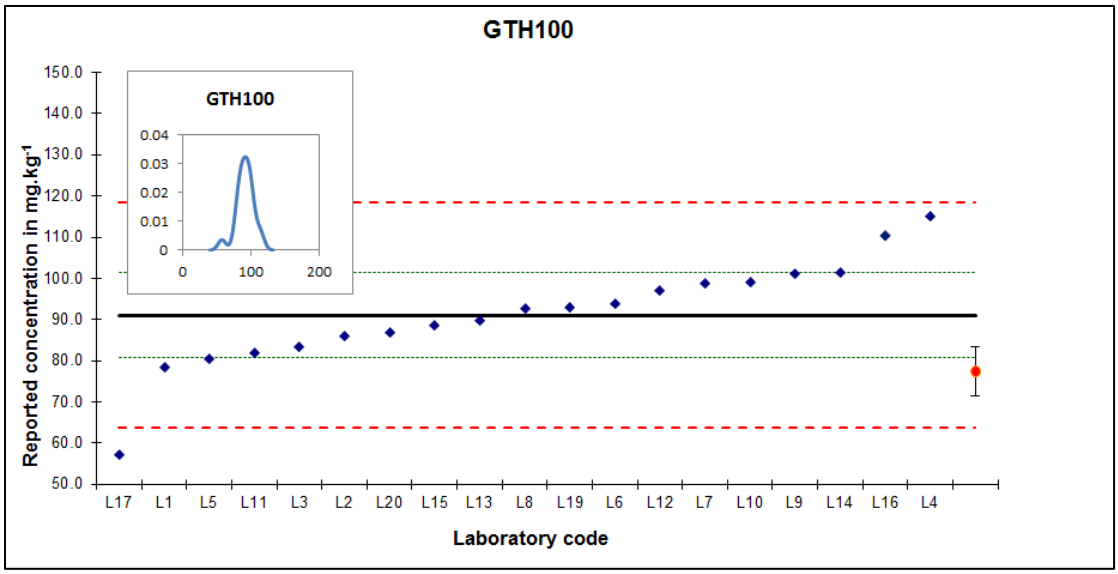
ANNEX VI

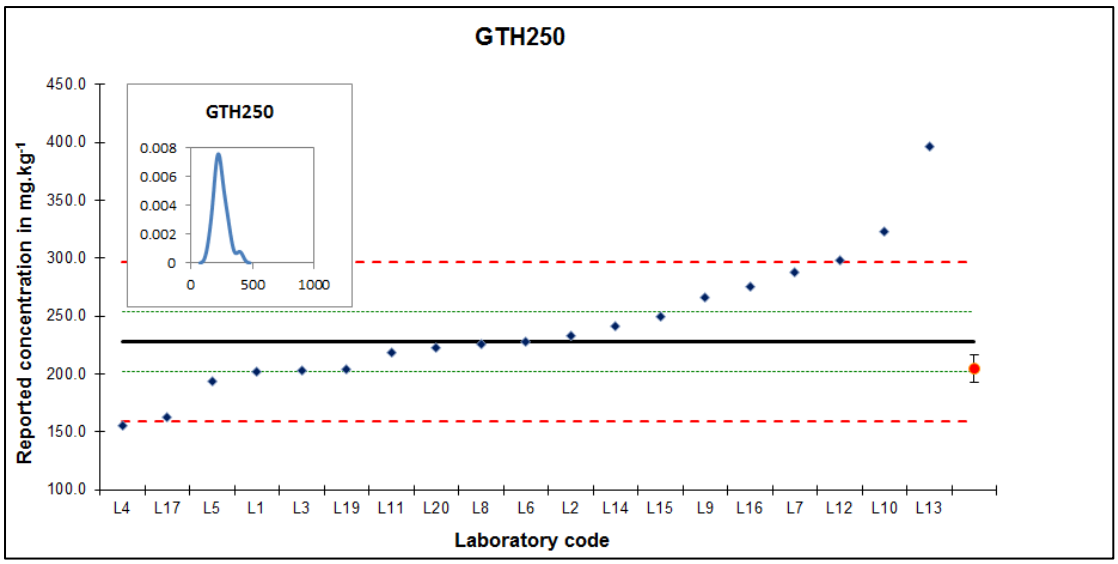
Graphical results presentation.

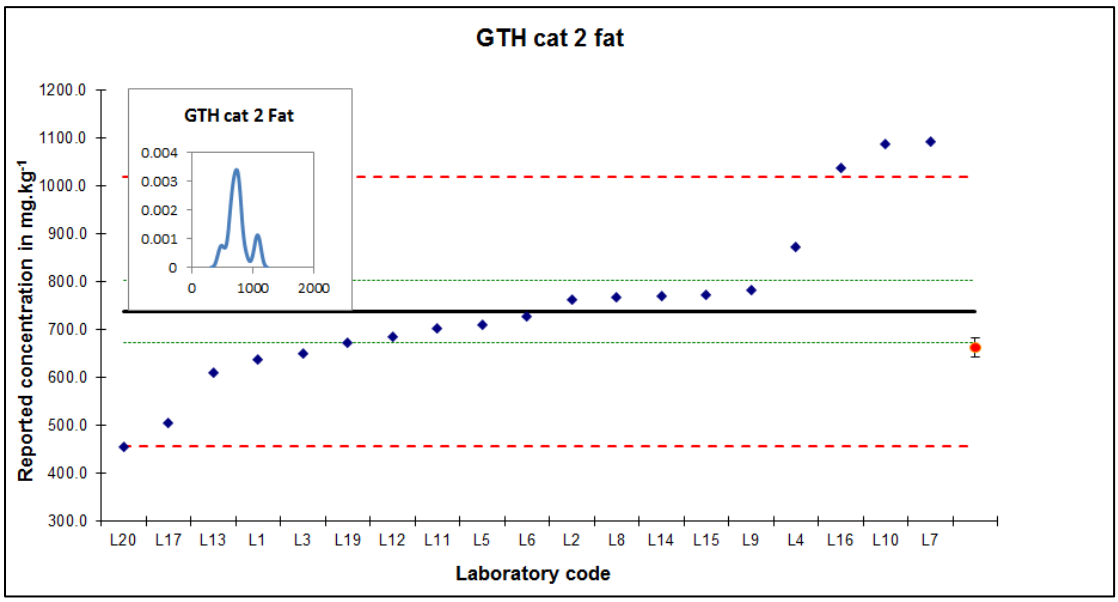
Assigned value (x_{pt}) : bold black line; Assigned range ($x_{pt} \pm u(x_{pt})$ (k=2)): green dotted lines; Acceptance range ($x_{pt} \pm 2\sigma_{pt}$): red dashed lines; Mean value from the homogeneity study (mean $_{hom} \pm 2u_{hom}$): red bullets on the right.






GTH100
GTH100
Reported concentration in mg.kg-1
150.0
140.0
130.0
120.0
110.0
100.0
90.0
80.0
70.0
60.0
50.0
0.04
0.03
0.02
0.01
0
0
100
200
L17 L1 L5 L11 L3 L2 L20 L15 L13 L8 L19 L6 L12 L7 L10 L9 L14 L16 L4
Laboratory code


GTH250
GTH250
Reported concentration in mg.kg-1
450.0
400.0
350.0
300.0
250.0
200.0
150.0
100.0
0.008
0.006
0.004
0.002
0
0
500
1000
L4 L17 L5 L1 L3 L19 L11 L20 L8 L6 L2 L14 L15 L9 L16 L7 L12 L10 L13
Laboratory code


GTH cat 2 fat
GTH cat 2 Fat
Reported concentration in mg.kg-1
1200.0
1100.0
1000.0
900.0
800.0
700.0
600.0
500.0
400.0
300.0
0.004
0.003
0.002
0.001
0
0
1000
2000
L20 L17 L13 L1 L3 L19 L12 L11 L5 L6 L2 L8 L14 L15 L9 L4 L16 L10 L7
Laboratory code

ANNEX VII

Summary of collected additional data.

reagents	purity (%)	Nb of Labs
GTH	94	8
	99	3
	> 99	7
α-cholestane	96	3
	97	9
	99	1
	> 99	5

solid phase extraction (SPE) cartridge:		
firm	type	Nb of Labs
Agilent	Bond Elut NH2	9
Phenomenex	Strata NH2	4
Macherey-Nagel	Chromabond NH2	2
Biotage	Isosolute NH2	1
Interchim	Upti-Clean NH2-S	1
no cartridge used		**1**

GC column:	Nb of Labs
DB-5MS	6
HP-5MS	6
VF-5MS	2
PAH	1
CP-Sil 24CB	1
Rxi-5MS	1
ZB-5MSI	1

column length [m]	diameter [mm]	film thickness [µm]	Nb of Labs
60	0.25	0.25	2
40	0.18	0.07	1
30	0.25	0.25	14
30	0.32	0.25	1
20	0.18	0.18	1

carrier gas :	helium only

injector type :	all of split/splitless type (except one PTV)

injected volume :	Nb of Labs
1 µl	9
2 µl	7
3 µl	2

injection °C :	Nb of Labs
250	9
265	1
270	2
280	2
300	3
unknown	1 (PTV inj. mode)

	GAS Flowrate [ml.min^{-1}]	Initial °C	duration [min]	RAMP RATE	Final °C	duration [min]	Column mode
L1	100	70	1	45	330	4	Ramped P
L3	1.4	70	1	45	325	8	Constant Fl
L4	0.4	90	1	45	330	6	Constant Fl
L5	1.1	60	1	25	320	2	Constant Fl
L6	1	75	1	45	345	5	Constant Fl
L7	0.91	70	1	45	345	45	Constant Fl
L8	2	160	0.5	14	280	15	Constant Fl
L9	1.2	80	2	45	330	5	Constant Fl
L10	1	75	1	15	345	5	Constant Fl
L11	1	70		45	320	15	Constant Fl
L12	0.8	90	1	25	325	19.6	Constant Fl
L13	1	75	1	45	290	1	Constant Fl
L14	1.4	90	1.5	10	330	4	Constant Fl
L15	2.1	70	1	45	325	4	Constant Fl
L16	2.6	70	2	45	325	8	Constant Fl
L17	1	130	1	30	345	2	Constant P
L19	1	80	1	20	300	10	Constant Fl
L20	1.2	50	1	15	300	16.33	Constant Fl

MS analyser type :	Nb of Labs
Quadrupole	13
Triple quadrupole	4
Ion trap	1

Ionization mode : Electron Ionization only

Ion source °C	Nb of Labs
175	1
200	3
220	1
230	8
250	4
280	1

Aquisition mode:	Nb of Labs
Scan	2
SIM	15
unknown	1

GTH ions [m/z]		α-cholestane ions [m/z]		
Quantif.	Conf.	Quantif.	Conf.	Nb of Labs
113	299	217	357	2
113	85	121	217	1
113	299	217	372	1
113	unknown	121	unknown	1
285	299.3	217	372.5	1
299	285	217	372	5
299	285	217	372.5	1
299	285	372	357	1
299	285	372	217	1
299.1	285	217	372.4	1
299.2	185.1	217	372	1
299.3	285	271	372.5	1
299.3	285.2	217.2	372.4	1