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Validation of an analytical method for the determination of glyceroltriheptanoate (GTH) in processed animal by-products: Results of a collaborative study

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ABSTRACT

The performance characteristics of a method for the determination of the marker substance glycerol triheptanoate (GTH) in processed animal by-products (ABPs) based on gas chromatography (GC) coupled to mass spectrometry (MS) were determined via a collaborative study. Within the European Union GTH needs to be added to the portion of processed ABPs that must not enter the feed and food chain (Categories 1 and 2) at a minimum concentration of 250 mg kg⁻¹ related to the fat fraction of the test samples analysed. The test materials included in the validation study consisted of three meat and bone meal (MBM) and three fat samples that contained GTH at different concentrations ranging from 61 to 455 mg kg⁻¹. The relative standard deviation of *repeatability* (RSD_r) varied from 3.4 to 7.8% and the relative standard deviation of *reproducibility* (RSD_R) varied from 9.0 to 16.5%, corresponding to HORRAT values that were in all cases equal or below the critical value of 2.0. The estimated trueness expressed in terms of average concentration compared to the target concentrations of GTH in all test materials varied from 95 to 107 % confirming acceptable values for the trueness of the method. Based on the acceptable values for the precision and trueness 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.

Keywords: marker; animal by-products; glyceroltriheptanoate; collaborative trial

INTRODUCTION

The European animal by-products Regulation (EC) No.1774/2002 (European Communities 2002) requires that certain raw materials and processed products that pose an elevated health risk need to be permanently marked with a marker substance to ensure identification and traceability of products to be disposed of. These materials belong to Category 1 or 2 as specified in this Regulation and must not enter the food and feed chain. Since mid of 2008 glyceroltriheptanoate (GTH) needs to be added as marker substance in plants processing animal by-products (ABPs) from these categories. The target concentration of GTH is at least 250 mg kg⁻¹ related to the fat fraction of the ABPs (European Union 2007). In order to enforce the proper use of GTH as marker, there is strong need for analytical method that can be applied by Member States' official feed laboratories. In a former publication (von Holst et al. 2009) we elaborated on the background of this new requirement and presented the performance profile of a single-laboratory validated method for the determination of GTH in meat and bone meal (MBM) and rendered fat, which are the main types processed animal by-products.

The primary aim of the study presented in this paper was to assess the performance characteristics of this analytical method via a collaborative trial designed according to the IUPAC harmonised protocol for method performance studies (Horwitz 1995). The method protocol comprises three main steps, which are (i) extraction of GTH from the samples, (ii) clean-up of the extracts using solid phase extraction (SPE) cartridges and (iii) determination of GTH by gas chromatography coupled to mass spectrometry (GC/MS).

The main method performance characteristics assessed in this study were the relative standard deviation of *repeatability* (RSD_r), which is the precision of the method when the method is performed *within* one laboratory and the relative standard deviation of *reproducibility* (RSD_R) which is the precision of the method when the method is performed *between* laboratories. In addition, the trueness of the method has been estimated by comparing the average values of the laboratories' results with the results obtained in the homogeneity study of the test material. Furthermore the specificity could also be evaluated, since the laboratories had to analyse MBM and fat without GTH (blank samples).

METHODS AND MATERIALS

Study design

Since the laboratories had to apply a specific gas chromatographic method, a step by step approach was applied including an initial training period prior to the validation study. In the training phase the laboratories had to analyse a set of known and unknown samples,

which were three MBM and one fat sample with a feedback questionnaire about the execution of the method protocol. This training period allowed the participants to get familiarised with the method protocol, to evaluate their effective performance on a limited number of samples and to check their rigorous application of the protocol. Feedback from the training period was used to adjust the method protocol and to facilitate correct application of the method. Based on the delivered results 19 out of 20 laboratories were finally invited to participate in the second phase of the study, which concerns the actual validation of the method.

The revised method protocol was sent to the laboratories that had four weeks to analyse a set of 14 samples composed of (i) three MBM and three fat as blind duplicate samples and (ii) two blank samples consisting of MBM and fat respectively. Six different test samples containing GTH at various levels were included in the study, thus fulfilling the minimum number of 5 different materials specified in the IUPAC harmonised protocol (Horwitz 1995).

Laboratories were asked to report on the GTH concentration in the samples expressed in mg kg^{-1} related to the fat fraction of the tests samples, since this reflects the legal requirement of adding at least 250 mg GTH per kg fat. Additional requested information were the 2 measured values of the GTH concentration obtained from 2 injections of the same purified extract expressed in $\mu\text{g ml}^{-1}$. Participants also had to provide detailed information on how the analytical method was performed and to report on the slightest deviation from the given protocol.

Statistics

The study design and the target performance characteristics for the evaluation of the GC method were selected according to the IUPAC harmonised protocol (Horwitz 1995). The study is based on the principle that the laboratories analyse a set of blind samples, which consists of duplicate samples of meat and bone meal and fat containing GTH at different concentration levels. After removal of outliers according to a fixed procedure, the laboratories' results were subjected to analysis of variance (ANOVA), in order to estimate the values for the RSD_f and the RSD_R for each type of the test materials. A mean value for the GTH concentration was also calculated from the reported GTH concentrations for each test material.

Extreme values were not included in the statistical evaluation,, especially when they were related to obvious experimental deviations from the protocol. Furthermore, as indicated by the IUPAC protocol (Horwitz 1995) the initial reported valid data must be purged of all outliers flagged by the harmonised outlier removal procedure. This procedure basically consists of the sequential application of the Cochran and Grubbs tests (at 2.5% probability level, 1 tail for Cochran and 2 tails for Grubbs) until no further

outliers are flagged or until a drop of 22.2% (= 2/9) in the original number of laboratories providing valid data would occur.

The precision data obtained from the collaborative study was then compared with predicted acceptable levels of precision. These levels are calculated from the Horwitz equation (Horwitz et al. 1980), which represents an empirical relation between the acceptable precision and the corresponding analyte concentration in the sample analysed. Thus the precision calculated with this equation provide an indication as to whether the method is sufficiently precise for the concentration level of the analyte being measured. This indication is expressed by the HORRAT value (Horwitz and Albert 2006). For the between-laboratories reproducibility the HORRAT value is a comparison of the measured reproducibility precision with the precision calculated by the Horwitz equation for a method measuring at that particular concentration level of analyte:

$$HORRAT = \frac{RSD_R}{RSD_{R,Horwitz}}$$

where RSD_R is the relative standard deviation of reproducibility obtained in the validation exercise and $RSD_{R,Horwitz}$ is the calculated standard deviation of reproducibility using the Horwitz equation:

$$RSD_{R,Horwitz} = 2^{(1-0.5\log C)}$$

where C is the mass concentration expressed in power of 10 (i.e. 1 mg/g = 10^{-3}). Here, the mean values of the GTH concentration obtained for each test material (Table 3) have been taken to calculate $RSD_{R,Horwitz}$. The target value for the HORRAT value is set at 1, the acceptance limit is 1.5 and the rejection level is 2.

Test materials

Twelve different materials processed under realistic rendering conditions consisted of MBM and fat from Categories 1 and 2 containing GTH at different concentration level including two blank samples were used in the study (Table 1). The levels of the target concentration in the test materials were selected to ensure that the laboratories had to analyse test materials containing GTH *at*, *below* and *above* of the legally binding minimum concentration of 250 mg GTH per kg fat. The GTH concentration in the test samples of the validation study varied from 61 to 455 mg kg⁻¹, established in the corresponding homogeneity study.

Mixtures of MBM samples from a former study (Boix et al. 2006) in which we demonstrated the applicability of the marker concept under realistic conditions were used for the preparation of materials 2, 3, 6, 7 and 8. Homogenisation of each material was achieved by splitting a bulk of test material into smaller portions using a commercial sample divider (Retsch PT 100, Hahn Germany) and pooling the portions again. This procedure was repeated 3 times. From the pooled and homogenised material after the third mixing step, 11 grams of material were packed into sealed aluminium bags. In total 647 units of MBM containing GTH at five different concentrations were produced accordingly. Fat materials (4, 10, 11 and 12) were used as produced by rendering plants for the implementation study (Boix et al. 2006). Hundred units of approximately 1.5 grams were prepared from each material and kept in glass vials for dispatching.

Materials 1 to 4 were used for the training period and were sent in duplicate to the participants. One of the replicates was sent as unknown sample while the concentration in the other replicate was known. Materials 5 to 12 were sent as blind samples to the participants for the validation phase of the study. All samples except the blanks (materials 5 and 9) were sent as duplicates.

Table 1 should be here

Sufficient homogeneity of all packed test materials was tested prior to their use in the collaborative study. According to the experimental design (Thompson and Wood 1993) 10 sample bags for each of the MBM test materials and 10 glass vials for the fat test materials were randomly selected. Two subsamples were taken from each of the selected containers, thus obtaining 20 samples to be analysed for GTH by GC/MS analysis. The analytical results were finally subjected to ANOVA to estimate the “within container” variability reflecting the analytical error and the “between container” variation reflecting potential heterogeneity. By calculating the F-value of both variances and comparing against the critical F-value (3.02, $\alpha=0.05$), it could be established whether both variances were significantly different or not. In addition, the average value for the GTH concentration was calculated for each test material and taken as target concentration in the validation study. F-values of the test materials presented in Table 1 showed that all test materials that were used in the validation study passed the test for sufficient homogeneity. The slightly elevated F-value for MAT 2 was accepted, since the reported results from the training period were not used to calculate method performance characteristics.

Method

Details of the method protocol are given in a previous publication of the single-laboratory validation of the method (von Holst et al. 2009). The method is based on the principle that GTH concentrates in the fat fraction of the samples, which is related to the specific physicochemical properties of this compound. When dealing with MBM samples,

a sub-sample of about 10 g is extracted using a soxhlet apparatus. Fat extracted from MBM or fat samples themselves was treated in an oven to get liquid and 0.5 g of the liquid fat was dissolved in n-hexane. The sample is subjected to clean-up using SPE aminopropyl-bonded solid-phase cartridges, in order to separate free fatty acids present in the fat and that may interfere with the gas chromatographic determination of the target analyte. Then, 5 α -cholestane is added as internal standard and finally, the purified extract is analysed with GC/MS operated in single ion monitoring mode and GTH is quantified by external calibration using the internal standard 5 α -cholestane. The method protocol did not include a specific GC column along with corresponding GC parameters to be applied but general criteria for the selection of appropriate GC conditions. The method protocol also requires that each batch of unknown samples contains a sample with a known GTH concentration in order to calculate the percentage recovery rate (RR). The system is considered to be under control if the measured RR is above 80 %.

Initially we intended to validate two methods based on the same extraction and clean-up procedures, but using different detectors, namely mass spectrometry and flame ionisation detection (FID). However, the results from the training period showed that only a small number of laboratories applied FID. Therefore we decided to validate exclusively the method using MS as detection mode.

RESULTS AND DISCUSSION

Training phase

In the training phase the laboratories were asked to determine the GTH concentration in the 8 training samples that included known and unknown samples of materials 1 to 4 (Table 1). In addition, laboratories had to provide precise information about how the analytical method was performed and to report comments on the protocol. Chromatograms of all analysed samples were also requested and scrutinised to ensure that the laboratories had selected appropriate GC conditions. The laboratories used different GC columns representing different polarity, but most of them used non polar columns such as Agilent DB 5 ms, Restek-5 ms or Varian CP Sil 8 CB. A minor portion of the laboratories conducted the analysis with medium polar columns such as Varian CP-TAP CB.

20 laboratories out of the 22 invited to participate in the study delivered complete results. One laboratory notified at the beginning of the training period their impossibility of taking part in the study, while a second laboratory delivered incomplete results produced in the training period only when the validation period was already launched. The low variation of the results reported by 20 laboratories indicated that most of the laboratories had no problem to implement the method and to report consistent values for the GTH concentration (data not shown).

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15 laboratories out of 20 reported MS results, 3 laboratories used FID and 2 laboratories reported results by using both detectors. The low number of laboratories delivering FID data did not allow for an appropriate statistical evaluation of the corresponding results; therefore only laboratories able to deliver MS results were invited to participate in the validation study. From the three laboratories only reporting FID data, two laboratories requested to take part in the validation study as they were able to provide data using MS as detection mode. 19 out of 20 laboratories that delivered results in the training period were therefore finally invited to participate in the validation phase.

Some considerations arisen during the training period were communicated to the participants mainly concerning the clean-up procedure and the GC conditions especially regarding the need of ensuring a good chromatographic separation of the GTH from the internal standard (5 α -Cholestane) and any other possible interfering compound. The importance of strictly following the protocol was highlighted. The method protocol was therefore slightly modified for the final validation phase and as guidance, the GC conditions set up at the IRMM laboratory were included as annex.

Validation phase

Nineteen laboratories reported results for the validation period of the collaborative study. In this phase, the participants analysed 14 blind samples (i.e. 3 MBM and 3 fat samples in duplicates, one blank MBM and one blank fat sample) as shown in Table 1. As in the training phase, information about the method and chromatograms of all analysed samples was requested. The results provided by the individual laboratories are summarised in Table 2. Figure 1 shows a typical chromatogram of one sample of Material 7 (target concentration 256 mg GTH per kg fat) submitted by one of the participating laboratories.

Figure 1 should be here

Regarding the analysis of the blank samples, 17 laboratories correctly reported negative results, whereas two laboratories reported false positive results for the blank samples. When checking the respective chromatograms an obvious problem due to automatic integration was observed. The laboratories were contacted and a new set of results was reported with correct negative results for the blanks. Therefore specificity of the method could be confirmed in this study.

During the execution of the study, the SPE cartridges proposed in the protocol for performing the clean-up procedure were withdrawn from the market. The alternative cartridge proposed by the supplier gave, according to some laboratories, low recovery rates. As stated in the protocol, cartridges from other suppliers are considered suitable if they are capable of retaining the free fatty acids in the extracted fat fraction. Complementary experiments carried out at the IRMM laboratory indicated that other

commercially available SPE cartridges that fulfil the criteria given in the literature (Ruiz et al. 2004) are suitable for the intended purpose as well.

Table 2 should be here

Prior to the calculation of the precision values, the data set was screened for the presence of results that need to be removed as specified in the harmonised protocol (Horwitz 1995). Criteria were major deviation from the method protocol and outlier detection by statistical tests.

Major deviation from the method protocol: The method protocol also contained a compulsory analysis of a sample with known GTH content (quality control sample) to demonstrate that the procedure as implemented in the different laboratories is under control. Average GTH concentration in the quality control samples expressed in terms of percentage recovery rate for the training and the validation periods are summarised in Figure 2. Four laboratories (No. 9, 15, 20 & 21) reported recovery rates (RR) for the quality control samples below 80%, which would require corrective measures to be taken prior a valid results could be reported. Since these laboratories proceeded with the analysis although the RR was below 80 %, the corresponding results for the GTH content in the unknown samples were therefore excluded from the statistical evaluation.

Figure 2 should be here

Outliers according to statistical tests: According to the IUPAC protocol (Horwitz 1995), outliers identified by the harmonised outlier removal procedure, should not be included in the statistical evaluation. This procedure consists of the sequential application of the Cochran and Grubbs tests on each test material set of results separately until no further outliers are identified. In short, the Cochran allows the identification of laboratories that report results from the duplicate samples of individual test materials, where the difference between the duplicates significantly deviates from the corresponding differences reported by the other laboratories. Complementary, the Grubbs checks whether the mean of the duplicate results from individual laboratories deviates significantly from the corresponding mean values from the other laboratories. After application of this procedure, *only one* laboratory was identified as outlier for MAT 12 and the corresponding pair of results was therefore excluded from the statistical assessment of the precision of the method.

In total, results from four laboratories for all test materials and one additional pair of results for MAT 12 have been removed, thus using results from 14 laboratories (Mat.12) and 15 laboratories (Mats 6, 7, 8, 10 and 11) for the statistical assessment of the precision

of the method. Therefore the important criterion of at least 8 laboratories as required from the IUPAC guideline (Horwitz 1995) has been fulfilled for all test materials.

Table 3 should be here

The results of the statistical evaluation are summarised in Table 3, showing the mean values of the each test material and the corresponding values of the precision of the method. The RSD_r ranged between 3.4 and 7.8%, which corresponds quite well with corresponding precision obtained in the single-laboratory validation of the method (von Holst et al. 2009). The obtained RSD_R varied from 9.0 to 16.5% and the corresponding HORRAT values were equal to or below 2.0. These results demonstrated that the precision values are acceptable and the method can therefore be considered as fit for the intended purpose.

Regarding the use of the Horwitz equation we were confronted with the specific situation that the matrix analysed i.e. MBM did not coincide with the reference matrix which was the fat fraction of the MBM and which was used to calculate the GTH concentrations. This aspect did not affect the calculated precision data expressed as percentage relative standard deviations but the concentration inserted in the Horwitz equation to estimate the reference relative standard deviation ($RSD_{R,Horwitz}$). In consequence also the Horrat values would differ. In detail, by taking into account the fat content of the test materials as indicated in Table 1, the mean GTH concentrations in MAT 6, 7 and 8 become 22, 28 and 46 mg/kg related to the MBM matrix, respectively. These are lower values compared to the GTH concentrations calculated on fat basis (Table 3). Consequently, the values for $RSD_{R,Horwitz}$ based on the MBM related GTH concentrations are higher compared to the situation when the GTH concentrations are related to the fat fraction of the samples. This leads to lower Horrat values, namely 1.2 for MAT 6, 0.9 for MAT 7 and 1.1 for MAT 8. Since in both cases the obtained Horrat values were below the critical value of 2 and in order to maintain consistency throughout the whole study, we did all calculations on fat basis. However, the situation might be different in future studies when conducting a proficiency test, which requires setting a target standard deviation for calculating z-scores. Most often the $RSD_{R,Horwitz}$ is used as target standard deviation and the organiser of this study could then decide whether to calculate this value based on the concentration related to the matrix or the fat fraction.

We also evaluated the trueness of the method by comparing the average values for the GTH concentration in the test materials obtained in the study with the target value for GTH in the test material calculated from the homogeneity study (Table 3). As shown in Figure 3, in all test materials the estimated trueness varied from 95 to 106 % confirming the acceptable values for the trueness of the method.

Figure 3 should be here

CONCLUSIONS

A GC method coupled to MS detection for the determination of GTH in processed products from Category 1 and Category 2 materials, has been successfully validated via a collaborative study, in which 19 laboratories from 11 EU Member States participated. The obtained relative standard deviation of *repeatability* (RSD_r) varied from 3.4 to 7.8% and the relative standard deviation of *reproducibility* (RSD_R) varied from 9.0 to 16.5%, corresponding to HORRAT values that were in all cases equal to or below the critical value of 2.0. Furthermore the standard deviation for reproducibility can be used by the laboratories to estimate the measured uncertainty of analytical results obtained with the present method. The estimated trueness expressed in terms of average concentration obtained in the study compared to the target concentrations of GTH in all test materials varied from 95 to 107 %. Based on the acceptable values for the precision and trueness 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. Specificity of the method has also been demonstrated, since the blank MBM and fat samples have been correctly identified as negative.

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Materials												
	Training period				Validation study							
	1	2	3	4	5	6	7	8	9	10	11	12
Processed Product	MBM	MBM	MBM	FAT	MBM	MBM	MBM	MBM	FAT	FAT	FAT	FAT
Fat content (%)		15.7	16.5			11.8	10.3	9.9				
GTH concentration (mg kg ⁻¹)	-	112	404	199	-	191	256	455	-	186	301	61
F-value		6.2	1.1	2.6		1.0	0.9	1.4		0.75	0.71	0.45
Samples per laboratory	2	2	2	2	1	2	2	2	1	2	2	2

Table 1. Target concentrations of GTH expressed as mg kg⁻¹ related to the fat content of the test materials. In addition F-values and the average GTH concentrations obtained in the homogeneity study that were used as target concentrations are also displayed. The test material was considered as homogeneous enough and therefore accepted when the calculated F-value was below the critical F-value (3.02, $\alpha=0.05$) MBM: meat and bone meal.

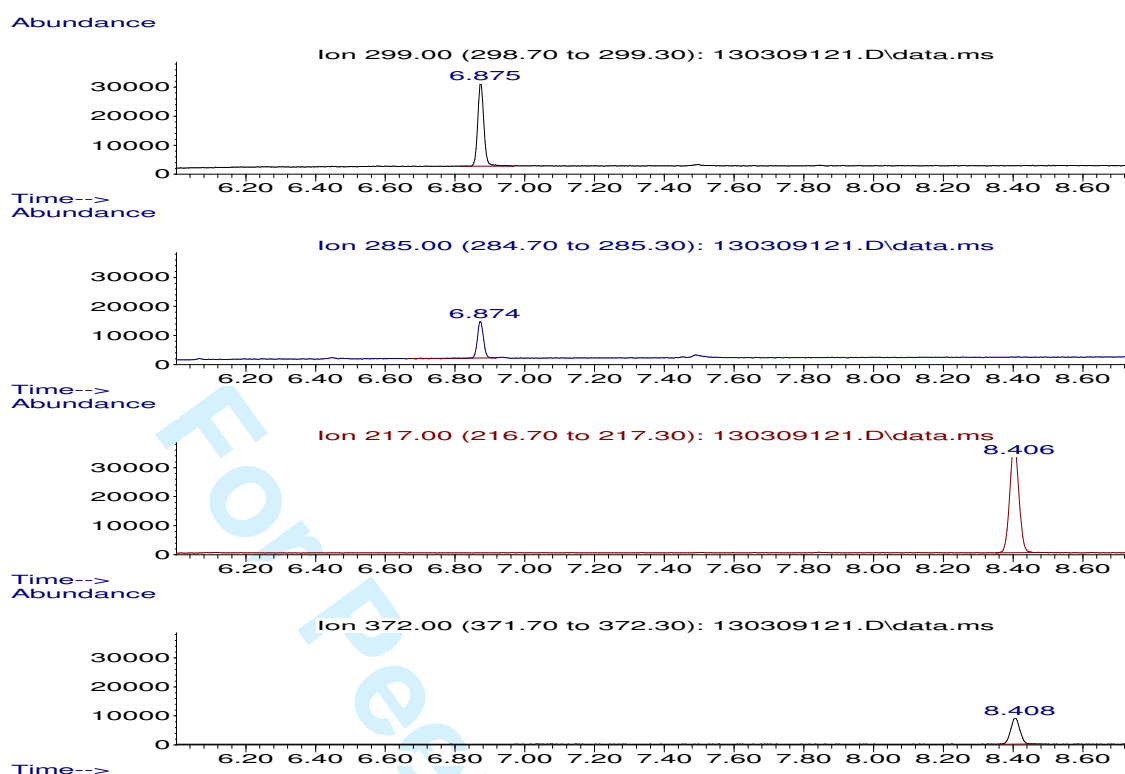


Figure 1. Chromatogram submitted by one of the participants corresponding to a blind sample of Material 7 (i.e. MBM with target concentration of 256 mg GTH kg⁻¹ fat). The GTH peak has a retention time of 6.87 and the internal standard has retention time of 8.41.

Lab No	Mat 5 blank	Mat 6, MBM (191)		Mat 7, MBM (256)		Mat 8, MBM (455)		Mat 9 blank	Mat 10, Fat (186)		Mat 11, Fat (301)		Mat 12, Fat (61)	
1	<13	176	198	245	276	431	454	<13	161	174	271	299	31	57
2	<7.5	169	163	246	248	447	413	<7.5	158	154	276	261	49	45
3	<20	179	197	290	309	543	585	<20	214	213	382	381	63	73
5	0	160	141	220	267	495	512	0	131	153	243	227	45	49
6	<5	186	197	281	281	493	498	<5	190	196	318	316	51	61
7	<15	169	160	235	240	392	394	<15	158	150	267	259	50	47
8	<1.0	170	160	245	239	401	390	<1.0	172	168	280	283	53	53
9	<10	84	99	141	120	191	233	<10	140	122	121	245	51	49
10	<10.0	205	180	299	309	527	529	<10.0	193	197	354	329	63	66
11	<5	189	185	273	277	467	486	<5	175	183	303	308	55	54
12	<10	200	203	278	298	489	482	<10	170	172	295	349	55	53
14	0	248	228	311	291	458	454	0	206	178	363	352	64	67
15	0	89	123	123	257	409	447	0	100	142	254	243	0	0
16	<10	193	192	275	288	471	420	<10	187	183	310	316	58	57
17	<7.5	198	191	278	280	488	466	<7.5	185	180	301	300	63	59
18	<0.8	217	187	291	294	450	449	<0.8	231	210	365	339	87	71
19	<16.0	196	196	256	286	477	459	<16.0	202	196	268	275	58	67
20	<1.7	131	130	178	206	334	308	<1.7	115	129	206	182	39	40
21	<7.0	156	148	224	223	367	383	<7.0	127	128	226	230	37	36

Table 2. Individual results of the blind duplicates from the validation phase as reported by the participants. All concentrations are given in mg kg⁻¹. The target concentration values for GTH are given in brackets next to the material number. Results from laboratories of which the identification number is given in grey background have been removed prior to statistical analysis due to deviation from the protocol (see text). One individual pair of results from one laboratory (in grey background) has been identified as outlier by the Cochran test and therefore excluded from the statistical assessment.

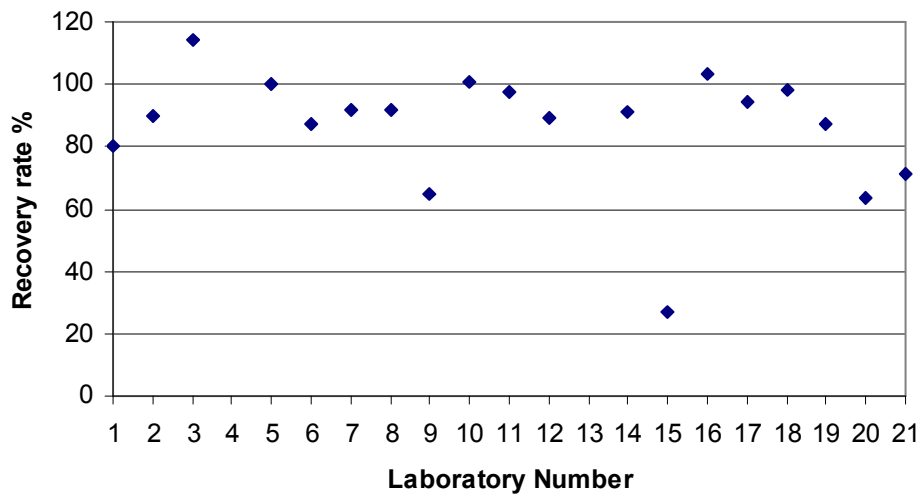


Figure 1. GTH concentration in the quality control samples expressed in terms of percentage recovery rate reported by the laboratories for the validation study.

Processed product	Mat 6 MBM	Mat 7 MBM	Mat 8 MBM	Mat 10 FAT	Mat 11 FAT	Mat 12 FAT
No. of laboratories	19 (4)	19 (4)	19 (4)	19 (4)	19 (4)	19 (5)
Target concentration (mg kg ⁻¹)	191	256	455	186	301	61
Mean (mg kg ⁻¹)	188	273	467	181	306	58
S _r (mg kg ⁻¹)	10.8	13.6	16.1	8.5	13.9	4.6
RSD _r (%)	5.7	5.0	3.4	4.7	4.5	7.8
S _R (mg kg ⁻¹)	22.1	24.5	47.1	22.8	41.4	9.6
RSD _R (%)	11.8	9.0	10.1	12.6	13.5	16.5
HORRAT value	1.6	1.3	1.6	1.7	2.0	1.9

Table 3. 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.

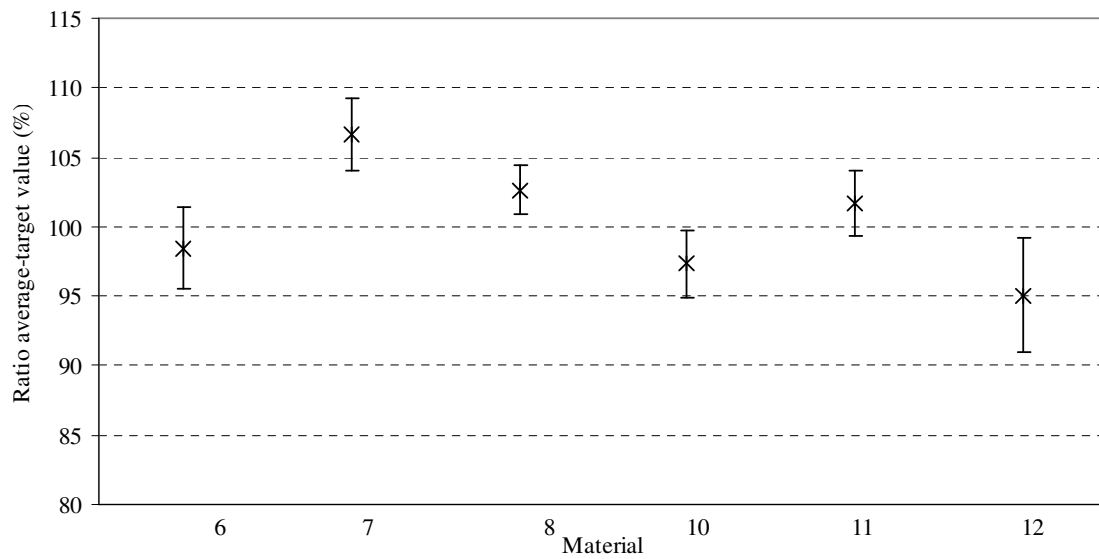


Figure 1. Estimated trueness of the method expressed in terms of average concentrations obtained in the study compared to the target concentrations of GTH. Error bars represent the expanded uncertainty which is twice the standard error, calculated from RSD_R (Table 3) and the number of laboratories (14 for MAT 12 and 15 for all other materials).