Event-specific Method for the Quantification of Cotton Line GHB614 Using Real-time PCR

Validation Report

5 September 2008

Joint Research Centre
Institute for Health and Consumer Protection
Biotechnology & GMOs Unit

Executive Summary

The JRC as Community Reference Laboratory for GM Food and Feed (CRL-GMFF), established by Regulation (EC) No 1829/2003, in collaboration with the European Network of GMO Laboratories (ENGL), has carried out a collaborative study to assess the performance of a quantitative event-specific method to detect and quantify the GHB614 transformation event in cotton DNA (unique identifier BCS-GHØØ2-5). The collaborative trial was conducted according to internationally accepted guidelines [1, 2].

In accordance with Regulation (EC) No 1829/2003 of 22 September 2003 on genetically modified food and feed and with Regulation (EC) No 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003, Bayer CropScience provided the detection method and the samples (genomic DNA from cotton plants containing the transformation event GHB614 and from conventional cotton plants). The JRC prepared the validation samples (calibration samples and blind samples at unknown GM percentage [DNA/DNA]). The collaborative trial involved twelve laboratories from ten European countries.

The results of the international collaborative trial met the ENGL performance requirements. The method is therefore considered applicable to the control samples provided, in accordance with the requirements of Annex I-2.C.2 to Commission Regulation (EC) No 641/2004.

The results of the collaborative study are made publicly available at http://gmo-crl.jrc.it/.

Drafted by:
C. Savini

Report Verification Team:
1) M. Ermolli

2) M. Querci

Scientific and technical approval:
M. Mazzara

Compliance with CRL Quality System:
S. Cordeil

Authorisation to publish:
G. Van den Eede

Address of contact laboratory:
European Commission, Joint Research Centre
Institute for Health and Consumer Protection (IHCP)
Biotechnology and GMOs Unit – Community Reference Laboratory for GM Food and Feed
Via Fermi 2749, 21027 Ispra (VA) - Italy

Report on Steps 1-3 of the Validation Process

Bayer CropScience submitted the detection method and control samples for cotton event GHB614 (unique identifier BCS-GHØØ2-5) under Article 8 and 20 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".

The Community Reference Laboratory for GM Food and Feed (CRL-GMFF), following reception of the documentation and material, including control samples, (step 1 of the validation process) carried out the scientific assessment of documentation and data (step 2) in accordance with Commission Regulation (EC) No 641/2004 "on detailed rules for the implementation of Regulation (EC) No 1829/2003 of the European Parliament and of the Council as regards the application for the authorisation of new genetically modified food and feed, the notification of existing products and adventitious or technically unavoidable presence of genetically modified material which has benefited from a favourable risk evaluation" and according to its operational procedures ("Description of the CRL-GMFF Validation Process", http://gmo-crl.jrc.it/guidancedocs.htm).

The scientific assessment focused on the method performance characteristics assessed against the method acceptance criteria set out by the European Network of GMO Laboratories and listed in the "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (http://gmo-crl.jrc.it/guidancedocs.htm) (see Annex 1 for a summary of method acceptance criteria and method performance requirements). During step 2, two scientific assessments were performed and requests of complementary information addressed to the applicant. Upon reception of complementary information, the scientific evaluation of the detection method for event GHB614 was positively concluded in March 2008.

In January 2008, the CRL-GMFF verified the purity of the control samples provided and in March 2008 the CRL-GMFF verified experimentally the method characteristics (step 3, experimental testing of samples and methods) by quantifying five blind GM levels within the range 0.09%-4.5% on a genome copy number basis. The experiments were performed under repeatability conditions and demonstrated that the PCR efficiency, linearity, accuracy and precision of the quantifications were within the limits established by the ENGL. The DNA extraction module of the method was previously tested on samples of food and feed and a report was published on the CRL-GMFF website on 14th March 2007 (http://gmo-crl.jrc.it/statusofdoss.htm).

A Technical Report summarising the results of tests carried out by the CRL-GMFF (step 3) is available on request.

Content

1. Introduction

Bayer CropScience submitted the detection method and control samples for cotton event GHB614 (unique identifier BCS-GHØØ2-5) under Article 8 and 20 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".

The Joint Research Centre (JRC, Biotechnology and GMOs Unit of the Institute for Health and Consumer Protection) as Community Reference Laboratory for GM Food and Feed (see Commission Regulation EC (No) 1829/2003) organised the international collaborative study for the event-specific method for the detection and quantification of GHB614 cotton. The study involved twelve laboratories, among those listed in Annex II ("National reference laboratories assisting the CRL for testing and validation of methods for detection") of Commission Regulation (EC) No 1981/2006 of 22 December 2006.

Upon reception of method, samples and related data (step 1), the JRC carried out the assessment of the documentation (step 2) and the in-house evaluation of the method (step 3) according to the requirements of Commission Regulation (EC) No 641/2004 and following its operational procedures.

The internal experimental evaluation of the method was carried out between January and March 2008.

Following the evaluation of the data and the results of the internal laboratory tests, the international collaborative study was organised (step 4) and took place in April 2008.

A method for DNA extraction from cotton seeds, submitted by the applicant, was evaluated by the CRL-GMFF in order to confirm its performance characteristics. The protocol for DNA extraction and a report on method testing is available at http://gmo-crl.jrc.it/.

The operational procedure of the collaborative study included the following module:

- ✓ Quantitative real-time PCR (Polymerase Chain Reaction). The method is an event-specific real-time quantitative TaqMan® PCR procedure for the determination of the relative content of event GHB614 DNA to total cotton DNA. The procedure is a simplex system, in which a cotton *adhC* (*alcohol dehydrogenase C*) endogenous assay (reference gene) and the target assay (GHB614) are performed in separate wells.

The international collaborative study was carried out in accordance with the following internationally accepted guidelines:

- ✓ ISO 5725 (1994).
- ✓ The IUPAC "Protocol for the design, conduct and interpretation of method-performance studies" (Horwitz, 1995).

2. List of participating laboratories

As part of the international collaborative study the method was tested in twelve laboratories to determine its performance.

In November 2007 the CRL-GMFF invited all National Reference Laboratories nominated under Commission Regulation (EC) No 1981/2006 of 22 December 2006 and listed in Annex II ("National reference laboratories assisting the CRL for testing and validation of methods for detection") of that Regulation to express the availability to participate in the validation study of the quantitative real-time PCR method for the detection and quantification of maize GM event GHB614.

Thirty-eight laboratories expressed in writing their willingness to participate, two declined the invitation, while thirty-one did not answer. The CRL-GMFF performed a random selection of twelve laboratories out of those that responded positively to the invitation, making use of a validated software application.

Clear guidance was given to the selected laboratories with regards to the standard operational procedures to follow for the execution of the protocol. The participating laboratories are listed in Table 1.

Table 1. Laboratories participating in the validation of the detection method for cotton line GHB614.

Laboratory	Country
Austrian Agency for Health and Food Safety, Competence Centre Biochemistry	AT
Central Agricultural Office, Food and Feed Safety Directorate, Central Feed Investigation Laboratory - National Reference Laboratory	HU
Central Agricultural Office, Food and Feed Safety Directorate, Lab. for GMO food	HU
Danish Plant Directorate, Laboratory for Diagnostics in Plants, Seed, and Feed	DK
Federal Institute for Risk Assessment	DE
Finnish Customs Laboratory	FI
Institute of Biochemistry and Biophysics Polish Academy of Sciences, Genetic Modifications Analysis Laboratory	PL
National Centre for Food, Spanish Food Safety Agency	ES
National Institute of Biology	SI
National Institute of Engineer, Technology and Innovation – Food Industry Laboratory	PT
State Institute of Chemical and Veterinarian Analysis	DE
Walloon Agricultural Research Centre (CRA-W) - Dept. of Quality of Agricultural Products	BE

3. Materials

For the validation of the quantitative event-specific method, control samples consisting of:

i) genomic DNA extracted from leaves of cotton plants harbouring the event GHB614 homozygously (32RRMM0225)
and
ii) genomic DNA extracted from leaves of a near-isogenic non-GM cotton plants (32RRMM0393)

Samples were provided by the applicant in accordance to the provisions of Commission Regulation (EC) No 1829/2003, Art 2.11 [control sample defined as the GMO or its genetic material (positive sample) and the parental organism or its genetic material that has been used for the purpose of the genetic modification (negative sample)].

Samples containing mixtures of 100% GHB614 cotton and non-GM cotton genomic DNA at different GMO concentrations were prepared by the CRL-GMFF, using the control samples provided, in a constant amount of total cotton DNA.

Participants received the following materials:

- ✓ Five calibration samples (200 µL of DNA solution each) labelled from S1 to S5.
- ✓ Twenty unknown DNA samples (80 µL of DNA solution each) labelled from U1 to U20.
- ✓ Reaction reagents as follows:
  - ❑ Universal PCR Master Mix, two bottles: 5 mL each
  - ❑ Distilled sterile water, one tube: 4 mL

- ✓ Primers and probes (1 tube each) as follows:

  adhC reference system

  - ❑ KVM157 (10 µM): 160 µL
  - ❑ KVM158 (10 µM): 160 µL
  - ❑ TM012 (10 µM): 160 µL

  GHB614 system

  - ❑ SHA007 (10 µM): 320 µL
  - ❑ SHA008 (10 µM): 320 µL
  - ❑ TM072 (10 µM): 160 µL

4. Experimental design

Twenty unknown samples (labelled from U1 to U20), representing five GM levels, were used in the validation study (Table 2). On each PCR plate, the samples were analysed for the GHB614 specific system and for the *adhC* reference system. In total, two plates were run per participating laboratory and four replicates for each GM level were analysed. PCR analysis was performed in triplicate for all samples. Participating laboratories carried out the

determination of the GM% according to the instructions provided in the protocol and using the electronic tool provided (Excel spreadsheet).

Table 2. GHB614 GM contents

GHB614 GM% (GM copy number/cotton genome copy number x 100)
0.09
0.40
0.90
2.00
4.50

5. Method

Description of operational steps followed

For the specific detection of event GHB614 DNA, a 119-bp fragment of the integration region of the construct inserted into the plant genome (3' insert-to-plant junction) is amplified using two specific primers. PCR products are measured at each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with FAM dye and TAMRA as quencher dye.

For the relative quantification of event GHB614 DNA, a cotton-specific reference system amplifies a 73-bp fragment of the cotton endogenous gene *adhC* (*alcohol dehydrogenase C*), using two *adhC* gene-specific primers and an *adhC* gene-specific probe labelled with VIC dye and TAMRA as quencher dye.

Standard curves are generated for both the GHB614 and the *adhC* specific systems by plotting the Ct values measured for the calibration points against the logarithm of the DNA copy numbers and by fitting a regression line into these data. Thereafter, the standard curves are used to estimate the copy numbers in the unknown sample DNA by interpolation from the standard curves.

For relative quantification of event GHB614 DNA in a test sample, the GHB614 copy number is divided by the copy number of the cotton reference gene (*adhC*) and multiplied by 100 to obtain the percentage value (GM% = GHB614 / *adhC* x 100).

Calibration sample S1 was prepared by mixing the appropriate amount of GHB614 DNA in control non-GM cotton DNA to obtain a 10% GM GHB614 in a total of 200 ng cotton DNA. Samples S2 was prepared by three-fold dilution from the S1 sample; sample S3 was prepared by five-fold dilution from S2 sample; sample S4 was prepared by four-fold dilution of sample S3 and sample S5 was prepared by five-fold dilution from the S4 sample.

The absolute copy numbers in the calibration curve samples are determined by dividing the sample DNA weight (nanograms) by the published average 1C value for cotton genome (2.33 pg) [(3)]. The copy number values used in the quantification, the GM contents of the calibration

samples and total DNA quantity used in PCR are provided in Table 3 (% GM calculated considering the 1C value for cotton genome as 2.33 pg) [(3)].

Table 3. % GM values of the standard curve samples.

Sample code	S1	S2	S3	S4	S5
Total amount of DNA in reaction	300	100	20	5	1
Cotton genome copies	128750	42917	8583	2146	429
GHB614 GM cotton copies	12875	4292	858	215	43

6. Deviations reported

Nine laboratories reported no deviations from the protocol.

One laboratory inverted the S2 and S5 samples on one plate with no consequences since sample label was correctly attributed in subsequent analysis.

One laboratory inverted the loading position for the reference and GM-specific systems in one plate. This had no consequences in terms of data analysis.

One laboratory performed PCR reactions in 20 µL of total volume because only a 384-well plate configuration of the ABI 9700HT instrument was available. Final concentrations of PCR reagents remained unchanged.

The regression parameters of the measured versus true values for one laboratory were inconsistent. Based on the F statistics by Dent and Blackie (1979), the hypothesis of intercept and slope simultaneously equal to 0 and 1 was rejected ($F = 789.36$, $p = 8.26011E^{-05}$); therefore this laboratory was excluded from data analysis.

7. Summary of results

PCR efficiency and linearity

The values of the slopes [from which the PCR efficiency is calculated using the formula ((10^(-1/slope))-1) x 100) of the reference curve and of the R^2 (expressing the linearity of the regression) reported by participating laboratories for the GHB614 system and the *adhC* reference system are summarised in Table 4.

Table 4. Values of reference curve slope, PCR efficiency and linearity (R^2)

		GHB614			*adhC*		
Lab	**Plate**	**Slope**	**PCR Efficiency (%)**	**R^2**	**Slope**	**PCR Efficiency (%)**	**R^2**
1	A	-3.40	97	1.00	-3.49	93	1.00
	B	-3.57	91	0.99	-3.53	92	1.00
2	A	-3.47	94	1.00	-3.59	90	1.00
	B	-3.50	93	1.00	-3.60	89	1.00
3	A	-	-	-	-	-	-
	B	-	-	-	-	-	-
4	A	-3.44	95	1.00	-3.44	95	1.00
	B	-3.57	91	0.99	-3.54	92	1.00
5	A	-3.50	93	1.00	-3.39	97	1.00
	B	-3.53	92	1.00	-3.52	93	1.00
6	A	-3.22	105	0.98	-3.64	88	0.99
	B	-3.24	104	0.99	-3.63	89	0.99
7	A	-3.55	91	1.00	-3.56	91	1.00
	B	-3.62	89	1.00	-3.51	92	1.00
8	A	-3.63	88	0.99	-3.53	92	1.00
	B	-3.56	91	1.00	-3.53	92	1.00
9	A	-3.53	92	1.00	-3.46	95	1.00
	B	-3.37	98	1.00	-3.52	92	1.00
10	A	-3.49	94	1.00	-3.49	93	1.00
	B	-3.48	94	1.00	-3.47	94	1.00
11	A	-3.51	93	1.00	-3.39	97	1.00
	B	-3.47	94	1.00	-3.49	93	1.00
	A	-3.64	88	1.00	-3.57	90	1.00
	B	-3.51	93	1.00	-3.58	90	1.00
	Mean	-3.49	94	1.00	-3.52	92	1.00

The mean PCR efficiency was 94% for the GHB614 system and 92% for the *adhC* system, with both values within the ENGL acceptance criteria. The linearity of the method was 1.00 for both systems.

Data reported confirm the appropriate performance characteristics of the method tested in terms of efficiency and linearity.

GMO quantification

Table 5 shows the mean values of the four replicates for each GM level as provided by all laboratories. Each mean value is the average of three PCR repetitions.

Table 5. GM% mean values determined by laboratories for unknown samples.

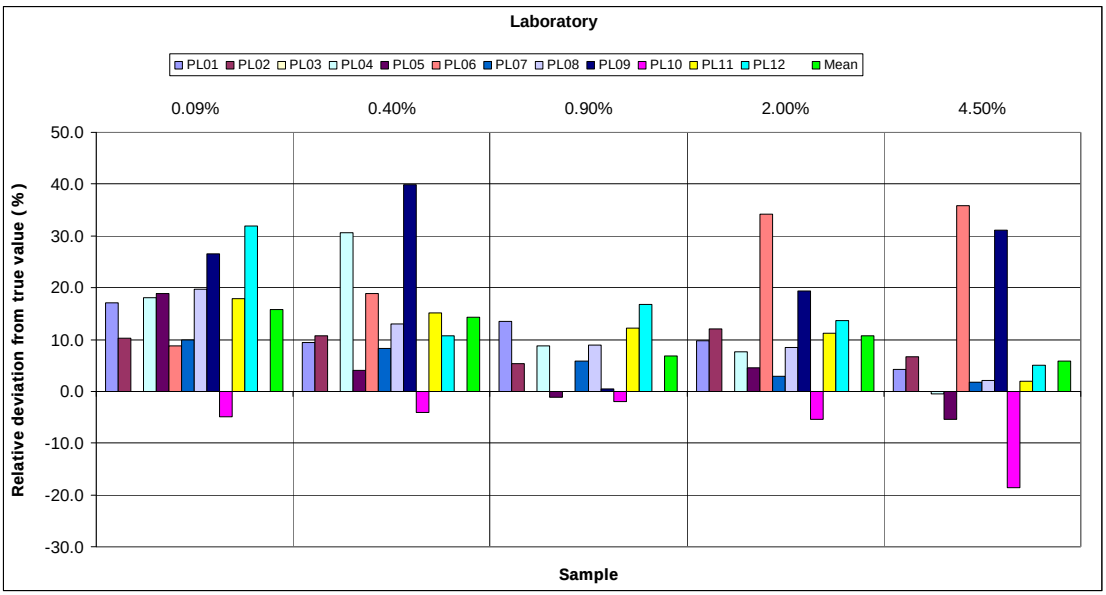
	GMO content (GMO% = GMO copy number/cotton genome copy number x 100)																			
LAB	0.1				0.4				0.9				2.0				4.5			
	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4
1	0.10	0.09	0.11	0.12	0.41	0.48	0.39	0.47	1.04	0.96	1.02	1.05	2.22	2.26	2.13	2.17	4.68	4.77	4.53	4.77
2	0.10	0.11	0.09	0.10	0.45	0.48	0.42	0.42	1.08	0.94	0.86	0.91	2.33	2.35	2.14	2.15	4.81	4.97	4.64	4.78
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	0.10	0.11	0.11	0.10	0.45	0.74	0.42	0.48	0.99	1.03	0.90	1.00	2.10	2.28	2.16	2.06	4.46	4.71	4.57	4.15
5	0.12	0.09	0.12	0.11	0.41	0.43	0.38	0.44	0.88	0.93	0.82	0.92	2.04	2.09	2.08	2.16	4.38	4.53	3.91	4.22
6	0.15	0.06	0.09	0.09	0.46	0.50	0.60	0.34	1.43	1.60	2.30	0.79	2.84	3.44	2.41	2.05	7.16	7.58	4.61	5.11
7	0.10	0.09	0.11	0.10	0.44	0.41	0.48	0.40	0.94	0.93	0.98	0.96	2.03	2.09	2.11	2.00	4.57	4.23	4.62	4.88
8	0.11	0.11	0.11	0.10	0.45	0.42	0.47	0.47	0.98	1.01	0.97	0.96	2.23	2.18	2.12	2.15	4.60	4.47	4.55	4.76
9	0.09	0.10	0.18	0.08	0.70	0.61	0.48	0.45	1.06	0.57	0.99	1.00	2.43	2.88	2.14	2.10	7.74	6.82	4.52	4.51
10	0.09	0.10	0.09	0.07	0.39	0.37	0.36	0.41	1.01	0.76	0.80	0.95	2.23	1.70	1.75	1.89	3.60	2.98	4.05	4.01
11	0.12	0.10	0.11	0.09	0.49	0.48	0.42	0.45	1.03	1.03	1.01	0.97	2.13	2.27	2.30	2.20	4.81	4.63	4.40	4.51
12	0.12	0.13	0.12	0.11	0.41	0.41	0.47	0.48	1.10	0.94	1.07	1.09	2.35	2.26	2.26	2.22	4.66	4.86	4.65	4.74

In Figure 1 the relative deviation from the true value for each GM level tested is shown for each laboratory. The coloured bars represent the relative GM quantification obtained by the participating laboratories; the green bar represents the overall mean for each GM level.

As observed in Figure 1, the mean relative deviations from the true values are positive for all GM levels, meaning that the GM content tends to be over-estimated at all GM levels. Only two laboratories showed a deviation from the true values above 25% at the GM levels of 0.09%, 0.4%, 2.0% and 4.5%.

Overall, the average relative deviation is within the acceptance criterion at all GM levels tested, indicating a satisfactory trueness of the method.

Figure 1. Relative deviation (%) from the true value of GHB614 for all laboratories



8. Method performance requirements

Among the performance criteria established by ENGL and adopted by the CRL-GMFF (http://gmo-crl.jrc.it/guidancedocs.htm, see also Annex 1), repeatability and reproducibility are assessed through an international collaborative trial, carried out with the support of twelve ENGL laboratories (see Table 1).

Table 6 illustrates the estimation of repeatability and reproducibility at various GM levels, according to the range of GM percentages tested during the collaborative trial.

The relative reproducibility standard deviation (RSD_R), that describes the inter-laboratory variation, should be below 33% at the target concentration and over the majority of the dynamic range, while it should be below 50% at the lower end of the dynamic range.

As it can be observed in Table 6, the method satisfies this requirement at all GM levels tested. In fact, the highest values of RSD_R (%) is 17% at the 0.4% GM level, thus well within the acceptance criterion.

Table 6 further documents the relative repeatability standard deviation (RSD_r), as estimated for each GM level. In order to accept methods for collaborative study evaluation, the CRL requires that the RSD_r value is below 25%, as indicated by ENGL (Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (http://gmo-crl.jrc.it/guidancedocs.htm).

Table 6. GHB614: summary of validation results.

	Expected value (GMO %)				
unknown sample GMO %	**0.09**	**0.4**	**0.9**	**2.0**	**4.5**
Laboratories having returned valid results	11	11	11	11	11
Samples per laboratory	4	4	4	4	4
Number of outliers	2	0	2	3	3
Reason for exclusion	2C	-	2C	3C	2C, 1G
Mean value	0.10	0.46	0.97	2.18	4.59
Relative repeatability standard deviation, RSDr (%)	9.4	15	6.8	3.3	4.1
Repeatability standard deviation	0.010	0.070	0.065	0.072	0.190
Relative reproducibility standard deviation, RSDR (%)	12	17	8.3	4.4	5.1
Reproducibility standard deviation	0.012	0.078	0.080	0.097	0.235
Bias (absolute value)	0.014	0.057	0.068	0.175	0.089
Bias (%)	15	14	7.5	8.8	2.0

C = Cochran's test; G = Grubbs' test; identification and removal of outliers through Cochran and Grubbs tests, according to ISO 5725-2.
Bias is estimated according to ISO 5725 data analysis protocol.

As can be observed from the values reported in Table 6, the method has a repeatability standard deviation below 25% at all GM levels, with the highest value of RSD_r (%) of 15% at the 0.4% GM level.

The trueness of the method is estimated using the measures of the method bias for each GM level. According to ENGL method performance requirements, trueness should be ± 25% across the entire dynamic range. In this case the method fully satisfies this requirement across the entire dynamic range tested; in fact, the highest deviation from true value (bias %) is 15% at the 0.09% level, thus well within the acceptance criterion.

9. Conclusions

The overall method performance has been evaluated with respect to the method acceptance criteria and method performance requirements recommended by the ENGL (as detailed at http://gmo-crl.jrc.it/guidancedocs.htm). The method acceptance criteria were reported by the applicant and used to evaluate the method prior to the international collaborative study (see Annex 1 for a summary of method acceptance criteria and method performance requirements).

The results obtained during the collaborative study, indicate that the analytical module of the method submitted by the applicant complies with ENGL performance criteria.

Therefore, the method is considered applicable to the control samples provided (see paragraph 3 "Materials"), in accordance with the requirements of Annex I-2.C.2 to Commission Regulation (EC) No 641/2004.

10. Quality assurance

The CRL-GMFF carries out all operations according to ISO 9001:2000 (certificate number: CH-32232) and ISO 17025:2005 (certificate number: DAC-PL-0459-06-00) [DNA extraction, qualitative and quantitative PCR in the area of Biology (DNA extraction and PCR method validation for the detection and identification of GMOs in food and feed materials)].

11. References

1. Horwitz W., 1995. Protocol for the design, conduct and interpretation of method performance studies, *Pure and Appl. Chem*, 67, 331-343.
2. International Standard (ISO) 5725, 1994. Accuracy (trueness and precision) of measurement methods and results. International Organization for Standardization, Genève, Swizerland.
3. Arumuganathan K. and Earle E. D., 1991. Nuclear DNA content of some important plant species. *Plant Molecular Biology Reporter* 9, 208-218.
4. Dent J.B. and Blackie M.J., 1979. Systems simulation in agriculture. Applied Science Publishers Ltd., London.

12. Annex 1: method acceptance criteria and method performance requirements as set by the European Network of GMO Laboratories (ENGL)

Method Acceptance Criteria should be fulfilled at the moment of submission of a method (Phase 1: acceptance for the collaborative study).

Method Performance Requirements should be fulfilled in a collaborative study in order to consider the method as fit for its purpose (Phase 2: evaluation of the collaborative study results).

Method Acceptance Criteria

Applicability

Definition: the description of analytes, matrices, and concentrations to which a method can be applied.

Acceptance Criterion: the applicability statement should provide information on the scope of the method and include data for the indices listed below for the product/s for which the application is submitted. The description should also include warnings to known interferences by other analytes, or inapplicability to certain matrices and situations.

Practicability

Definition: the ease of operations, the feasibility and efficiency of implementation, the associated unitary costs (e.g. Euro/sample) of the method.

Acceptance Criterion: the practicability statement should provide indication on the required equipment for the application of the method with regards to the analysis *per se* and the sample preparation. An indication of costs, timing, practical difficulties and any other factor that could be of importance for the operators should be indicated.

Specificity

Definition: property of a method to respond exclusively to the characteristic or analyte of interest.

Acceptance Criterion: the method should be event-specific and be functional only with the GMO or GM based product for which it was developed. This should be demonstrated by empirical results from testing the method with non-target transgenic events and non-transgenic material. This testing should include closely related events and cases where the limit of the detection is tested.

Dynamic Range

Definition: the range of concentrations over which the method performs in a linear manner with an acceptable level of accuracy and precision.

Acceptance Criterion: the dynamic range of the method should include the 1/10 and at least 5 times the target concentration. Target concentration is intended as the threshold relevant for legislative requirements. the acceptable level of accuracy and precision are described below. The range of the

standard curve(s) should allow testing of blind samples throughout the entire dynamic range, including the lower (10%) and upper (500%) end.

Accuracy

Definition: the closeness of agreement between a test result and the accepted reference value.

Acceptance Criterion: the accuracy should be within ± 25% of the accepted reference value over the whole dynamic range.

Amplification Efficiency

Definition: the rate of amplification that leads to a theoretical slope of – 3.32 with an efficiency of 100% in each cycle. The efficiency of the reaction can be calculated by the following equation: Efficiency = $[10^{(-1/slope)}] - 1$

Acceptance Criterion: the average value of the slope of the standard curve should be in the range of (- 3.1 ≥ slope ≥ - 3.6)

R^2 Coefficient

Definition: the R^2 coefficient is the correlation coefficient of a standard curve obtained by linear regression analysis.

Acceptance Criterion: the average value of R^2 should be ≥ 0.98.

Repeatability Standard Deviation (RSD_r)

Definition: the standard deviation of test results obtained under repeatability conditions. Repeatability conditions are conditions where test results are obtained with the same method, on identical test items, in the same laboratory, by the same operator, using the same equipment within short intervals of time.

Acceptance Criterion: the relative repeatability standard deviation should be below 25% over the whole dynamic range of the method.

Note: estimates of repeatability submitted by the applicant should be obtained on a sufficient number of test results, at least 15, as indicated in ISO 5725-3 (1994).

Limit of Quantitation (LOQ)

Definition: the limit of quantitation is the lowest amount or concentration of analyte in a sample that can be reliably quantified with an acceptable level of precision and accuracy.

Acceptance Criterion: LOQ should be less than $1/10^{th}$ of the value of the target concentration with an $RSD_r \leq 25\%$. Target concentration should be intended as the threshold relevant for legislative requirements. The acceptable level of accuracy and precision are described below.

Limit of Detection (LOD)

Definition: the limit of detection is the lowest amount or concentration of analyte in a sample, which can be reliably detected, but not necessarily quantified, as demonstrated by single laboratory validation.

Acceptance Criterion: LOD should be less than $1/20^{th}$ of the target concentration. Experimentally, quantitative methods should detect the presence of the analyte at least 95% of the time at the LOD, ensuring $\leq$ 5% false negative results. Target concentration should be intended as the threshold relevant for legislative requirements.

Robustness

Definition: the robustness of a method is a measure of its capacity to remain unaffected by small, but deliberate deviations from the experimental conditions described in the procedure.

Acceptance Criterion: the response of an assay with respect to these small variations should not deviate more than $\pm$ 30%. Examples of factors that a robustness test could address are: use of different instrument type, operator, brand of reagents, concentration of reagents, and temperature of reaction.

Method Performance Requirements

Dynamic Range

Definition: in the collaborative trial the dynamic range is the range of concentrations over which the reproducibility and the trueness of the method are evaluated with respect to the requirements specified below.

Acceptance Criterion: the dynamic range of the method should include the 1/10 and at least five times the target concentration. Target concentration should be intended as the threshold relevant for legislative requirements.

Reproducibility Standard Deviation (RSD_R)

Definition: the standard deviation of test results obtained under reproducibility conditions. Reproducibility conditions are conditions where test results are obtained with the same method, on identical test items, in different laboratories, with different operators, using different equipment. Reproducibility standard deviation describes the inter-laboratory variation.

Acceptance Criterion: the relative reproducibility standard deviation should be below 35% at the target concentration and over the entire dynamic range. An RSD_R < 50% is acceptable for concentrations below 0.2%.

Trueness

Definition: the closeness of agreement between the average value obtained from a large series of test results and an accepted reference value. The measure of trueness is usually expressed in terms of bias.

Acceptance Criterion: the trueness should be within $\pm$ 25% of the accepted reference value over the whole dynamic range.