Report on the Verification of the Performance of MON 89034, 1507 and NK603 Event-specific Methods on the Maize Event MON 89034 x 1507 x NK603 Using Real-Time PCR

9 July 2010

Joint Research Centre
Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit

Executive Summary

The European Union Reference Laboratory for GM Food and Feed EURL-GMFF), established by Regulation (EC) No 1829/2003, has carried out a verification study to assess the performance of three quantitative event-specific methods on the maize event MON 89034 x 1507 x NK603 (unique identifier MON-89Ø34-3 x DAS-Ø15Ø7-1 x MON-ØØ6Ø3-6) which combines the MON 89034, 1507 and NK603 transformation events. The three methods have been validated individually on single-trait events, to detect and quantify each event in maize samples. This study was conducted according to internationally accepted guidelines [1, 2].

In accordance to Regulation (EC) No 1829/2003 of 22 September 2003 on genetically modified food and feed and to Regulation (EC) No 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003, Monsanto and Dow AgroSciences provided the detection methods and the control samples: genomic DNA from homogenised seeds of MON 89034 x 1507 x NK603 maize (60-B) and from homogenised seeds of conventional maize (10001262-V). The EURL-GMFF prepared the verification samples (calibration samples and blind samples at different GM percentages [DNA/DNA]).

The results of the verification study were evaluated with reference to ENGL method performance requirements (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm) and to the validation results on the individual parental events (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm)

The results of this EURL-GMFF verification study are publicly available at http://gmo-crl.jrc.ec.europa.eu/.

Drafted by:
E. Luque-Perez (scientific officer)

Report review:
1) M. Querci (scientific officer)

2) M. Ermolli (scientific officer)

Scientific and technical approval:
M. Mazzara (scientific officer)

Compliance with EURL Quality System:
S. Cordeil (quality manager)

Authorisation to publish:
G. Van den Eede (head of MBG unit)

Address of contact laboratory:
European Commission, Joint Research Centre (JRC)
Institute for Health and Consumer Protection (IHCP)
Molecular Biology and Genomics Unit
European Union Reference Laboratory for GM Food and Feed
Via E. Fermi 2749, I-21020 Ispra (VA) - Italy

Report on Steps 1-3 of the Validation Process

Monsanto and Dow AgroSciences submitted the detection methods and control samples of the maize event MON 89034 x 1507 x NK603 (unique identifier MON-89Ø34-3 x DAS-Ø15Ø7-1 x MON-ØØ6Ø3-6) under Articles 5 and 17 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".

The European Union Reference Laboratory for GM Food and Feed (EURL-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 procedures ("Description of the CRL-GMFF Validation Process", http://gmo-crl.jrc.ec.europa.eu/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.ec.europa.eu/doc/Method%20requirements.pdf) (see Annex 1 for a summary of method acceptance criteria and method performance requirements). During step 2, three scientific assessments were performed and two requests of complementary information were addressed to the applicant. Upon reception of the complementary information, the scientific assessment of the detection method for maize MON 89034 x 1507 x NK603 was positively concluded in June 2009.

The event-specific detection methods for the three maize lines hosting the single events MON 89034, 1507 and NK603 were validated by the EURL-GMFF following the conclusion of the respective international collaborative studies and the publication of the validation reports (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm). Hence, the detection methods applied on the maize event MON 89034 x 1507 x NK603 did not undergo a full validation process. The EURL-GMFF performed a verification of the detection methods to asses whether they exhibit a comparable performance on samples of event MON 89034 x 1507 x NK603 combining the three traits (as provided in accordance to Annex I.2.C.2 of Commission Regulation (EC) No 641/2004).

In August 2009, the EURL-GMFF concluded the verification of the method characteristics (step 3, experimental testing of the samples and methods) by quantifying, with each specific method, five blind GM levels within the range 0.09%-8%. The experiments were performed under repeatability conditions and demonstrated that the PCR efficiency, linearity, trueness and repeatability of the quantification were within the limits established by the ENGL.

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

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1. Introduction

Monsanto and Dow AgroSciences submitted the detection methods and control samples of the maize event MON 89034 x 1507 x NK603 (unique identifier MON-89Ø34-3 x DAS-Ø15Ø7-1 x MON-ØØ6Ø3-6) under Articles 5 and 17 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".

The European Union Reference Laboratory for GM Food and Feed (EURL-GMFF), established by Regulation (EC) No 1829/2003, carried out a verification of the three event-specific methods for the detection and quantification of MON 89034, 1507 and NK603 in the MON 89034 x 1507 x NK603 maize event combining the three traits. The single methods were previously validated by international collaborative studies on the single-trait maize events (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm).

Upon reception of methods, samples and related data (step 1), the EURL-GMFF carried out the assessment of the documentation (step 2) and the verification of the methods (step 3) according to the requirements of Regulation (EC) 641/2004 and following EURL-GMFF procedures. The EURL-GMFF method verification was concluded in August 2009.

A method for DNA extraction from maize seeds, submitted by the applicant, was evaluated by the EURL-GMFF in order to confirm its performance characteristics. The protocol for DNA extraction is available at http://gmo-crl.jrc.ec.europa.eu/.

The procedure of verification consisted of quantitative real-time polymerase chain reaction (PCR). The methodology consists of three event-specific real-time quantitative TaqMan® PCR procedures for the determination of the relative content of events MON 89034, 1507 and NK603 DNA to total maize DNA in the MON 89034 x 1507 x NK603 maize event. The procedures are simplex systems, in which the events MON 89034, 1507 and NK603 were quantified in reference to the maize *hmg* (high mobility group) endogenous gene.

The study was carried out in accordance to the following internationally accepted guidelines:

- ISO 5725: 1994.
- The IUPAC "Protocol for the design, conduct and interpretation of method-performance studies".

2. Materials

The following samples were provided by the applicant:

- genomic DNA extracted from homogenised seeds of MON 89034 x 1507 x NK603 maize (60-B),
- genomic DNA extracted from homogenised seeds of conventional maize (10001262-V),

in accordance to the provisions of 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 MON 89034 x 1507 x NK603 and non-GM maize genomic DNA at different GMO levels were prepared by the EURL-GMFF in a constant amount of total maize DNA.

The validated methods for the individual MON 89034, 1507 and NK603 events were applied in the verification as published and available at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm.

In Table 1 are reported the five GM levels used in the verification of the MON 89034, 1507 and NK603 methods.

Table 1. MON 89034, 1507 and NK603 GM contents (%) in maize event MON 89034 x 1507 x NK603

MON 89034 (GM DNA / Non-GM DNA x 100)	**1507** (GM DNA / Non-GM DNA x 100)	**NK603** (GM DNA / Non-GM DNA x 100)
0.09	0.1	0.1
0.4	0.5	0.5
0.9	0.9	0.9
3.0	2.0	2.0
8.0	5.0	5.0

3. Experimental design

Eight runs for each event-specific method were carried out. In each run, samples were analysed in parallel with both the GM-specific system and the reference system (*hmg*). Five GM levels per run were examined and two replicates for each GM level were analysed. PCR analysis was performed in triplicate for all samples. In total, for each method (MON 89034, 1507 and NK603), the quantification of the five GM levels was performed as an average of sixteen replicates per GM level. An Excel spreadsheet was used for determination of GM%.

4. Method

To detect MON 89034, 1507 and NK603 in maize event MON 89034 x 1507 x NK603, three specific fragments, corresponding to the integration regions of the constructs inserted into the plant genome, of 77 bp, 58 bp and 108 bp respectively, were amplified using specific primers.

For relative quantification of events MON 89034, 1507 and NK603, a maize-specific target-taxon system, amplifying a 79 bp fragment of the maize endogenous gene *hmg* (high mobility group), was used.

Standard curves are generated for each GM specific system (MON 89034, 1507 and NK603), by plotting Ct values of the calibration samples against the logarithm of the DNA copy numbers of MON 89034, 1507 or NK603, and fitting a linear regression into these data. Thereafter, the normalised Ct values of the blind samples are measured and, by mean of the regression function, the relative amount of MON 89034, 1507 or NK603 DNA, respectively, is estimated.

Detailed information on the preparation of the standard curve samples is available at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm.

5. Deviations reported

For the 1507 event-specific method buffer 10x (without Rox) instead buffer 10x (including Rox) was used. Passive dye Rox 50x was added independently to the reaction mixture.

6. Summary of results

PCR efficiency and linearity

PCR efficiency was calculated using the formula $[10^{(-1/slope)} - 1] \times 100$, and of the R^2 (expressing the linearity of the regression) is reported for all PCR systems in the eight runs. Values of the standard curves slopes for MON 89034, 1507 and NK603 methods are presented in Tables 2, 3 and 4, respectively.

Table 2. Values of slope, PCR efficiency and linearity (R^2) of the MON 89034 method on event MON 89034 x 1507 x NK603.

Run	MON 89034			*hmg*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.56	91	0.99	-3.31	100	1.00
2	-3.43	96	1.00	-3.26	103	1.00
3	-3.48	94	1.00	-3.27	102	1.00
4	-3.43	96	1.00	-3.28	102	1.00
5	-3.38	98	1.00	-3.27	102	1.00
6	-3.46	95	1.00	-3.21	105	1.00
7	-3.53	92	1.00	-3.24	104	1.00
8	-3.39	97	1.00	-3.22	104	1.00
Mean	-3.46	95	1.00	-3.26	103	1.00

Table 3. Values of slope, PCR efficiency and linearity (R^2) of the 1507 method on event MON 89034 x 1507 x NK603.

Run	1507			*hmg*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.23	104	1.00	-3.22	105	1.00
2	-3.30	101	0.99	-3.24	103	1.00
3	-3.32	100	0.99	-3.18	106	1.00
4	-3.16	107	1.00	-3.20	105	1.00
5	-3.36	98	0.99	-3.37	98	0.99
6	-3.35	99	0.99	-3.23	104	1.00
7	-3.45	95	1.00	-3.18	106	1.00
8	-3.26	103	0.99	-3.18	106	1.00
Mean	-3.30	101	0.99	-3.23	104	1.00

Table 4. Values of slope, PCR efficiency and linearity (R^2) of the NK603 method on event MON 89034 x 1507 x NK603.

Run	NK603			*hmg*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.68	87	0.99	-3.27	102	1.00
2	-3.72	86	0.99	-3.33	100	1.00
3	-3.60	90	0.99	-3.34	99	1.00
4	-3.56	91	0.99	-3.29	101	1.00
5	-3.85	82	0.99	-3.29	101	1.00
6	-3.53	92	0.99	-3.28	102	1.00
7	-3.79	84	0.99	-3.25	103	1.00
8	-3.75	85	0.99	-3.27	102	1.00
Mean	-3.68	87	0.99	-3.29	101	1.00

The mean PCR efficiencies of the GM and target taxon-specific systems were 95% and 103% for MON 89034, 101% and 104% for 1507, and 87% and 101% for NK603, respectively. The R^2 of the methods was above 0.99 for both systems in all cases. Overall, the data reported in Tables 2, 3 and 4 confirm the appropriate performance characteristics of the three methods tested on MON 89034 x 1507 x NK603 maize samples in terms of PCR efficiency and linearity.

7. Method performance requirements

The results of the verification study for the MON 89034, 1507 and NK603 detection methods applied to event MON 89034 x 1507 x NK603 maize DNA are reported in Tables 5, 6 and 7, respectively. Results were evaluated with respect to the method acceptance criteria, as established by ENGL and adopted by the EURL-GMFF (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm, see also Annex 1). In addition, Tables 5 to 7 report the trueness and precision for each GM level and for all methods.

Table 5. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the MON 89034 method on event MON 89034 x 1507 x NK603 maize DNA.

MON 89034					
Unknown sample GM%	Expected value (GMO%)				
	0.09	0.4	0.9	3.0	8.0
Mean	0.09	0.37	0.87	2.75	7.82
SD	0.01	0.04	0.04	0.24	0.38
RSD_r (%)	9.3	11	4.9	8.8	4.8
Bias (%)	-4.9	-6.8	-3.4	-8.2	-2.3

Table 6. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation of the 1507 method on event MON 89034 x 1507 x NK603 maize DNA.

1507					
Unknown sample GM%	**Expected value (GMO%)**				
	0.1	**0.5**	**0.9**	**2.0**	**5.0**
Mean	0.11	0.44	0.86	1.69	4.66
SD	0.01	0.02	0.07	0.09	0.41
RSD_r (%)	14	5.6	7.9	5.4	8.7
Bias (%)	5.0	-12	-5.0	-15	-6.9

Table 7. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the NK603 method on event MON 89034 x 1507 x NK603 maize DNA.

NK603					
Unknown sample GM%	**Expected value (GMO%)**				
	0.1	**0.5**	**0.9**	**2.0**	**5.0**
Mean	0.13	0.58	1.00	1.83	4.91
SD	0.01	0.10	0.05	0.13	0.27
RSD_r (%)	11	18	5.4	7.3	5.6
Bias (%)	27	15	11	-8.5	-1.8

The trueness of the method is estimated using the measurements of the method bias for each GM level. According to the ENGL acceptance criteria and method performance requirements, the trueness of the method should be ± 25% across the entire dynamic range. As shown in Tables 5, 6 and 7, the values ranged from -8.2% to -2.3% for MON 89034, from -15% to 5.0% for 1507, and from -8.5% to 27% for NK603. Therefore, with the only exception of NK603 at the lowest (0.1%) GM level, which shows a slightly higher value, the three methods satisfied the above mentioned requirement throughout their respective ranges of applicability.

Tables 5, 6 and 7 report on the relative repeatability standard deviation (RSD_r %) estimated for each GM level. As indicated by ENGL (Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing", http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm), the EURL-GMFF requires that the RSD_r % is below 25%. As it can be observed in Tables 5, 6 and 7, the values ranged between 4.8% and 11% for MON 89034, between 5.4% and 14% for 1507, and between 5.4% and 18% for NK603. Therefore, the three methods satisfied this requirement throughout their respective ranges of applicability.

8. Comparison of method performance between event MON 89034 x 1507 x NK603 and the single trait events

An indicative comparison of the three method performances on the maize event MON 89034 x 1507 x NK603 and the single trait events is shown in Tables 8, 9 and 10. The performance of the methods on the single lines was previously assessed though international collaborative trials.

Table 8. Trueness (bias %) and relative repeatability standard deviation (RSD_r %) of the MON 89034 detection method on event MON 89034 x 1507 x NK603 and on event MON 89034.

Trueness and repeatability of MON 89034 quantification on MON 89034 x 1507 x NK603			**Trueness and repeatability of MON 89034 quantification on single event MON 89034***		
GM %	**Bias (%)**	**RSD_r (%)**	**GM %**	**Bias (%)**	**RSD_r (%)**
0.09	-4.9	9.3	**0.09**	25	18
0.4	-6.8	11	**0.4**	6.4	13
0.9	-3.4	4.9	**0.9**	4.3	17
3.0	-8.2	8.8	**3.0**	-5.8	12
8.0	-2.3	4.8	**8.0**	-11	9.5

*method validated (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm)

Table 9. Trueness (bias %) and relative repeatability standard deviation (RSDr %) of the 1507 detection method on event MON 89034 x 1507 x NK603 and on event 1507.

Trueness and repeatability of 1507 quantification on MON 89034 x 1507 x NK603			**Trueness and repeatability of 1507 quantification on single event 1507***		
GM %	**Bias (%)**	**RSD_r (%)**	**GM %**	**Bias (%)**	**RSD_r (%)**
0.1	5.0	14	**0.1**	6.0	18
0.5	-12	5.6	**0.5**	-4.0	12
0.9	-5.0	7.9	**0.9**	3.7	7.7
2.0	-15	5.4	**2.0**	-1.7	8.5
5.0	-6.9	8.7	**5.0**	8.4	14

*method validated (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm)

Table 10. Trueness (bias %) and relative repeatability standard deviation (RSD_r %) of the NK603 detection method on event MON 89034 x 1507 x NK603 and on event NK603.

Trueness and repeatability of NK603 quantification on MON 89034 x 1507 x NK603			**Trueness and repeatability of NK603 quantification on single event NK603***		
GM %	**Bias (%)**	**RSD_r (%)**	**GM %**	**Bias (%)**	**RSD_r (%)**
0.1	27	11	**0.1**	83	24
0.5	15	18	**0.49**	73	15
0.9	11	5.4	**0.98**	47	17
2.0	-8.5	7.3	**1.96**	14	7.7
5.0	-1.8	5.6	**4.91**	22	22

*method validated (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm)

Regarding trueness, the MON 89034 method (Table 8) showed generally similar or lower bias when applied to event MON 89034 x 1507 x NK603, compared to the single trait event. The 1507 event-specific method (Table 9) shows slightly lower bias at both ends of the quantification range studied (0.1% and 5.0%), when applied to event MON 89034 x 1507 x NK603 and compared to the single line event, and higher at 0.5%, 0.9% and 2.0% GM levels. The NK603 event-specific method (Table 10) showed lower bias over the whole range of applicability when applied to the combined event and compared to the single trait. In all cases, the trueness of the three event-specific methods, when applied to MON 89034 x 1507 x NK603, were within the acceptance range set by ENGL (± 25%) for the whole dynamic ranges studied.

Concerning the relative repeatability standard deviation (RSD_r %), the three event-specific methods (Tables 8, 9 and 10) showed lower or similar values when applied to MON 89034 x 1507 x NK603 and compared to the single events. In all cases, the relative repeatability standard deviation (RSD_r %) of the three event-specific methods when applied to MON 89034 x 1507 x NK603, were below the ENGL acceptance level established at maximum 25%.

Therefore, the method verification has demonstrated that the MON 89034, 1507 and NK603 detection methods developed to detect and quantify the single events can be equally applied for the quantification of the respective events combined in MON 89034 x 1507 x NK603.

9. Conclusions

The method performance of the three event-specific methods for the quantitative detection of events MON 89034, 1507 and NK603 combined in maize event MON 89034 x 1507 x NK603 have been evaluated with respect to the method acceptance criteria and the method performance requirements recommended by the ENGL (as detailed under http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm), and to the validation results obtained for the single trait events (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm).

The results obtained during the verification study indicated that the analytical modules of the methods submitted by the applicant comply with ENGL performance criteria. The methods are

therefore 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 EURL-GMFF is accredited 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.

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 ≤ 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.