Report on the Verification of the Performance of 59122, 1507 and NK603 Event-specific Methods on the Hybrid Maize Line 59122x1507xNK603 Using Real-Time PCR

28 January 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, has carried out an in-house verification study to assess the performance of three quantitative event-specific methods on the hybrid maize line 59122x1507xNK603 (unique identifier DAS-59122-7xDAS-Ø15Ø7-1xMON-ØØ6Ø3-6) which combines the 59122, 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, Pioneer Hi-Bred Intl. Inc. provided the detection methods and the control samples (59122x1507xNK603 ground maize flour and conventional ground maize flour). The JRC prepared the in-house verification samples (calibration samples and blind samples at different GM percentages).

The results of the in-house verification study were evaluated with reference to ENGL method performance requirements (http://gmo-crl.jrc.it/doc/Method%20requirements.pdf) and to the validation results on the individual parental events (http://gmo-crl.jrc.it/statusofdoss.htm).

The results of this CRL-GMFF in-house verification studies are made publicly available at http://gmo-crl.jrc.it/.

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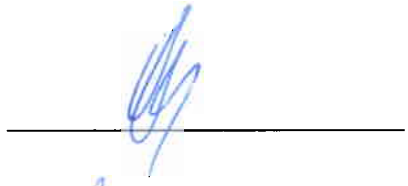

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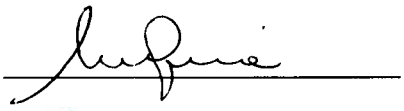

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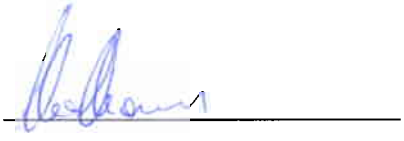

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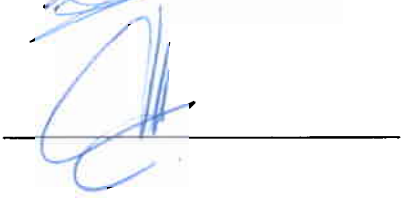

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Report on Steps 1-3 of the Validation Process

Pioneer Hi-Bred submitted the detection methods and control samples of the hybrid maize line 59122x1507xNK603 (unique identifier DAS-59122-7xDAS-Ø15Ø7-1xMON-ØØ6Ø3-6) under Article 5 and 17 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 to 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/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 last complementary information, the scientific assessment of the detection method for the 59122x1507xNK603 maize was positively concluded in June 2006.

The event-specific detection methods for the three maize lines hosting the single events 59122, 1507 and NK603 were validated by the CRL-GMFF following the conclusion of the respective international collaborative studies and the publication of the validation reports (http://gmo-crl.jrc.it/statusofdoss.htm). Hence, the detection methods applied on the hybrid 59122x1507xNK603 maize did not undergo a full validation process. The CRL-GMFF performed an in-house verification of the detection methods to verify that they exhibit a comparable performance on samples of hybrid 59122x1507xNK603 combining the three traits (as provided in accordance to Annex 1.2.C.2 of Commission Regulation (EC) No 641/2004).

In September 2006, the CRL-GMFF concluded the experimental 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 0.1%-5% (0.1%-5% for 59122), on a DNA copy number basis. 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 CRL-GMFF (step 3) is available on request.

Content

1. Introduction

Pioneer Hi-Bred submitted the detection methods (for 59122, 1507 and NK603) and the control samples of the hybrid maize line 59122x1507xNK603 (unique identifier DAS-59122-7xDAS-Ø15Ø7-1xMON=ØØ6Ø3-6) under Article 5 and 17 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 of Health and Consumer Protection) as Community Reference Laboratory for GM Food and Feed, established by Regulation (EC) 1829/2003, carried out an in-house verification of the three event-specific methods for the detection and quantification of 59122, 1507 and NK603 in the 59122x1507xNK603 hybrid maize line combining the three traits derived through traditional breeding techniques between progeny of the three genetically modified lines. The single methods had been previously validated by international collaborative studies on the single-trait maize events (http://gmo-crl.jrc.it/statusofdoss.htm).

Upon reception of methods, samples and related data (step 1), the CRL-GMFF carried out the assessment of the documentation (step 2) and the in-house evaluation of the methods (step 3) according to the requirements of Regulation (EC) 641/2004 and following CRL-GMFF operational procedures. The CRL-GMFF method verification was concluded in September 2006.

The validated methods of DNA extraction from single-trait maize seeds were used by the CRL-GMFF to extract DNA from the control samples received. The protocols for DNA extraction are available at http://gmo-crl.jrc.it/.

The operational procedure of the in-house verification included the following module:

- ✓ Quantitative real-time PCR (Polymerase Chain Reaction). The methodology consists of three event-specific real-time quantitative TaqMan® PCR procedures for the determination of the relative content of events 59122, 1507 and NK603 DNA to total maize DNA in the 59122x1507xNK603 hybrid maize line. The procedures are simplex systems, in which the events 59122 and 1507 were quantified in reference to the maize *hmg* (high mobility group) endogenous gene; the NK603 event was quantified in reference to the maize *adh1* (Alcohol dehydrogenase-1) endogenous gene.

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

- ✓ ISO 5725:1994 (1).
- ✓ The IUPAC "Protocol for the design, conduct and interpretation of method-performance studies" (2).

2. Materials

For the verification of the quantitative event-specific methods, control samples consisting of semi-ground grains of hybrid 59122x1507xNK603 (lot PW04512037PWN12CR200) and of conventional maize (Lot PIV3CON11011-00) were provided by the applicant. The genomic DNA was extracted from such control samples.

Samples containing mixtures of 100% 59122x1507xNK603 and non-GM maize genomic DNA at different GMO concentrations were prepared by the CRL-GMFF, using the control samples provided, in a constant amount of total maize DNA.

The protocols (reagents, concentrations, primer/probe sequences) followed in the in-house verification are those already published as validated methods for the individual 59122, 1507 and NK603 events and available at http://gmo-crl.jrc.it/statusofdoss.htm.

Table 1 shows the five GM levels of unknown samples used in the verification of the 59122, 1507 and NK603 methods.

Table 1. 59122, 1507 and NK603 GM contents in hybrid 59122x1507xNK603

59122 GM % (GM copy number/maize genome copy number *100)	**1507 GM %** (GM copy number/maize genome copy number *100)	**NK603 GM %** (GM copy number/maize genome copy number *100)
0.1	0.1	0.1
0.4	0.5	0.5
0.9	0.9	1.0
2.0	2.0	2.0
4.5	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* or *Adh1*). 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 (59122, 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

Description of the operational steps

For specific detection of events 59122, 1507 and NK603 genomic DNA, an 86-bp, 58-bp and 108-bp fragment of the integration region of the construct inserted into the plant genome is amplified using two specific primers.

PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with two fluorescent dyes: FAM is used as reporter dye at its 5′ end and TAMRA as a quencher dye at its 3′ end.

For the relative quantification of event 59122 DNA and event 1507 DNA, a maize-specific reference system which amplifies a 79-bp fragment of the *hmg* (High mobility group) gene, using a pair of *hmg* gene-specific primers, and an *hmg* gene-specific probe labelled with FAM and TAMRA, were used. For the relative quantification of event NK603 DNA, a maize-specific reference system which amplifies a 70-bp fragment of the *adh1* (alcohol dehydrogenase 1) gene, using a pair of *adh1* gene-specific primers and an *adh1* gene-specific probe labelled with FAM and TAMRA, was used.

Standard curves are generated for each GM specific system (59122, 1507 or NK603) and its respective reference gene (*hmg* or *adh1*), by plotting the Ct-values measured for the calibration samples against the logarithm of the DNA copy number, and by fitting a linear 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 the determination of the amount of 59122, 1507 or NK603 DNA in the unknown sample, the 59122, 1507 or NK603 copy number is divided by the copy number of the maize reference gene *hmg* (59122, 1507) or *adh1* (NK603) and multiplied by 100 to obtain the percentage value (GM% = GM-specific system/maize reference system * 100).

For detailed information on the preparation of the standard curve calibration samples please refer to the protocols of the validated methods at http://gmo-crl.jrc.it/statusofdoss.htm.

5. Deviations reported

No deviations from the protocols of the three previously validated methods were introduced.

6. Summary of results

PCR efficiency and linearity

The values of the slopes of the standard curves, from which the PCR efficiency is calculated using the formula [10^(-1/slope)-1]*100, and of the R^2 (expressing the linearity of the

regression) reported for all PCR systems in the eight runs, are presented in Table 2, 3 and 4 for 59122, 1507 and NK603 methods, respectively.

Table 2. Values of standard curve slope, PCR efficiency and linearity (R^2) for the 59122 method (59122 assay and endogenous *hmg* assay) on hybrid 59122x1507xNK603

Run	59122			*Hmg*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.464	94.4	0.994	-3.312	100.4	0.999
2	-3.680	87.0	0.977	-3.476	93.9	0.999
3	-3.547	91.4	0.996	-3.444	95.2	0.997
4	-3.604	89.4	0.997	-3.569	90.7	0.998
5	-3.586	90.0	0.997	-3.320	100.1	0.998
6	-3.635	88.4	0.997	-3.496	93.2	0.997
7	-3.608	89.3	0.998	-3.319	100.1	0.999
8	-3.685	86.8	0.998	-3.487	93.6	0.998
Mean	-3.601	90	0.994	-3.428	96	0.998

Table 3. Values of standard curve slope, PCR efficiency and linearity (R^2) for the 1507 method (1507 assay and endogenous *hmg* assay) on hybrid 59122x1507xNK603

Run	1507			*Hmg*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.338	99.4	0.994	-3.292	101.2	0.997
2	-3.362	98.4	0.996	-3.472	94.1	0.999
3	-3.407	96.6	0.995	-3.134	108.5	0.999
4	-3.285	101.5	0.998	-3.372	98.0	0.997
5	-3.396	97.0	0.990	-3.194	105.6	0.994
6	-3.274	102	0.991	-3.363	98.3	0.996
7	-3.384	97.5	0.997	-3.164	107	0.996
8	-3.332	99.6	0.997	-3.353	98.7	0.998
Mean	-3.347	99	0.995	-3.293	101	0.997

Table 4. Values of standard curve slope, PCR efficiency and linearity (R^2) for the NK603 method (NK603 assay and endogenous *Adh1* assay) on hybrid 59122x1507xNK603

Run	NK603			*Adh1*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.785	83.7	0.994	-3.175	106.5	0.990
2	-3.630	88.6	0.991	-3.146	107	0.997
3	-3.688	86.7	0.986	-3.205	105.1	0.996
4	-3.822	82.7	0.994	-3.193	105.7	0.993
5	-3.758	84.6	0.995	-3.295	101.1	0.997
6	-3.822	82.6	0.993	-3.137	108.3	0.997
7	-3.779	83.9	0.993	-3.147	107.8	0.996
8	-3.744	85.0	0.995	-3.124	109	0.993
Mean	-3.753	85	0.993	-3.178	106	0.995

The mean PCR efficiencies of the GM specific systems were equal or higher than 90%, with the exception of the NK603 specific system (84.7%); the efficiency of the *hmg* endogenous assay was close to 100% (96% and 101%), while the mean efficiency of the *Adh1* endogenous assay was 106%. The linearity of all methods (R^2 value) was above 0.99. Overall, data reported in Table 3, 4 and 5 confirm the appropriate performance characteristics of the three methods tested on 59122x1507xNK603 hybrid maize samples in terms of PCR efficiency and linearity.

7. Method performance requirements

The results of the in-house verification study for the 59122, 1507 and NK603 detection methods applied to hybrid 59122x1507xNK603 maize DNA are reported in Tables 6, 7 and 8, respectively. Results were evaluated with respect to the method acceptance criteria, as established by ENGL and adopted by the CRL-GMFF (http://gmo-crl.jrc.it/guidancedocs.htm, see also Annex 1). In addition, Tables 5, 6 and 7 report estimates of accuracy and precision for each GM level and for all methods.

Table 5. Estimates of trueness (expressed as bias %) and repeatability standard deviation (RSDr %) of the 59122 method on hybrid 59122x1507xNK603 maize DNA

59122					
Unknown sample GM%	**Expected value (GMO %)**				
	0.1	**0.4**	**0.9**	**2.0**	**4.5**
Mean	0.12	0.40	0.90	1.84	4.3
SD	0.01	0.06	0.06	0.12	0.37
RSDr (%)	12	14	7.2	6.8	8.5
Bias%	23	-0.4	0.3	-7.9	-4.6

Table 6. Estimates of trueness (expressed as bias %) and repeatability standard deviation (RSDr %) of the 1507 method on hybrid 59122x1507xNK603 maize DNA

1507					
Unknown sample GM%	**Expected value (GMO %)**				
	0.1	**0.5**	**0.9**	**2.0**	**5.0**
Mean	0.11	0.48	0.91	1.87	4.81
SD	0.02	0.07	0.05	0.21	0.44
RSDr (%)	14	14	5.7	11	9.1
Bias%	6.5	-4.4	0.9	-6.3	-3.7

Table 7. Estimates of trueness (expressed as bias %) and repeatability standard deviation (RSDr %) of the NK603 method on hybrid 59122x1507xNK603 maize DNA

NK603					
Unknown sample GM%	**Expected value (GMO %)**				
	0.1	**0.5**	**1.0**	**2.0**	**5.0**
Mean	0.11	0.49	1.14	1.83	5.44
SD	0.02	0.07	0.09	0.32	0.40
RSDr (%)	22	14	8.1	18	7.3
Bias%	8.4	-2.5	14	-8.4	8.9

The *trueness* of the method is estimated using the measures of the method bias for each GM level. According to the ENGL acceptance criteria and method performance requirements, the accuracy of the quantification, measured as bias from the accepted value, should be ± 25% across the entire dynamic range. As shown in Tables 5, 6 and 7, all methods satisfy the above requirement throughout their respective dynamic ranges.

Tables 5, 6 and 7 further document the *relative repeatability standard deviation (RSD_r)* as estimated for each GM level. In order to accept methods for collaborative trial evaluation, the CRL-GMFF requires that RSD_r values be below 25%, as indicated by ENGL (Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" [http://gmo-crl.jrc.it/guidancedocs.htm]).

As it can be observed from the values reported in Tables 6, 7 and 8, the three methods satisfy this requirement throughout their respective dynamic ranges.

8. Comparison of method performance between hybrid 59122x1507xNK603 and the single trait events

A synoptic comparison of the three method performances on the maize hybrid 59122x1507xNK603 and the ingle 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 (expressed as bias %) and repeatability standard deviation (RSDr %) of the 59122 detection method on hybrid 59122x1507xNK603 and on line 59122 maize DNA

Trueness and repeatability of 59122 quantification on 59122x1507xNK603			Trueness and repeatability of 59122 quantification on single line 59122*		
GM %	**Bias (%)**	**RSDr (%)**	**GM %**	**Bias (%)**	**RSDr (%)**
0.1	23	12	**0.1**	29	25
0.4	-0.4	14	**0.4**	15	22
0.9	0.3	7.2	**0.9**	9.0	22
2.0	-7.9	6.8	**2.0**	7.0	15
4.5	-4.6	8.5	**4.5**	-1.0	13

*method validated (http://gmo-crl.jrc.it/statusofdoss.htm)

Table 9. Trueness (expressed as bias %) and repeatability standard deviation (RSDr %) of the 1507 detection method on hybrid 59122x1507xNK603 and on line 1507 maize DNA

Trueness and repeatability of 1507 quantification on 59122x1507xNK603			Trueness and repeatability of 1507 quantification on single line 1507*		
GM %	**Bias (%)**	**RSDr (%)**	**GM %**	**Bias (%)**	**RSDr (%)**
0.1	6.5	14	**0.1**	6.0	18
0.5	-4.4	14	**0.5**	-4.0	12
0.9	0.9	5.7	**0.9**	3.7	7.7
2.0	-6.3	11	**2.0**	-1.7	8.5
5.0	-3.7	9.1	**5.0**	8.4	14

*method validated (http://gmo-crl.jrc.it/statusofdoss.htm)

Table 10. Trueness (expressed as bias %) and repeatability standard deviation (RSDr %) of the NK603 detection method on hybrid 59122x1507xNK603 and on line NK603 maize DNA

Trueness and repeatability of NK603 quantification on 59122x1507xNK603			Trueness and repeatability of NK603 quantification on single line NK603*		
GM %	**Bias (%)**	**RSDr (%)**	**GM %**	**Bias (%)**	**RSDr (%)**
0.1	8.4	22	**0.10**	83	24
0.5	-2.5	14	**0.49**	73	15
1.0	14	8.1	**0.98**	47	17
2.0	-8.4	18	**1.96**	14	7.7
5.0	8.9	7.3	**4.91**	22	22

*method validated (http://gmo-crl.jrc.it/statusofdoss.htm)

The individual methods for maize lines 59122 and 1507 show very comparable results in terms of trueness and repeatability of quantification when applied to the single-trait events samples and to the hybrid maize line combining the three events.

The NK603 method shows a lower or virtually identical RSDr (%) when validated on the single-trait events samples and when applied to the hybrid maize line combining the three events. In terms of accuracy, the method verification provided lower bias (%) at all GM levels tested, in comparison to the bias (%) obtained in the full validation.

Therefore, the in-house method verification has demonstrated that the 59122, 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 the hybrid line 59122x1507xNK603.

9. Conclusions

The overall method performance of the three event-specific methods for the quantitative detection of events 59122, 1507 and NK603 combined in the maize hybrid 59122x1507xNK603 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.it/guidancedocs.htm), and to the validation results obtained for the single trait events (http://gmo-crl.jrc.it/statusofdoss.htm).

The results obtained during the present verification study indicate 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 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.

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.