

# Report on the Verification of the Performance of MON89034 and MON88017 Event-specific Methods on the Maize Event MON89034 x MON88017 Using Real-Time PCR

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**Joint Research Centre  
Institute for Health and Consumer Protection  
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## Executive Summary

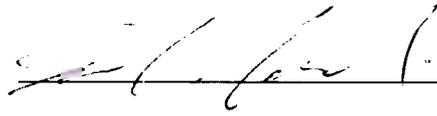
The JRC as Community Reference Laboratory for GM Food and Feed (CRL-GMFF), established by Regulation (EC) No 1829/2003, has carried out an in-house verification study to assess the performance of two quantitative event-specific methods on the maize event MON89034 x MON88017 (unique identifier MON-89Ø34-3 x MON-88Ø17-3) which combines the MON89034 and MON88017 transformation events. The two 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 <sup>(1, 2)</sup>.

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 Company provided the detection methods and the control samples: genomic DNA extracted from seeds of MON89034 x MON88017 maize, genomic DNA extracted from seeds of non-GM maize, seeds of MON89034 x MON88017 and seed of conventional maize. 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.ec.europa.eu/guidancedocs.htm>) and to the validation results on the parental events (<http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>).

The results of this CRL-GMFF in-house verification studies are made publicly available at <http://gmo-crl.jrc.ec.europa.eu/>.

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## Report on Steps 1-3 of the Validation Process

Monsanto Company submitted the detection methods and control samples of the maize event MON89034 x MON88017 (unique identifier MON-89Ø34-3 x MON-88Ø17-3) 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 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.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, two 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 the MON89034 x MON88017 maize was positively concluded in June 2007.

The event-specific detection methods for the two maize lines hosting the single events MON89034 and MON88017 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.ec.europa.eu/statusofdoss.htm>). Hence, the detection methods applied on the maize event MON89034 x MON88017 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 event MON89034 x MON88017 combining the two traits (as provided in accordance to Annex 1.2.C.2 of Commission Regulation (EC) No 641/2004).

In January 2008, 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 0.09%-8%, on a DNA/DNA ratio. The experiments were performed under repeatability conditions and demonstrated that the PCR efficiency, linearity, trueness and repeatability of the quantification were mostly 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.

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## 1. Introduction

Monsanto Company submitted the detection methods for MON89034 and MON88017 and the control samples of the maize event MON89034 x MON88017 (unique identifier MON 89034-3 x MON-88017-3) 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, Molecular Biology and Genomics 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 two event-specific methods for the detection and quantification of MON89034 and MON88017 in the MON89034 x MON88017 maize event combining the two traits. The single methods had been 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 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 January 2008.

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

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

- ✓ Quantitative real-time PCR (Polymerase Chain Reaction). The methodology consists of two event-specific real-time quantitative TaqMan<sup>®</sup> PCR procedures for the determination of the relative content of events MON89034 and MON88017 DNA to total maize DNA in the MON89034 x MON88017 maize event. The procedures are simplex systems, in which the events MON89034 and MON88017 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 <sup>(1)</sup>.
- ✓ The IUPAC "Protocol for the design, conduct and interpretation of method-performance studies" <sup>(2)</sup>.

## 2. Materials

For the verification of the methods, control samples consisting of:

- genomic DNA extracted from homogenised seeds of MON89034 x MON88017 maize (lot GLP-0701-17957-S),
- genomic DNA extracted from homogenised seeds of non-GM maize (lot GLP-0612-17871-S),

were provided by the applicant, 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 100% MON89034 x MON88017 and non GM maize genomic DNA at different GMO contents were prepared by the CRL-GMFF in a constant amount of total maize DNA, using the control samples provided.

The protocols (reagents, concentrations, primer/probe sequences) followed in the in-house verification are those already published as validated methods for the individual MON89034 and MON88017 events and available at <http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>.

Table 1 shows the five GM levels of unknown samples used in the verification of the MON89034 and MON88017 methods.

Table 1. MON89034 and MON88017 GM contents in maize event MON89034 x MON88017

MON89034 GM% (GM DNA / Non-GM DNA x 100)	MON88017 GM% (GM DNA / Non-GM DNA x 100)
0.09	0.09
0.40	0.50
0.90	0.90
3.00	5.00
8.00	8.00

## 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 (MON89034 and MON88017), the quantification of the five GM levels was performed as an average of sixteen replicates per GM level.

## 4. Method

### *Description of the operational steps*

For detection of events MON89034 and MON88017 in maize event MON89034 x MON88017, two fragments of 77 bp and 95 bp respectively, covering the 3' and the 5' insert-to-plant junctions), are amplified using specific primers.

PCR products are measured during each cycle (real-time) by means of target-specific oligonucleotide probes labelled with two fluorescent dyes: FAM is used as reporter dye at its 5' end and TAMRA (for MON88017) or MGBNFQ (for MON89034) as a quencher dye at its 3' end.

For relative quantification of events MON89034 and MON88017 DNA, a maize-specific reference system which amplifies a 79 bp fragment of the maize endogenous gene *hmg* (high mobility group, accession number AJ131373), using two *hmg* gene-specific primers and an *hmg* gene-specific probe labelled with FAM and TAMRA, is used.

Standard curves are generated for each system (*hmg*, MON89034 and MON88017), 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 copy numbers in the unknown sample DNA are estimated by interpolation from the standard curves.

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.ec.europa.eu/statusofdoss.htm>.

## 5. Summary of results

### *PCR efficiency and linearity*

The values of the slopes of the standard curves, from which the PCR efficiency was calculated using the formula  $[10^{(-1/\text{slope})}-1]*100$ , 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 and 3 for MON89034 and MON88017 methods, respectively.

Table 2. Values of standard curve slope, PCR efficiency and linearity ( $R^2$ ) for MON89034 and *hmg* methods on event MON89034 x MON88017.

Run	MON89034			<i>hmg</i>		
	Slope	PCR Efficiency (%)	Linearity ( $R^2$ )	Slope	PCR Efficiency (%)	Linearity ( $R^2$ )
1	-3.5	92	0.991	-3.1	111	0.996
2	-3.5	92	0.994	-3.3	102	0.998
3	-3.7	88	0.994	-3.2	107	0.996
4	-3.4	95	0.996	-3.1	109	0.990
5	-3.6	91	0.993	-3.2	106	0.993
6	-3.4	95	0.996	-3.1	109	0.993
7	-3.4	95	0.996	-3.1	110	0.995
8	-3.4	98	0.998	-3.1	112	0.990
Mean	-3.5	93	0.995	-3.1	108	0.994

Table 3. Values of standard curve slope, PCR efficiency and linearity ( $R^2$ ) for MON88017 and *hmg* methods on event MON89034 x MON88017.

Run	MON88017			<i>hmg</i>		
	Slope	PCR Efficiency (%)	Linearity ( $R^2$ )	Slope	PCR Efficiency (%)	Linearity ( $R^2$ )
1	-3.3	102	0.994	-3.1	111	0.992
2	-3.4	96	0.997	-3.1	108	0.998
3	-3.5	94	0.995	-3.1	110	0.992
4	-3.5	93	0.995	-3.2	107	0.995
5	-3.5	94	0.993	-3.4	98	0.998
6	-3.6	90	0.998	-3.3	101	0.998
7	-3.5	94	0.993	-3.2	104	0.996
8	-3.4	97	0.991	-3.3	101	0.997
Mean	-3.4	95	0.994	-3.2	105	0.996

The mean PCR efficiencies of the MON89034 and MON88017 detection methods on event MON89034 x MON88017 were 93% and 95% respectively. The linearity was 0.995 and 0.994 for the MON89034 and MON88017 specific systems. The mean PCR efficiency of the *hmg* reference system was 108% and 105% when tested in conjunction with the MON89034 and the MON88017 specific systems, respectively; likewise, the linearity of the *hmg* was 0.994 and 0.996.

Overall, data reported in Table 2 and 3 confirm the appropriate performance characteristics in terms of PCR efficiency and linearity of the two methods tested on MON89034 x MON88017 maize samples.

## 6. Method performance requirements

The results of the verification study for the MON89034 and MON88017 detection methods applied to event MON89034 x MON88017 maize DNA are reported in Tables 4 and 5, 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.ec.europa.eu/guidancedocs.htm>, see also Annex 1). In addition, Tables 4 and 5 report the estimates of the trueness and repeatability standard deviation for each GM level and for both methods.

Table 4. Estimates of trueness (expressed as bias%) and relative repeatability standard deviation (RSDr%) of the MON89034 method on event MON89034 x MON88017 maize DNA.

MON89034					
Unknown sample GM%	Expected value (GMO%)				
	0.09	0.4	0.9	3.0	8.0
Mean	0.10	0.41	0.92	2.97	7.92
SD	0.01	0.06	0.08	0.25	0.64
RSDr (%)	14	15	8.4	8.5	8.1
Bias (%)	10	3.4	2.6	-1.0	-0.9

Table 5. Estimates of trueness (expressed as bias%) and relative repeatability standard deviation (RSDr%) of the MON88017 method on event MON89034 x MON88017 maize DNA

MON88017					
Unknown sample GM%	Expected value (GMO%)				
	0.09	0.5	0.9	5.0	8.0
Mean	0.10	0.52	0.93	4.97	8.03
SD	0.02	0.10	0.12	0.72	0.71
RSDr (%)	18	19	13	14	8.9
Bias (%)	12	3.6	3.8	-0.5	0.4

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 trueness of the method, measured as bias from the accepted value, should be  $\pm 25\%$  across the entire dynamic range. As shown in Tables 4 and 5, both methods satisfy the above requirement throughout their respective dynamic ranges.

Tables 4 and 5 further document the relative repeatability standard deviation (RSD<sub>r</sub>) as estimated for each GM level. In order to accept methods for collaborative trial evaluation, the CRL-GMFF requires that RSD<sub>r</sub> values are below 25%, as indicated by ENGL (Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" [<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>]). As it can be observed from the values reported in Tables 4 and 5, the two methods satisfy this requirement throughout their respective dynamic ranges.

## 7. Comparison of methods performance between event MON89034 x MON88017 and the single trait events

An indicative comparison of the performances of the two methods on the maize event MON89034 x MON88017 and the single trait events is shown in Tables 6 and 7. The performance of the methods on the single lines was previously assessed through international collaborative trials.

Table 6. Trueness (bias%) and relative repeatability standard deviation (RSD<sub>r</sub>%) of the MON89034 detection method on event MON89034 x MON88017 and on event MON89034.

Trueness and repeatability of MON89034 quantification on MON89034 x MON88017			Trueness and repeatability of MON89034 quantification on single event MON89034*		
GM%	Bias (%)	RSD <sub>r</sub> (%)	GM%	Bias (%)	RSD <sub>r</sub> (%)
0.09	10	14	0.09	25	18
0.4	3.4	15	0.4	6.4	13
0.9	2.6	8.4	0.9	4.3	17
3.0	-1.0	8.5	3.0	-5.8	12
8.0	-0.9	8.1	8.0	-11	9.5

\*method validated in collaborative trial (<http://gmo-crl.jrc.it/statusofdoss.htm>)

Table 7. Trueness (bias%) and relative repeatability standard deviation (RSDr%) of the MON88017 detection method on event MON89034 x MON88017 and on event MON88017.

Trueness and repeatability of MON88017 quantification on MON89034 x MON88017			Trueness and repeatability of MON88017 quantification on single event MON88017*		
GM%	Bias (%)	RSDr (%)	GM%	Bias (%)	RSDr (%)
0.09	12	18	0.09	-2.6	28
0.5	3.6	19	0.5	2.9	13
1.0	3.8	13	1.0	-9.6	19
5.0	-0.5	14	5.0	-4.8	19
8.0	0.4	8.9	8.0	-7.6	18

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

The MON89034 event-specific method (Table 6), when is applied to event MON89034 x MON88017 shows lower bias than the single line at all GM contents. The MON88017 event-specific method (Table 7), when applied to event MON89034 x MON88017, shows a higher bias at GM content of 0.09% and comparable or lower values at higher GM contents. Moreover, in three cases (0.09%, 1.0% and 8.0%), the bias observed on MON89034 x MON88017 was positive while it was originally negative on the single line.

Regarding the relative repeatability standard deviation (RSDr%), the MON89034 method shows slightly lower or similar values when applied to MON89034 x MON88017 hybrid, with the exception of a reduction by half of the RSDr% at GM content of 0.9% (Table 6). The MON88017 method shows lower values of RSDr% on MON89034 x MON88017 event compared to the single line, with the exception of a higher RSDr% at GM content of 0.5%.

In all cases, the values of trueness and RSDr% are within the ENGL acceptance levels established at a maximum of 25%.

Therefore, the method verification has demonstrated that the MON89034 and MON88017 detection methods developed to detect and quantify the single events can be equally applied for the quantification of the respective events combined in event MON89034 x MON88017.

## 8. Conclusions

The overall method performance of the two event-specific methods for the quantitative detection of events MON89034 and MON88017 combined in maize event MON89034 x MON88017 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 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.

## 9. 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)].

## 10. 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.

## 11. 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  $\pm 25\%$  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 =  $[10^{-(1/\text{slope})}] - 1$

Acceptance Criterion: The average value of the slope of the standard curve should be in the range of  $(- 3.1 \geq \text{slope} \geq - 3.6)$

### ***R<sup>2</sup> Coefficient***

Definition: The R<sup>2</sup> coefficient is the correlation coefficient of a standard curve obtained by linear regression analysis.

Acceptance Criterion: The average value of R<sup>2</sup> should be  $\geq 0.98$ .

### ***Repeatability Standard Deviation (RSD<sub>r</sub>)***

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<sup>th</sup> of the value of the target concentration with an RSD<sub>r</sub>  $\leq 25\%$ . 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<sup>th</sup> 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<sub>R</sub>)***

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.