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# JRC VALIDATED METHODS, REFERENCE METHODS AND MEASUREMENTS REPORT

## Report on the Verification of the Performance of DP4114, MON89034, MON87411 and DAS-40278-9 event-specific PCR-based Methods applied to DNA extracted from GM Stack DP4114 × MON89034 × MON87411 × DAS-40278-9 maize

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EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Directorate F - Health, Consumers and Reference Materials (Geel)

**Food and Feed Compliance**

# Report on the Verification of the Performance of DP4114, MON89034, MON87411 and DAS-40278-9 event-specific PCR-based Methods applied to DNA extracted from GM Stack DP4114 × MON89034 × MON87411 × DAS-40278-9 maize

**15 June 2022**

## European Union Reference Laboratory for GM Food and Feed

## Executive Summary

An application was submitted by Pioneer Overseas Corporation to request the authorisation of the genetically modified stack (GM stack) DP4114 × MON89034 × MON87411 × DAS-40278-9 maize (resistance to insects, rootworms and tolerance to herbicides) and all sub-combinations of the individual events as present in the segregating progeny, for food and feed uses, import and processing, in accordance with articles 5 and 17 of Regulation (EC) N° 1829/2003 GM Food and GM Feed. The unique identifier assigned to the GM stack DP4114 × MON89034 × MON87411 × DAS-40278-9 maize is DP-ØØ4114-3-MON-89Ø34-3-MON-87411-9-DAS-4Ø278-9.

The GM stack DP4114 × MON89034 × MON87411 × DAS-40278-9 maize was obtained by conventional crossing between the genetically modified maize events DP4114, MON89034, MON87411 and DAS-40278-9, without any new genetic modification.

The EURL GMFF has previously validated individually, and declared fit for purpose, the detection methods for the single events DP4114, MON89034, MON87411 and DAS-40278-9 (see http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx). In line with the approach defined by the ENGL (http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf) the EURL GMFF has carried out an *in-house* verification of the performance of each validated method when applied to genomic DNA extracted from GM stack DP4114 × MON89034 × MON87411 × DAS-40278-9 maize.

The results of the *in-house* verification led to the conclusion that the individual methods meet the ENGL performance criteria also when applied to genomic DNA extracted from the GM stack DP4114 × MON89034 × MON87411 × DAS-40278-9 maize.
This report is published at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx.

# Content

# Quality assurance

The EURL GMFF is ISO/IEC 17025:2017 accredited [certificate number: BELAC 268 TEST (Flexible Scope for determination of Genetically Modified content in % (m/m) and % (cp/cp) in food and feed by DNA extraction, DNA identification and Real-time PCR and for determination of Genetically Modified content in % (cp/cp) in food and feed by DNA extraction and digital PCR)].

The original version of the document containing evidence of internal checks and authorisation for publication is archived within the EURL GMFF quality system.

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# 1. Introduction

The EU legislative system [1, 2] for genetically modified food and feed foresees that any GMO for food and feed use shall undergo the authorisation process before it can be placed on the market. This holds true also for a GMO containing more than one single GM event obtained by conventional crossing, co-transformation or re-transformation (genetically modified stack).

Consequently, the application for authorisation of a GM stack shall be accompanied, among others, by an event-specific method for detection, identification and quantification for each GM event composing the stack, and by samples of the stack and food and feed derived from it. The EURL GMFF shall validate the event specific methods of detection proposed by the applicant with regard to their performance when applied to DNA extracted from the stack, and shall report to the European Food Safety Authority, who will include the EURL GMFF report in the overall opinion concerning the risk assessment and potential authorisation of the assessed stack. In line with the approach defined by the ENGL (http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf), the EURL GMFF carries out an *in-house* verification of the performance of each event-specific method if this method has previously been validated by the EURL GMFF for the parental single-line event and these events have been stacked by conventional crossing. These criteria are met for the GM stack DP4114 × MON89034 × MON87411 × DAS-40278-9 maize.

Upon reception of methods, samples and related data (step 1), the EURL GMFF carried out the assessment of the documentation (step 2) and the *in-house* verification of the methods (step 3) according to the requirements of Regulation (EU) No 503/2013 (Annex III).

The results of the *in-house* verification study were evaluated with reference to ENGL method performance requirements [3] and to the validation results on the individual events.

# 2. Dossier reception and acceptance (step 1)

Pioneer Overseas Corporation submitted the detection methods, data demonstrating their adequate performance when applied to genomic DNA extracted from the stack, and the corresponding control samples of DNA extracted from the GM stack maize DP4114 × MON89034 × MON87411 × DAS-40278-9 and from non-GM maize.

The dossier was found to be complete and thus was moved to step 2.

# 3. Dossier scientific assessment (step 2)

The data provided by the applicant were assessed against the method acceptance criteria set out by the ENGL[3] and with regard to their documentation and reliability.

## *3.1 DNA extraction*

A method for DNA extraction from maize was previously evaluated by the EURL GMFF with regard to its performance characteristics and was considered valid, i.e. fit the purpose of providing maize DNA of appropriate quality and amount for being used in subsequent PCR experiments.

Annex III to Reg. (EU) No 503/2013 [2] requires the applicant to discuss the validity and limitations of the detection methods in the various types of foods and feeds (matrices) that are expected to be placed on the market. To this regard, the applicant stated that the applicability of the quantitative real-time PCR methods developed for DP4114, MON89034, MON87411 and DAS-40278-9 were pre-validated on maize seed and tissues. These methods can, in principle, be applied to any sample from which sufficient quantities of maize DNA, free of PCR inhibitors, can be purified.

However, the processing of maize grain involves varying degrees of mechanical, enzymatic, solvent, heat, acid, pressure treatment, or combinations of these steps [...]. These steps influence the quality and intactness of DNA contained in the final processed maize products [4-7] which may result in significant degradation of high molecular weight DNA and failure to PCR amplify products greater than a few 100 base pairs [4,5]. Random DNA fragmentation is known to lead to variability in quantifying DNA by qPCR [8], thus affecting the ability to accurately quantify the presence of a GM event and taxon-specific target in processed fractions. Moreover, the DNA extraction procedure necessary for some of these processed matrices may need additional rounds of processing to clean-up the DNA, to eliminate PCR inhibitors in order to achieve quality genomic DNA suitable for PCR testing [9-10]. These extraction methods are widely used for plant-based materials, are economical and can be easily scaled [11].

The EURL GMFF recommends that laboratories using this validated method for testing complex or difficult matrices always verify that the extracted genomic DNA is of sufficient quality.

The protocol for the DNA extraction method is available at https://gmo-crl.jrc.ec.europa.eu/summaries/TC1507-DNAextrc.pdf.

Consequently, the EURL GMFF did not verify the DNA extraction method proposed by the applicant.

## 3.2 PCR methods

Table 1 shows values of trueness (expressed as bias %) and precision (expressed as $RSD_r$ %) calculated by the applicant for the four methods applied to DP4114 × MON89034 × MON87411 × DAS-40278-9 maize genomic DNA. Means are the average of sixteen replicates obtained on eight runs for events DP4114, MON87411 and DAS-40278-9, and ten runs for event MON89034, performed with the Bio-Rad CFX96 Touch™ real-time PCR equipment for both DP4114 and MON89034, and with the Applied Biosystems™ 7500 real-time PCR equipment for MON87411 and DAS-40278-9. Percentages are expressed as GM-maize DNA copy numbers relative to haploid maize genome copy numbers.

*Note: Numerical values presented in the following tables were rounded keeping two digits for values ≤ 1, one digit for values between 1 and 10 and no digit for values ≥ 10, unless otherwise indicated. The calculations in the MS Excel files however were done over not rounded data. This approach might create small inconsistencies in the numerical values reported in the tables but it allows a higher precision in the final results.*

Table 1. Trueness (expressed as bias %) and precision (expressed as relative repeatability standard deviation, $RSD_r$ %) provided by the applicant for the DP4114, MON89034, MON87411 and DAS-40278-9 methods applied to GM stack DP4114 × MON89034 × MON87411 × DAS-40278-9 maize.

| **DP4114 *** | | | | | |
|---|---|---|---|---|---|
| **Sample GM %** | **Expected value (GMO %)** | | | | |
| | **5.00** | **2.61** | **0.900** | **0.520** | **0.090** |
| **Mean** | 5.10 | 2.71 | 0.933 | 0.545 | 0.053 |
| $RSD_r$ (%) | 6.0 | 7.0 | 7.3 | 6.3 | 12.3 |
| Bias (%) | 2.0 | 3.8 | 3.7 | 4.8 | 1.9 |
| **MON89034 *** | | | | | |
| **Sample GM %** | **Expected value (GMO %)** | | | | |
| | **8.00** | **3.00** | **0.900** | **0.400** | **0.085** |
| **Mean** | 7.77 | 2.94 | 0.869 | 0.378 | 0.079 |
| $RSD_r$ (%) | 5.3 | 4.6 | 6.5 | 5.6 | 11.0 |
| Bias (%) | -2.9 | -2.0 | -3.4 | -5.5 | -7.1 |
| **MON87411 *** | | | | | |
| **Sample GM %** | **Expected value (GMO %)** | | | | |
| | **5.00** | **2.00** | **0.900** | **0.550** | **0.085** |
| **Mean** | 4.84 | 2.02 | 0.912 | 0.558 | 0.046 |
| $RSD_r$ (%) | 4.5 | 5.0 | 4.2 | 5.0 | 15.4 |
| Bias (%) | -3.2 | 1.0 | 1.3 | 1.5 | -6.1 |
| | | | | | |
| **DAS-40278-9 *** | | | | | |
| **Sample GM %** | **Expected value (GMO %)** | | | | |
| | **5.00** | **2.00** | **0.900** | **0.400** | **0.080** |
| **Mean** | 5.32 | 2.14 | 0.941 | 0.418 | 0.076 |
| $RSD_r$ (%) | 7.2 | 6.4 | 5.2 | 6.0 | 13.9 |
| Bias (%) | 6.4 | 7.0 | 4.6 | 4.5 | -5.0 |

* Numbers are not rounded but are presented as reported by the applicant

### 3.2.1 Deviations from the validated methods introduced by the applicant

The event-specific methods for MON89034 maize [12], DAS-40278-9 maize [13], DP4114 maize [14] and MON87411 maize [15] were applied as published, with the following modifications:

- **MON89034**: the *hmg* reference method from MON87460 was used (with TaqMan® Universal Master Mix, instead of TaqMan® Buffer A and with 25 µL final volume of reaction). The performance of the *hmg* reference systems of MON89034 and MON87460 were compared in the 1507 x 59122 x MON 810 x NK603 verification study (EURL-VL-01/11VR) and both *hmg* methods fulfilled the EURL GMFF acceptance criteria. See the validation of event MON87460 for more information (EURL-VL-04/13VR).
- **MON87411:** the *hmg* reaction volume was reduced from 50 µL to 25 µL. The performance of the MON87411 method with reduced reaction volume was tested in the MON87427 x MON89034 x MIR162 x MON87411 verification study (EURL-VL-05/17VR).

The EURL GMFF verified the data and concluded that they were reliable and seemed to confirm that the methods meet the ENGL performance criteria [3].

The dossier was therefore moved to step 3.

# 4. EURL GMFF experimental testing (step 3)

In step 3 the EURL GMFF implemented the four methods in its own laboratory and performed a verification of their performance when applied to genomic DNA extracted from GM stack DP4114 × MON89034 × MON87411 × DAS-40278-9 maize.

## *4.1 Materials*

The following control samples were provided by the applicant:

- genomic DNA extracted from homogenized hybrid seeds of GM stack DP4114 × MON89034 × MON87411 × DAS-40278-9 maize, as positive control sample.
- genomic DNA extracted from homogenized seeds of non-GM NIL maize , as negative control sample.

The EURL GMFF prepared test samples of different GMO concentrations by mixing genomic DNA extracted from GM stack DP4114 × MON89034 × MON87411 × DAS-40278-9 maize with the non-GM maize genomic DNA, in a constant amount of total maize genomic DNA. The same GM concentrations as in the validation of the methods for the single lines were obtained. Table 2 shows the five GM concentrations used in the verification of the DP4114, MON89034, MON87411 and DAS-40278-9 methods when applying them to genomic DNA extracted from the GM stack DP4114 × MON89034 × MON87411 × DAS-40278-9 maize.

Table 2. Percentage (GM %) of DP4114, MON89034, MON87411 and DAS-40278-9 in DP4114 × MON89034 × MON87411 × DAS-40278-9 stack genomic DNA contained in the verification samples.

| **DP4114 GM %*** (GM DNA / total maize DNA x 100) | **MON89034 GM %*** (GM DNA / total maize DNA x 100) | **MON87411 GM %*** (GM DNA / total maize DNA x 100) | **DAS-40278-9 GM %*** (GM DNA / total maize DNA x 100) |
|---|---|---|---|
| 0.05 | 0.085 | 0.05 | 0.10 |
| 0.52 | 0.40 | 0.55 | 0.40 |
| 0.90 | 0.90 | 0.90 | 0.90 |
| 2.6 | 3.0 | 2.0 | 2.0 |
| 5.0 | 8.0 | 5.0 | 5.0 |

* percentage expressed in copy number ratio.

The protocols described by the applicant were implemented precisely in the EURL GMFF laboratory and were in accordance with the protocols already published for the individual DP4114, MON89034, MON87411 and DAS-40278-9 GM events (available at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx).

## 4.2 Experimental design

Eight PCR runs were carried out for each method. In each run, samples were analysed in parallel with both the GM-specific system and the reference system *hmg*, high mobility group. Five GM levels were examined per run, each GM level in duplicate. PCR analysis was performed in triplicate for all samples. In total, for each method DP4114, MON89034, MON87411 and DAS-40278-9, the quantification of the five GM levels was performed as an average of sixteen replicates per GM level (8 runs x 2 replicated levels per run). An Excel spreadsheet was used for determination of the GM %.

## 4.3 PCR methods

During the verification study, the EURL GMFF carried out parallel tests on DNA extracted from GM stack DP4114 × MON89034 × MON87411 × DAS-40278-9 maize using the single detection methods previously validated for the respective single GM events DP4114, MON89034, MON87411 and DAS-40278-9.

For detection of GM maize events DP4114, MON89034, MON87411 and DAS-40278-9, DNA fragments of 80-bp, 77-bp, 109-bp and 98-bp respectively 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 (6-carboxyfluorescein) as reporter dye at their 5'-end and the following as quencher dyes at their 3'-end: BHQ (Black Hole quencher) for DP4114, MGBNFQ (minor groove binding non-fluorescent quencher) for MON89034 and TAMRA (6-carboxytetramethylrhodamine) for both MON87411 and DAS-40278-9.

For the quantification of GM maize events DP4114, MON89034, MON87411 and DAS-40278-9, a taxon-specific reference method amplifies a 79-bp fragment of high mobility group (*hmg*), a maize endogenous gene (GenBank accession number AJ131373.1), using two *hmg* gene-specific primers and a gene-specific probe labelled with FAM (6-carboxyfluorescein) as reporter dye at their 5′-end, and respectively Minor Groove Binding (MGB) for DP4114 and TAMRA (6-carboxytetramethylrhodamine) for MON89034, MON87411 and DAS-40278-9 as quencher dyes at their 3′-end.

For the relative quantification of GM maize events DP4114, MON89034, MON87411 and DAS-40278-9 standard curves are generated both for the DP4114, MON89034, MON87411 and DAS-40278-9 and for the *hmg* reference system specific system by plotting Cq values of the calibration standards against the logarithm of the DNA amount and by fitting a linear regression into these data. Thereafter, the Cq values of the unknown samples are measured and, by means of the regression formula, the relative amount of DP4114, MON89034, MON87411 and DAS-40278-9 DNA is estimated.
For detailed information on the preparation of the respective standard curve calibration samples please refer to the protocols of the validated methods at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx.

### 4.3.1 Deviations from the validated methods introduced by the EURL GMFF

The following deviations from the original validated methods were introduced, in accordance with the applicant (see paragraph 3.2.1):

- MON89034: the *hmg* reference system from MON87460 was used, in line with EURL-VL-04/13VR;
- MON87411: the *hmg* reaction volume was reduced from 50 µL to 25 µL, in line with EURL-VL-05/17VR.

## *4.4 Results*

Tables 3, 4, 5 and 6 present the values of the slopes of the different standard curves generated by the EURL GMFF when using DNA extracted from the GM stack, from which the PCR efficiency is calculated using the formula $[10^{(-1/slope)} - 1] \times 100$, and of the coefficient of determination ($R^2$) reported for all PCR systems in the eight runs, for GM maize events DP4114, MON89034, MON87411 and DAS-40278-9. Slope values were rounded to two digits.

Table 3. Values of standard curve slope, PCR efficiency and $R^2$ coefficient for the DP4114 method on GM stack DP4114 × MON89034 × MON87411 × DAS-40278-9 maize.

| Run | DP4114 | | | *hmg* | | |
|---|---|---|---|---|---|---|
| | Slope | PCR Efficiency (%) | $R^2$ coefficient | Slope | PCR Efficiency (%) | $R^2$ coefficient |
| **1** | -3.44 | 95 | 1.00 | -3.39 | 97 | 1.00 |
| **2** | -3.37 | 98 | 1.00 | -3.38 | 98 | 1.00 |
| **3** | -3.39 | 97 | 1.00 | -3.40 | 97 | 1.00 |
| **4** | -3.52 | 92 | 1.00 | -3.37 | 98 | 1.00 |
| **5** | -3.46 | 94 | 1.00 | -3.39 | 97 | 1.00 |
| **6** | -3.41 | 96 | 1.00 | -3.41 | 96 | 1.00 |
| **7** | -3.34 | 99 | 1.00 | -3.38 | 97 | 1.00 |
| **8** | -3.41 | 96 | 1.00 | -3.42 | 96 | 1.00 |
| **Mean** | -3.42 | 96 | 1.00 | -3.39 | 97 | 1.00 |

Table 4. Values of standard curve slope, PCR efficiency and $R^2$ coefficient for the MON89034 method on GM stack DP4114 × MON89034 × MON87411 × DAS-40278-9 maize.

| Run | MON89034 | | | *hmg* | | |
|---|---|---|---|---|---|---|
| | Slope | PCR Efficiency (%) | $R^2$ coefficient | Slope | PCR Efficiency (%) | $R^2$ coefficient |
| **1** | -3.54 | 91 | 1.00 | -3.35 | 99 | 1.00 |
| **2** | -3.59 | 90 | 1.00 | -3.38 | 98 | 1.00 |
| **3** | -3.51 | 93 | 1.00 | -3.44 | 95 | 1.00 |
| **4** | -3.54 | 92 | 1.00 | -3.38 | 98 | 1.00 |
| **5** | -3.53 | 92 | 1.00 | -3.37 | 98 | 1.00 |
| **6** | -3.50 | 93 | 1.00 | -3.38 | 98 | 1.00 |
| **7** | -3.43 | 96 | 1.00 | -3.43 | 96 | 1.00 |
| **8** | -3.51 | 93 | 1.00 | -3.37 | 98 | 1.00 |
| **Mean** | -3.52 | 92 | 1.00 | -3.39 | 97 | 1.00 |

Table 5. Values of standard curve slope, PCR efficiency and $R^2$ coefficient for the MON87411 method on GM stack DP4114 × MON89034 × MON87411 × DAS-40278-9 maize.

| Run | MON87411 | | | *hmg* | | |
|---|---|---|---|---|---|---|
| | Slope | PCR Efficiency (%) | $R^2$ coefficient | Slope | PCR Efficiency (%) | $R^2$ coefficient |
| **1** | -3.44 | 95 | 1.00 | -3.36 | 98 | 1.00 |
| **2** | -3.34 | 99 | 1.00 | -3.38 | 98 | 1.00 |
| **3** | -3.47 | 94 | 1.00 | -3.46 | 94 | 1.00 |
| **4** | -3.38 | 98 | 1.00 | -3.39 | 97 | 1.00 |
| **5** | -3.40 | 97 | 1.00 | -3.37 | 98 | 1.00 |
| **6** | -3.30 | 101 | 1.00 | -3.35 | 99 | 1.00 |
| **7** | -3.37 | 98 | 1.00 | -3.41 | 96 | 1.00 |
| **8** | -3.54 | 92 | 1.00 | -3.38 | 98 | 1.00 |
| **Mean** | -3.40 | 97 | 1.00 | -3.39 | 97 | 1.00 |

Table 6. Values of standard curve slope, PCR efficiency and $R^2$ coefficient for the DAS-40278-9 method on GM stack DP4114 × MON89034 × MON87411 × DAS-40278-9 maize.

| Run | DAS-40278-9 | | | *hmg* | | |
|---|---|---|---|---|---|---|
| | Slope | PCR Efficiency (%) | $R^2$ coefficient | Slope | PCR Efficiency (%) | $R^2$ coefficient |
| **1** | -3.32 | 100 | 1.00 | -3.38 | 97 | 1.00 |
| **2** | -3.40 | 97 | 1.00 | -3.37 | 98 | 1.00 |
| **3** | -3.42 | 96 | 1.00 | -3.37 | 98 | 1.00 |
| **4** | -3.26 | 103 | 1.00 | -3.39 | 97 | 1.00 |
| **5** | -3.27 | 102 | 1.00 | -3.35 | 99 | 1.00 |
| **6** | -3.43 | 96 | 1.00 | -3.40 | 97 | 1.00 |
| **7** | -3.38 | 97 | 1.00 | -3.40 | 97 | 1.00 |
| **8** | -3.34 | 99 | 1.00 | -3.40 | 97 | 1.00 |
| **Mean** | -3.35 | 99 | 1.00 | -3.38 | 98 | 1.00 |

The mean PCR efficiencies of the GM and species-specific systems were equal or above 90 % (96 % for DP4114, 92 % for MON89034, 97 % for MON87411 and 99 % for DAS-40278-9 respectively). The mean PCR efficiencies were 97-98 % for the *hmg* maize-specific reference method. The mean $R^2$ coefficient of the methods was 1.00 for all systems in all cases. The data presented in Tables 3, 4, 5 and 6 confirm the appropriate performance characteristics of the four methods when tested on GM stack DP4114 × MON89034 × MON87411 × DAS-40278-9 maize in terms of PCR efficiency and $R^2$ coefficient.

The EURL GMFF also assessed the values of trueness (expressed as bias %) and precision (expressed as relative repeatability standard deviation, $RSD_r$ %) of the four methods applied

to samples of DNA extracted from GM stack DP4114 × MON89034 × MON87411 × DAS-40278-9 maize, presented in Tables 7, 8, 9 and 10.

Table 7. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation ($RSD_r$ %) of the DP4114 method applied to genomic DNA extracted from GM stack DP4114 × MON89034 × MON87411 × DAS-40278-9 maize.

| DP4114 | | | | | |
|---|---|---|---|---|---|
| **Unknown sample GM %** | **Expected value (GMO %)** | | | | |
| | **5.0** | **2.6** | **0.90** | **0.52** | **0.05** |
| **Mean** | 5.2 | 2.8 | 0.96 | 0.55 | 0.06 |
| SD | 0.20 | 0.15 | 0.06 | 0.04 | 0.01 |
| $RSD_r$ (%) | 3.9 | 5.4 | 5.9 | 7.8 | 11 |
| Bias (%) | 3.8 | 7.4 | 7.2 | 4.9 | 16 |

Table 8. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation ($RSD_r$ %) of the MON89034 method applied to genomic DNA extracted from GM stack DP4114 × MON89034 × MON87411 × DAS-40278-9 maize.

| MON89034 | | | | | |
|---|---|---|---|---|---|
| **Unknown sample GM %** | **Expected value (GMO %)** | | | | |
| | **8.0** | **3.0** | **0.90** | **0.40** | **0.09** |
| **Mean** | 8.1 | 2.8 | 0.90 | 0.39 | 0.09 |
| SD | 0.27 | 0.12 | 0.02 | 0.02 | 0.01 |
| $RSD_r$ (%) | 3.4 | 4.2 | 2.6 | 4.0 | 9.2 |
| Bias (%) | 1.9 | -5.5 | -0.18 | -1.7 | 1.8 |

Table 9. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation ($RSD_r$ %) of the MON87411 method applied to genomic DNA extracted from GM stack DP4114 × MON89034 × MON87411 × DAS-40278-9 maize.

| MON87411 | | | | | |
|---|---|---|---|---|---|
| **Unknown sample GM %** | **Expected value (GMO %)** | | | | |
| | **5.0** | **2.0** | **0.90** | **0.55** | **0.05** |
| **Mean** | 4.8 | 1.9 | 0.83 | 0.52 | 0.04 |
| SD | 0.14 | 0.21 | 0.04 | 0.05 | 0.01 |
| $RSD_r$ (%) | 2.8 | 11 | 4.7 | 9.3 | 12 |
| Bias (%) | -3.5 | -5.6 | -8.0 | -5.6 | -12 |

Table 10. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation ($RSD_r$ %) of the DAS-40278-9 method applied to genomic DNA extracted from GM stack DP4114 × MON89034 × MON87411 × DAS-40278-9 maize.

| DAS-40278-9 | | | | | |
|---|---|---|---|---|---|
| **Unknown sample GM %** | **Expected value (GMO %)** | | | | |
| | **5.0** | **2.0** | **0.90** | **0.40** | **0.10** |
| **Mean** | 4.6 | 2.0 | 0.81 | 0.38 | 0.08 |
| SD | 0.12 | 0.07 | 0.04 | 0.03 | 0.01 |
| $RSD_r$ (%) | 2.6 | 3.4 | 4.6 | 8.4 | 10 |
| Bias (%) | -7.7 | -0.41 | -9.5 | -4.4 | -19 |

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 less or equal to ± 25 % across the entire dynamic range. As shown in Tables 7, 8, 9 and 10, the values range from 3.8 % to 16 % for DP4114, from -5.5 % to 1.9 % for MON89034, from -12 % to -3.5 % for MON87411, and from -19 % to -0.41 % for DAS-40278-9. Therefore, the four methods satisfy the above-mentioned requirement throughout their respective dynamic ranges, also when applied to DNA extracted from GM stack DP4114 × MON89034 × MON87411 × DAS-40278-9 maize.

Tables 7, 8, 9 and 10 also show the relative repeatability standard deviation ($RSD_r$) estimated for each GM level. According to the ENGL acceptance criteria and method performance requirements, the $RSD_r$ values should be equal to or below 25 %. As the values range between 3.9 % and 11 % for DP4114, between 2.6 % and 9.2 % for MON89034, between 2.8 % and 12 % for MON87411, and between 2.6 % and 10 % for DAS-40278-9, the four methods satisfy this requirement throughout their respective dynamic ranges when applied to DNA extracted from GM stack DP4114 × MON89034 × MON87411 × DAS-40278-9 maize.

# 5. Conclusions

The performance of the four event-specific methods for the detection and quantification of maize single line events DP4114, MON89034, MON87411 and DAS-40278-9, when applied to genomic DNA extracted from GM stack DP4114 × MON89034 × MON87411 × DAS-40278-9 maize, meets the ENGL performance requirements, as assessed on the control samples provided by the applicant.

Therefore these methods, developed and validated to detect and quantify the single maize events DP4114, MON89034, MON87411 and DAS-40278-9, can be equally applied for the detection and quantification of the respective events in DNA extracted from the GM stack DP4114 × MON89034 × MON87411 × DAS-40278-9 maize or any of its sub-combinations, supposed that sufficient genomic DNA of appropriate quality is available.

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