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Comparative Testing Report on the Quantification of Soybean GM Event 40-3-2 in Chicken Feed

Comparative testing round: ILC-EURL-GMFF-CT-02/14 - Part II

European Union Reference Laboratory for Genetically Modified Food and Feed

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Joint Research Centre

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Abstract

The European Union Reference Laboratory for Genetically Modified Food and Feed (EURL GMFF), accredited under ISO/IEC 17043, organised a comparative testing (CT) round for National Reference Laboratories (NRLs) nominated under Regulation (EC) No 882/2004 (NRL/882), with voluntary participation of other official control laboratories.
The CT round included two GM events and two different test items. The first report on the qualitative results obtained in this CT round and on the quantitative results for GM maize event 40278 was published on the 6th May 2015. The quantitative results for 40-3-2 soybean required a further in-depth investigation, it was therefore decided to evaluate the results for this event in a separate report. The current report describes the quantitative results obtained for soybean event 40-3-2 in test item 1 (T1).
The results reported by the participants for the quantification of soybean event 40-3-2 in T1 did not follow a normal distribution but rather a left-skewed multimodal distribution. More detailed information on the analytical methodology used was sought from the participants to understand the causes of this non-normal data distribution and deviant results. Additionally, the EURL GMFF re-tested some T1 bottles retrieved from participants together with DNA extracted from T1 by participants. Also a number of participants repeated their own analysis of T1.
Re-testing of T1 flour by the EURL GMFF indicated that the test material remained homogeneous and stable during shipment to the participants since the results were comparable to those originally obtained during homogeneity testing. Re-testing by the EURL GMFF of the DNA extracted by participants and repeated analysis by a number of participants provided evidence that the deviating results were most probably caused by poor DNA extractions, rather than in the subsequent PCR amplification. In some cases inhibition may also have caused a deviating result; however, in other cases, no signs of inhibition were observed. Taking all this information together, it was concluded that the use of an appropriate DNA extraction procedure with adequate sample intake, and the careful verification of the absence of PCR inhibitors in the extracts, were crucial steps for obtaining reliable quantification results for the entrained event 40-3-2 in T1, a complex feed material.
The performance of the laboratories was evaluated as follows: based on the results of the NRL/882 "expert laboratories", which followed a normal distribution, the EURL GMFF calculated a robust mean (μR) for event 40-3-2. The robust mean and target standard deviation, agreed by the Advisory Board of Comparative Testing, were used to derive z-scores for all participants' results. Based on these parameters the quantification of soybean event 40-3-2 resulted in a satisfactory performance ($|z| \leq 2.0$) for 54 out of 70 laboratories (77 %) that provided a quantitative result. Further follow-up actions are ongoing for some laboratories that received an unsatisfactory performance score.
Eight other participants did not test for event 40-3-2, hence their performance for analysis of this event could not be evaluated.

EUROPEAN COMMISSION
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Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit

EURL European Union Reference Laboratory for GM Food & Feed

Comparative Testing Report on the Quantification of Soybean GM Event 40-3-2 in Chicken Feed

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Executive Summary

The European Union Reference Laboratory for Genetically Modified Food and Feed (EURL GMFF), accredited under ISO/IEC 17043, organised a comparative testing (CT) round for National Reference Laboratories (NRLs) nominated under Regulation (EC) No 882/2004 (NRL/882), with voluntary participation of other official control laboratories.

The CT round included two GM events and two different test items. The first report on the qualitative results obtained in this CT round and on the quantitative results for GM maize event 40278 was published on the 6th May 2015. The quantitative results for 40-3-2 soybean required a further in-depth investigation, it was therefore decided to evaluate the results for this event in a separate report. The current report describes the quantitative results obtained for soybean event 40-3-2 in test item 1 (T1).

The results reported by the participants for the quantification of soybean event 40-3-2 in T1 did not follow a normal distribution but rather a left-skewed multimodal distribution. More detailed information on the analytical methodology used was sought from the participants to understand the causes of this non-normal data distribution and deviant results. Additionally, the EURL GMFF re-tested some T1 bottles retrieved from participants together with DNA extracted from T1 by participants. Also a number of participants repeated their own analysis of T1.

Re-testing of T1 flour by the EURL GMFF indicated that the test material remained homogeneous and stable during shipment to the participants since the results were comparable to those originally obtained during homogeneity testing. Re-testing by the EURL GMFF of the DNA extracted by participants and repeated analysis by a number of participants provided evidence that the deviating results were most probably caused by poor DNA extractions, rather than in the subsequent PCR amplification. In some cases inhibition may also have caused a deviating result; however, in other cases, no signs of inhibition were observed. Taking all this information together, it was concluded that the use of an appropriate DNA extraction procedure with adequate sample intake, and the careful verification of the absence of PCR inhibitors in the extracts, were crucial steps for obtaining reliable quantification results for the entrained event 40-3-2 in T1, a complex feed material.

The performance of the laboratories was evaluated as follows: based on the results of the NRL/882 "expert laboratories", which followed a normal distribution, the EURL GMFF calculated a robust mean (μ_R) for event 40-3-2. The robust mean and target standard deviation, agreed by the Advisory Board of Comparative Testing, were used to derive z-scores for all participants' results. Based on these parameters the quantification of soybean event 40-3-2 resulted in a satisfactory performance ($|z| \leq 2.0$) for 54 out of 70 laboratories (77 %) that provided a quantitative result. Further follow-up actions are ongoing for some laboratories that received an unsatisfactory performance score.

Eight other participants did not test for event 40-3-2, hence their performance for analysis of this event could not be evaluated.

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1. Introduction

The Joint Research Centre (JRC) of the European Commission was established as European Union Reference Laboratory for GM Food and Feed (EURL GMFF) by Regulation (EC) No 1829/2003[(1)]. The EURL GMFF is also mandated by Regulation (EC) No 882/2004[(2)].

Article 32 of Regulation (EC) No 882/2004 tasks the EURLs with the organisation of comparative testing (CT) for National Reference Laboratories (NRLs), designated under Regulation (EC) No 882/2004) and an appropriate follow-up of such testing. The EURL GMFF is accredited under ISO/IEC 17043[(3)] to organise CT rounds. The aim of this activity is 'to contribute to a high quality and uniformity of analytical results'[(2)]. Article 12 of the said Regulation requires that the designated NRLs should be accredited under ISO/IEC 17025 on 'General Requirements for the Competence of Testing and Calibration Laboratories' and 17025-accredited laboratories must prove their competence, *e.g.* by taking part in comparative testing.

Regulations (EC) No 1829/2003 and (EU) No 619/2011[(4)] establish a threshold for labelling of food and feed products (0.9 %) and a minimum required performance limit (0.1 m/m %) for detecting low level presence of listed Genetically Modified Organisms (GMOs) in feed. Since these values are used by the Member States of the European Union in the official control of food and feed, an accurate and reliable determination of the GMO content is of paramount importance.

The EURL GMFF organised a CT round for NRLs designated under Regulation (EC) No 882/2004 (NRL/882), whose participation was mandatory. EURL GMFF also recommended the participation of NRLs nominated under Regulation (EU) No 120/2014[(5)] (NRL/120) and participation was open and free of charge for any official control laboratory worldwide. Two test items were prepared by the EURL GMFF and were shipped to the participants in plastic containers containing approximately 5 g of powder. The EURL GMFF managed the on-line laboratory registration, the submission of results and the evaluation and reporting of the results. This activity was supported by an Advisory Board for CT. This CT round meets the requirements of ISO/IEC 17043.

Most of the results obtained in this CT round ILC-EURL-GMFF-CT-02/14 have already been published in the final report, dated the 6th May 2015[1], however, the current report summarises the additional quantitative results and laboratory performance for soybean event 40-3-2 in test item 1 (T1).

2. Test item

Test item T1 was prepared in-house by the EURL GMFF. It was a complex, real-life feedstuff material composed of chicken feed already containing event 40-3-2, which was then spiked with maize event 40278, and non-GM soybean flour. Its production and characterisation have been described previously (see footnote 1).

3. Tasks to be performed by participants

Participants in this CT round were required to screen T1 for the presence of three soybean and three maize events and quantify any events detected (more details can be found in the report cited in footnote 1).

[1] Comparative Testing Report on the Detection and Quantification of 40278 Maize in Chicken Feed and Maize Flour. Comparative testing round: ILC-EURL-GMFF-CT-02/14, published 6 May 2015.

4. Results

For soybean event 40-3-2 in T1, 70 participants reported a quantitative result (for the other results, see the report cited in footnote 1). One participant provided a semi-quantitative value (below 0.07), which was excluded from the calculations below. The remaining seven participants in this CT round did not provide a numerical value for this event.

The quantitative results were expressed in m/m % (62 laboratories) or cp/cp % (8 laboratories). For the calculations, all values were converted to m/m % using a conversion factor of 1 (since soybean is a homozygous crop there will be no significant difference between results expressed in either unit).

4.3.1 Data distribution for soybean event 40-3-2 in T1

Following evaluation of the results reported for 40-3-2 soybean on both the original and $\log_{10}$-transformed scale, it appeared that the data distribution was non-normal and skewed towards the left, with a smaller bump at the lower end (see Figure 1, nonparametric representation of the probability density function of a random variable). As a result of this data distribution, calculation of a consensus value and z-scores reflecting the performance of the participants would not be meaningful. A more detailed evaluation of the unexpected variability in the results determined that there was no apparent relationship between the results and any method-related parameter, as reported in the questionnaire of the CT round, e.g. the DNA extraction method employed, number of replicates, qPCR method used, real-time PCR instrument, etc. Furthermore it was noted that more deviating results were reported by laboratories that were in Categories b and c, i.e. NRL/120 and non-EU control laboratories, compared to laboratories designated as NRL/882 (Category a).

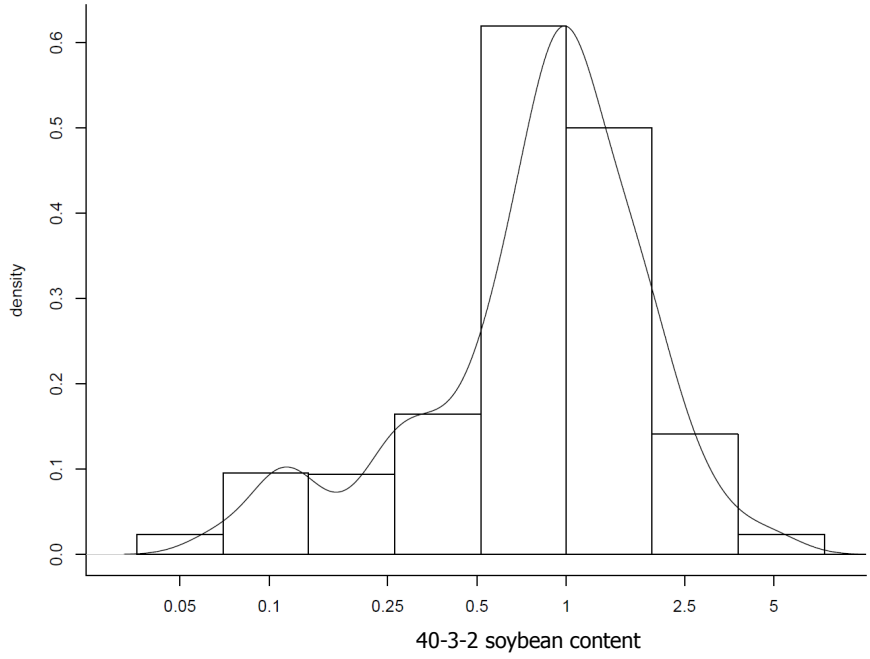

Figure 1. Kernel density distribution of soybean event 40-3-2 results reported by participants (in m/m %).

4.3.2 Evaluation of additional information from laboratories on 40-3-2 analysis

As a result of the deviating 40-3-2 soybean results received from some participants, further details on the analysis methods used by participants were sought. Information was received from 17 laboratories, 13 of which had reported a low value (between 0.07 and 0.41 m/m %) and 4 a high value (> 2.2 m/m %). It was found that half of these participants had performed some sort of PCR inhibition test and had found no inhibition, whereas the other half had not performed an inhibition test. Slopes and R^2 values were within the ENGL acceptance limits for all but one laboratory.

All the participating laboratories were subsequently contacted to provide further information on their analysis using a more extensive follow-up questionnaire. This information was received from a total of 49 of the 70 laboratories that had originally provided a quantitative result for this event. The results were evaluated per group of results data, with 12 laboratories in the "low" GM content group (< 0.55 m/m %), 4 in the "high" GM content group (> 2.22 m/m %), and 33 in the "acceptable" GM content group (0.55 – 2.22 m/m %). This grouping corresponded to below 50 % (low group) and above 100 % (high group) of the expected GM content (average of the NRL/882 participants, see below), and within an interval around the average for the "acceptable" group. The results are summarised in Table 1. Based on this evaluation, there was no clear correlation apparent between the quantitative results obtained and any method-related parameters.

Table 1. Summary of method-related parameters for the testing of soybean event 40-3-2 used by the laboratories.

Quantitative PCR Result Group		Low	High	Acceptable
Number of Laboratories		12	4	33
Inhibition tests	None	4	1	7
	Regression on dilutions	4	0	8
	Comparing CTs of 2 dilutions	4	3	14
Calibrant	ERM-BF410gk	12	1	31
	In-house developed	0	1	2
	Eurofins kit	0	1	0
Slopes and R^2	Within acceptance limits	11	4	28
	Outside acceptance limits (slope)	1	0	5
QC material	1% CRM IRMM	7	2	24
	Proficiency test sample	2	0	3
	Other CRM IRMM (0.1%, 0.5%, 10%)	7	1	7
	In-house developed	0	1	0
	None used	0	0	2
Result QC	OK	12	2	28
	Not OK	0	1	2
Reported result	Adjusted based on QC result	3	2	7
	Not adjusted	10	2	23
Taxon-specific assay (lectin)	EURL method	10	1	24
	ISO	2	1	3
	Other	0	3	6
Event-specific assay (40-3-2)	EURL method	10	1	25
	ISO	0	2	2
	Other	2	1	6
Annealing temp. 40-3-2 assay	55°C	5	1	16
	60°C	7	3	15
Modifications	Volume 25 µL	8	3	19
	Other mastermix	4	3	11
	Other modifications	6	4	15

4.3.3 Re-testing of T1 bottles from participants by the EURL GMFF

To exclude the possibility that the homogeneity and/or stability of the T1 flour had been affected by shipment, the EURL GMFF retrieved T1 bottles from a number of laboratories in the "low" and "high" group for re-testing. DNA was extracted from the flour using a NucleoSpin methodology (note that EURL GMFF characterisation studies on T1 had previously been performed using CTAB). PCR inhibition tests confirmed the absence of inhibition in these DNA extracts. Real-time PCR analysis for event 40-3-2 confirmed the expected GM content in the materials (average 1.12 m/m %) and contrasted with the either low or high results previously reported by the participants for these bottles of T1 (see Table 2). This confirmed that the quantification of event 40-3-2 in T1 flour had been unaffected by the shipment and storage at the participant's premises.

Table 2. Results of 40-3-2 soybean testing by EURL GMFF on the same T1 bottles previously analysed by participants.

Sample Code	GM Content Reported by Participant (m/m %)	GM Content Measured by EURL GMFF (m/m %)		
		Result per extract	Average per bottle	Average overall
(L18)_A-1	0.25	0.81	0.83	1.12
(L18)_A-2		0.85		
(L28)_B-1	2.28	1.42	1.28	
(L28)_B-2		1.13		
(L66)_C-1	0.30	0.94	1.03	
(L66)_C-2		1.11		
(L81)_D-1	0.41	1.55	1.43	
(L81)_D-2		1.31		
(L27)_E-1	2.26	1.55	1.38	
(L27)_E-2		1.21		
(L60)_F-1	2.90	1.26	1.00	
(L60)_F-2		0.74		
(L19)_G-1	0.35	1.29	1.24	
(L19)_G-2		1.19		
(L03)_H-1	3.17	0.75	0.80	
(L03)_H-2		0.85		
(L76)_I-1	0.11	1.04	1.13	
(L76)_I-2		1.22		
QC_1%_1	/	1.04	1.05	/
QC_1%_2		1.05		

Note: QC_1%_1 and 2 are quality control samples prepared by the EURL GMFF from ERM-BF410dk (1 % 40-3-2 soybean)

4.3.4 Testing of DNA extracts from participants by the EURL GMFF

Some of the participants that had reported a low or high 40-3-2 soybean content in T1 returned an aliquot of the DNA extracted from T1 to the EURL GMFF. The EURL GMFF planned to re-test the extracts to determine if both the EURL GMFF and the participant would obtain similar results when applying the PCR module of the analytical procedure to the same DNA extracts. The EURL GMFF first verified the double-stranded DNA content in the extracts returned by six participants using PicoGreen; it was noted that the DNA concentration was significantly lower than the DNA concentration reported by the participants. This was caused by the use of spectrophotometric methods to measure the DNA content by at least some of the participants, which often overestimate the DNA content, particularly if the extracts also contain impurities, including RNA or denatured DNA. Because of the very low double-stranded DNA concentration in the extracts, the EURL GMFF only re-tested four DNA extracts from the same laboratory (L81) for their 40-3-2 content, two of which were extracted using the CTAB method, the other two by the Wizard method (Promega). The DNA concentration in these extracts ranged from 15 to 19 ng/µL, less than half the concentration recommended in the EURL-validated 40-3-2 method (40 ng/µL). No inhibition tests were performed by the EURL GMFF because of the low sample volume. The results, shown in Table 3, are comparable to the result previously reported by the laboratory (average of 0.41 m/m %), and about half of what the EURL GMFF had previously measured in other aliquots of T1. To verify the absence of PCR inhibitors, the EURL GMFF also tested the same DNA extracts diluted ten times and obtained similar results (data not reported). These results indicated that the low GM content measured in the extracts was correct, hence suggesting that the DNA extraction methodology used by the participant was not reliable as regards the

quantification of event 40-3-2 (although quantification of the spiked maize event 40278 in the same matrix appeared reliable – see the report in footnote 1). This was also confirmed by the participant (L81) who re-tested one of the CTAB extracts and obtained a low GM content again (see Table 4 below). One possible explanation could be that the sample intake for extraction was too low if there were small in-homogeneities in the composite flour. Since only a few DNA extracts from one participant were re-tested by the EURL GMFF, care should be taken when drawing general conclusions from these results.

Table 3. Results of 40-3-2 soybean testing by EURL GMFF on the same DNA extracts previously analysed by participant L81 (reporting an average value of 0.41 m/m %).

Sample Code	GM Content Measured by EURL GMFF (m/m %)	Average GM Content (m/m %)
L81_Wizard-A1	0.52	0.54
L81_Wizard-B1	0.56	
L81_CTAB-A1	0.43	0.53
L81_CTAB-B1	0.63	
QC_1%	1.06	/

Note: QC_1% is a quality control sample prepared by the EURL GMFF from ERM-BF410dk (1 % 40-3-2 soybean)

4.3.5 Re-testing results from the participants

The 40-3-2 soybean density data distribution (as in Figure 1) was included in a technical report sent to all participants that had reported a quantitative value for this event on the 28th April 2015. The skewed data distribution was shown in this report without revealing the actual values, however the laboratory codes were displayed at the corresponding positions on the X-axis. To understand the problems which laboratories faced when quantifying this event in the chicken feed matrix, the participants which had reported a quantitative result in the extremities of the distribution (arbitrarily set at <0.5 and >2.1 m/m %) were asked to voluntarily repeat their testing and report the results to the EURL GMFF. Thirteen out of 21 laboratories in this "extreme" group reported their re-testing results, together with one additional laboratory (L43, original result 2.1 m/m %). Nine of these laboratories had originally reported a value <0.5 m/m % whereas 5 a value ≥2.1 m/m % (Table 4).

Following re-testing, 11 of the 14 laboratories reported a value within the range 0.5 – 2.1 m/m %, although the values of three of these laboratories were only slightly improved compared to the previous analysis (L81, L73 and L27; note that L73 did not perform new DNA extractions but re-tested the original extracts). Another 3 laboratories (L03, L41 and L49) obtained a result that was comparable to the extreme result of their first analysis.

Table 4. Evaluation of the results of the repeated analysis for soybean event 40-3-2 by the participants.

Lab Code	Category	First Analysis		Re-Testing		
		DNA Extraction	Result	DNA Extraction	Result	Comments
L78	c	Biotecon kit	0.07	CTAB	1.00	Good mixing of flour
L76	b	CTAB	0.11	NucleoSpin kit	1.19	Original extracts seemed bad quality
L49	c	CTAB	0.12	CTAB+Wizard clean-up	0.19	Slopes too low (-3.71 and -3.94); 1:4 dilution similar result, but no inhibition tests done
L18	c	QiaAmp+DNA extractor clean-up	0.25	QiaAmp+DNA extractor clean-up	1.08	
L41	c	NucleoSpin kit	0.33	CTAB lysis+NucleoSpin (2 g)	0.30	No inhibition in both tests (3 dilutions)
L19	b	CTAB+Wizard clean-up	0.35	NucleoSpin kit	1.15	Repeating the extraction with CTAB gave similar result. Changing to NucleoSpin seemed better. Remarkably, effect of mastermix was found.
L81	a	CTAB	0.41	CTAB	0.63	No inhibition. Note: re-testing original extract confirmed low value (0.37%).
L73	a	DNeasy kit (0.1 g)	0.45	(same extracts re-tested)	0.52	Delta CT method; 1 % and 10 % QC samples OK
L82	c	Promega Wizard kit	0.48	Promega Wizard kit	0.93	No inhibition (regression); previous Wizard kit was expired
L43	c	Mericon Food kit	2.10	NucleoSpin kit	0.92	Inhibition first time as seen for 40278 maize
L27	a	CTAB+QIAEX II	2.27	NucleoSpin (0.2 g intake)	1.90	No inhibition in both tests
L28	a	NucleoSpin kit	2.28	NucleoSpin kit	1.42	Re-testing done the same; QC samples too high (first test) or too low (re-testing)
L60	a	NucleoSpin kit (2.0 g intake)	2.90	NucleoSpin kit (0.2 g intake)	0.86	Probably inhibition due to too high sample intake first time
L03	c	NucleoSpin kit	3.17	NucleoSpin+NucleoSpin clean-up	2.54	Inhibition not tested, but similar result for two-fold dilution

From the additional information received from the participants the following conclusions were drawn with regards to the causes of the deviating results originally reported:

1. The poor DNA quality of the original DNA extracts, with an observed or likely PCR inhibition, seemed to be the explanation for the extremely low or high results reported by L76, L19, L82, L43 and L60. Changing the extraction method from CTAB (L19, L73) or Mericon Food (L43) to NucleoSpin apparently resulted in an improved DNA quality and results closer to the expected value. L82 had used an expired Promega Wizard extraction kit, and replacing it with a new kit, resulted in an improved result. In the case of L60, reducing the sample intake (from 2 g to 0.2 g) resolved the problem (this laboratory also used the NucleoSpin method). Another laboratory (L52), reporting a very good result the first time, commented that following CTAB extraction on a complex soybean matrix it is important to use a clean-up column (e.g. Qiaquick) for further purification of the DNA; similarly, a sufficiently long lysis time is important to extract a representative share of all DNA fragments when particle sizes of GM and non-GM materials may not be exactly identical.

2. Mixing of the flour before taking the samples, perhaps in combination with the use of another extraction method, seemed the solution for L78, who used a CTAB method for the re-testing while a Biotecon kit was used in the first analysis.

3. For some participants it remains unclear why the results are not closer to the expected value. For example, for L41, L81, L27 and L03, no signs of PCR inhibition were observed in the DNA extracts, yet the results were unsatisfactory (low or high). There are different ways to test for the presence of PCR inhibitors in the DNA extracts, and some of these participants only compared the results obtained for two dilutions of the same extracts; this may not be accurate enough to conclude on the absence

of PCR inhibition. This may also explain why L49 did not succeed in obtaining a better quantitative result (used CTAB plus clean-up by a Wizard column), although the slopes of both calibration curves were also outside the ENGL acceptance criteria (http://gmo-crl.jrc.ec.europa.eu/doc/MPR%20Report%20Application%2020_10_2015.pdf) for this participant. L28 repeated the whole analysis exactly as before (original result 2.28 m/m %) and obtained a better result (1.42 m/m %); the QC control material, however, was quantified as either too high (first test) or too low (re-testing) and it remains unclear why the results were so different. Additionally, L78 reported more reliable results using the standard curve method compared to the delta Ct method (also used by L73). Use of the delta Ct method is discouraged by the ENGL in its most recent guidance document (see link above). Surprisingly, L19 noted an effect of the mastermix brand used during PCR, with the Universal Mastermix giving a value of 1.75 m/m % (N = 2) for the same DNA extracts that tested 1.15 m/m % with Buffer I from Life Technologies.

In conclusion, it seems that there may be different reasons for the deviating results observed by the participants. However, one conclusion which can be drawn is that ensuring an optimal DNA purity with minimal inhibitors is paramount for obtaining reliable results in quantitative real-time PCR. It is particularly important to select a DNA extraction method that is suitable for the matrix, as exemplified for the complex matrix of the chicken feed flour and soybean mixture used in T1. The extraction seemed less problematic for maize event 40278, which was spiked into the flour, than for soybean event 40-3-2, which was already present in the chicken feed material. In the absence of any information on the source of the soybean GM event in this material and how the feed has been treated in the past, it can only be said that the extractability of these GM events from the compound matrix was different, perhaps due to their specific physicochemical characteristics.

Of similar importance for reliable analytical measurements is to test the resulting extracts for inhibition, using a robust inhibition test based on several dilutions (regression test) and only comparing the Ct values measured for two dilutions of the extract may not be sufficient in all cases. Furthermore, the sample intake for extraction should be sufficiently large to give a good representation of all particles in the flour, and, as a routine procedure, the flour should be mixed before taking the samples. In addition the extraction of at least two test portions should be performed.

4.3.6 Calculation of the consensus value

Despite the variable quantitative results obtained for soybean event 40-3-2, the data distribution was normal when only the data from the NRL/882 laboratories was considered. These laboratories, all of which are also assigned as NRL under Regulation (EU) No 120/2014 and regularly participate in ring trials for validation of the real-time PCR methods for GMO analysis, could be considered as experts in the field of quantitative GMO analysis. Therefore, and in line with ISO 13528:2005 on "*Statistical methods for use in proficiency testing by interlaboratory comparisons*"[6], the Advisory Board for Comparative Testing decided that the consensus value for 40-3-2 soybean (μ_R) should be calculated on the basis of the results from the group of 28 expert laboratories (NRL/882) that provided a result for this event.

The consensus value (μ_R) for the data from NRL/882 participants for soybean event 40-3-2 in T1 was calculated using robust statistics[7,8]. This approach minimises the influence of outlying values.

The expanded uncertainty on the results obtained (U) comprises standard uncertainty (u) contributions from the characterisation of the material (u_{char}) and the between-test item homogeneity (u_{bb})[9], and is estimated according to:

$$U = k\sqrt{u_{char}^2 + u_{bb}^2} \quad (1)$$

A coverage factor (k) of 2 was used to calculate the expanded uncertainty corresponding to a 95 % level of confidence[10]. The standard uncertainty (u_{char}) on the characterisation was calculated using the formula:

$$u_{char} = \frac{\sigma}{\sqrt{N}} \quad (2)$$

where: σ = robust Relative Standard Deviation of the robust mean expressed in m/m %
N = number of data points

The robust mean (μ_R) for data on the non-transformed scale, and associated uncertainties, as calculated by the EURL GMFF, are reported in Table 5.

Table 5. Overview of robust mean (μ_R) and expanded uncertainty.

Test Item	GM Event	*N*	μ_R (m/m %)	*U* (m/m %)
T1	40-3-2 soybean	70	1.11	0.22

4.3.7 Performance of the laboratories

To evaluate laboratory performance, z-scores were calculated on the basis of the consensus value determined for the data as described in Section 4.3.6 (see Annex 1, formula A1.1). Based on the experience in previous CT rounds and taking into account the results of previous CTs, the target standard deviation for CT was fixed by the Advisory Board for Comparative Testing at 0.2 for this complex matrix. For consistency, all decimal numbers were rounded to two digits.

Z-scores were calculated for all results using the formula obtained for NRL/882 data and applying this to all results, including those of non-NRL/882 participants (category b and c participants).

Sixteen laboratories received a z-score outside the acceptable range (*i.e.* |z| > 2.0) for this event (Table 6); two of these are NRL/882 (L60 and L66). Detailed results are reported in Annex 2, Tables A2.1 to A2.3 and Figure A2. As outlined previously, 8 other participants did not report results for event 40-3-2, hence their performance for analysis of this event was not evaluated.

Table 6. Performance of laboratories in comparative test ILC-EURL-GMFF-CT-02/14 for quantification of soybean event 40-3-2 in T1[a].

Test Item	GM Event	No Quantitative Result Submitted[b]	Satisfactory Z-score	Unsatisfactory Z-score
T1	40-3-2 soybean	L01, L08, L16, L25, L40, L50, L53, L83	L04, *L06*, L09, L10, L11, L12, L13, L14, L17, L20, *L21*, L22, L23, L24, L26, L27, L28, L29, L30, L31, L32, L35, L37, L38, L39, L43, L44, L45, L46, L47, L48, L52, L54, L55, L56, L57, L58, *L59*, L61, L64, L67, L69, L70, L71, L72, L73, L74, L75, L77, L79, L80, L81, *L82*, L84	L03, L05, L18, L19, L33, L34, L36, L41, *L42*, L49, L51, L60, *L62*, L66, L76, L78

[a] Laboratories that reported their results in cp/cp % are shown in italics, these results were considered as values in m/m % for the subsequent calculations (see Section 4.3.2).
[b] The underlined laboratory code refers to a registered participant that reported a result as a semi-quantitative value (<LOQ).

5. Conclusions

Participants in CT 02/14 were required to quantify soybean event 40-3-2 in T1. The complexity of the test matrix, a compound feed mixture processed from chicken feed and soybean flour, was reflected in the performance of the laboratories. Quantification of the 40-3-2 soybean in T1 resulted in a wide range of reported results, and a non-normal data distribution that was skewed towards the left. Follow-up investigations were initiated to understand the causes of the variable results, including re-testing by the EURL GMFF and by several participants. The conclusions lend support to the importance of using an appropriate sample intake and DNA extraction methodology and to performing an inhibition test on the DNA extracts before real-time PCR analysis to determine the GM content.

The approach used to evaluate the performance of the laboratories with regards to the quantification of the 40-3-2 soybean content of T1 consisted of calculating a consensus value on the basis of the values reported by the NRL/882 "expert" laboratories. This consensus value was then used to calculate z-scores for all other laboratories. Fifty-four laboratories received a satisfactory performance score for quantification of soybean event 40-3-2 (77 %). The remaining laboratories must endeavour to improve their analytical procedures, particularly when handling complex food or feed matrices.

The participants which did not report a full set of quantitative results for some or all GM events to be tested during this CT round should implement the corresponding event-specific methods in their laboratories. Specifically, it is imperative under EU legislation that NRL/882 laboratories are able to identify and quantify all GM events that are authorised in the EU or for which the authorisation is pending or has expired and that they should ensure that the resources (including but not limited to appropriately validated primer and probe sets, CRM, quality control material and experienced staff) are available to perform these analyses.

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The laboratories listed below are kindly acknowledged for their participation in this exercise.

COUNTRY	ORGANISATION	DEPARTMENT	CITY
	CATEGORY[1] a		
AT	Umweltbundesamt GmbH	Landuse & Biosafety	Vienna
AT	Agentur für Gesundheit und Ernährungssicherheit GmbH (AGES)		Vienna
BE	Centre Wallon de Recherches Agronomiques	Valorisation des Productions	Gembloux
BE	Scientific Institute of Public Health	PBB	Brussels
BE	Institute for Agricultural and Fisheries Research	Technology and Food - PI	Merelbeke
BG	National Center of Public Health and Analyses	GMO unit	Sofia
CY	State General Laboratory	GMO & Allergens Laboratory	Nicosia
DE	Bundesamt für Verbraucherschutz und Lebensmittelsicherheit		Berlin
DK	Danish Veterinary and Food Administration	Plant diagnostics	Ringsted
ES	Laboratorio Arbitral Agroalimentario, LAA-MAGRAMA	OGM	Madrid
ES	Centro Nacional de Alimentación (Agencia Española de Consumo Seguridad Alimentaria y Nutrición	Biotechnology Unit	Madrid
FI	Finnish Customs Laboratory		Espoo
FR	BioGEVES		Surgeres
FR	Service Commun des Laboratoires		Illkirch-Graffenstad
FR	Agence Nationale de Sécurité Sanitaire de l'Alimentation, de l'Environnement et du Travail (ANSES)	Plant Health Laboratory (LSV)	Angers Cedex
GR	Ministry of Finance, General Chemical State Laboratory	A' Chemical Service of Athens	Athens
HR	Croatian National Institute of Public Health		Zagreb
HU	National Food Chain Safety Office		Budapest
IT	Istituto Zooprofilattico Sperimentale Delle Regioni Lazio e Toscana	Stuttutura di Biotecnologie	Rome
LT	National Food and Veterinary Risk Assessment Institute	Molecular Biology and GMO	Vilnius
LU	Laboratoire National de Santé	Food Control	Dudelange
LV	Institute of Food Safety, Animal Health and Environment	Virology	Riga
NL	RIKILT Wageningen UR		Wageningen
PL	Instytut Zootechniki PIB KLP Pracownia w Szczecinie		Szczecin
PL	National Veterinary Research Institute	Feed Hygiene	Pulawy
PL	Regional Laboratory of Genetically Modified Food		Tarnobrzeg
RO	Institute for Diagnosis and Animal Health	Molecular Biology and GMO	Bucharest
SE	National Food Agency		Uppsala
SI	National Institute of Biology		Ljubljana
SK	State Veterinary and Food Institute		Dolny Kubin
SK	Central Control and Testing Institute in Agriculture	Dptm. of Molecular Biology	Bratislava
UK	LGC		Teddington

COUNTRY	ORGANISATION	DEPARTMENT	CITY
CATEGORY b			
DE	Landesbetrieb Hessisches Landeslabor		Kassel
DE	CVUA Freiburg	GMO	Freiburg
DE	Landesuntersuchungsanstalt für das Gesundheits- und Veterinärwesen Sachsen	Amtliche Lebensmitteluntersuchung	Dresden
DE	Bundesinstitut für Risikobewertung (BfR)	Food Safety	Berlin
DE	Landesamtes für Landwirtschaft, Lebensmittelsicherheit und Fischerei (LALLF) Mecklenburg-Vorpommern (LALLF M-V)	Dez. 200	Rostock
DE	Landeslabor Schleswig-Holstein		Neumünster
DE	Landeslabor Berlin-Brandenburg	Fachbereich I-6	Berlin
DE	Landesuntersuchungsamt	Institut f. Lebensmittelchemie	Trier
DE	LAVES - Food- and Veterinary Institute Braunschweig/Hannover	FB12	Braunschweig
DE	Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft	GB 6, Fachbereich 63	Nossen
DE	Landesamt für Verbraucherschutz Sachsen-Anhalt	Fachbereich 3	Halle
DE	Thüringer Landesamt für Verbraucherschutz (TLV)	Lebensmitteluntersuchung	Bad Langensalza
DE	Bavarian Health and Food Safety Authority (LGL)		Oberschleissheim
FI	Finnish Food Safety Authority		Helsinki
IT	CRA-SCS	Sede di Tavazzano, Laboratorio	Tavazzano (LO)
NL	Netherlands Food and Consumer Product Safety Authority (NVWA)	Consument en Veiligheid	Wageningen
PL	Institute of Biochemistry and Biophysics PAS		Warszawa
SI	Agricultural Institute of Slovenia		Ljubljana
UK	Fera		York
UK	Scottish Government	SASA	Edinburgh
CATEGORY c			
BE	Federal Laboratory for Food Safety Melle	Department of GMO	Melle
BG	SGS Bulgaria Ltd	Laboratory of SGS Bulgaria	Varna
CH	Agroscope, Institute for Livestock Sciences		Posieux
CH	Federal Food Safety and Veterinary Office FSVO	Risk Assessment Division	Bern
CO	National Institute for Food and Drug Surveillance - INVIMA	OLCC	Bogotá
DE	Chemical and Veterinary Analytical Institute Muensterland-Emscher-Lippe (CVUA-MEL)		Muenster
DE	Thüringer Landesanstalt für Landwirtschaft	Untersuchungswesen	Jena
HU	BIOMI Ltd		Gödöllő
ID	National Quality Control Laboratory of Drug and Food	Biotechnology Laboratory	Jakarta Pusat
IN	National Bureau of Plant Genetic Resources	Division of Genomic Resources	New Delhi
IT	Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (IZSLER)	Reparto Genomica	Brescia
IT	Istituto Zooprofilattico Sperimentale Umbria e Marche	GMO laboratory	Perugia
LB	American University of Science and Technology	Laboratory Science &Technology	Achrafieh-Beirut
MX	SENASICA	CNRDOGM	Tecámac
MY	Department of Chemistry Malaysia		Selangor
RS	A Bio Tech Lab	Laboratory for biotechnology	Sremska Kamenica
RS	SP Laboratorija A.D.	Genetical and physico-chemical	Bečej
RS	Institute of Molecular Genetics and Genetic Engineering	Lab. for Plant Molec. Biology	Belgrade
SG	Agri-Food and Veterinary Authority of Singapore	Veterinary Public Health Lab	Singapore
TR	Ankara Food Control Laboratory	Biogenetics	Ankara
TR	National Food Reference Laboratory	Biotechnology and GMO Unit	Ankara
UK	Worcestershire Scientific Services	-	Worcester
VN	National Institute for Food Control	Quality management department	Hanoi
VN	Quality Assurance and Testing Centre 3 (QUATEST 3)	Microbiology - GMO Lab	Ho Chi Minh City
VN	Agricultural Genetics Institute	GMO Detection Laboratory	Hanoi
ZA	University of the Free State	Haematology and Cell Biology	Bloemfontein

[1] Category a includes NRLs designated under Regulation (EC) No 882/2004; Category b includes NRLs nominated under Regulation (EU) No 120/2014; Category c includes official control laboratories from EU or non-EU countries that are not NRLs according to the Regulations mentioned above.

Annex 1: Performance statistics

The aim of performance statistics is to provide participants with a meaningful result that can be easily interpreted. The procedure followed for the evaluation of the participants' performance was agreed by the Members of the Advisory Board and assumes a normal distribution of the data (see below).

In general, the approach relies on the calculation of z-scores from $\log_{10}$-transformed data[7,8] based on the robust means[11,12] (μ_R) of the participants' results.

The distribution of the data received for event 40-3-2 soybean deviated from normality, i.e. it was skewed towards the left and showed a second smaller bump. An alternative approach was therefore used to assign a performance score to the participants' results. The consensus value was calculated as a robust mean from the results of the expert laboratories only (NRLs assigned under Regulation (EC) No 882/2204 [NRL/882], i.e. Category a participants), which followed a normal distribution. The EURL GMFF calculated the consensus value from the results of NRL/882, taking the robust means (μ_R) (all data in m/m %) on both original and $\log_{10}$-transformed scale, taking into account the agreed standard deviation ($\hat{\sigma}$) for comparative testing, set to 0.2 based on previous experience.

The z-scores (z_i) for participant i reporting measurement result x_i are calculated in comparison to the robust mean as follows:

$$z_i = \left(\log_{10} x_i - \log_{10} \mu_R\right) / \hat{\sigma} \qquad \text{(A1.1)}$$

Z-scores were determined for the group of expert laboratories using equation A1.1. The z-score boundaries ($|z| \leq 2.0$) for soybean event 40-3-2 in T1 corresponded to rounded quantitative values in the range of 0.4 – 2.6 m/m %, with the robust mean being 1.11 m/m %. These z-score boundaries were then used to assign z-scores to the results of the other laboratories for this event (non-NRL/882, i.e. Category b and c participants).

Annex 2: Participants' results

The z-scores of all laboratories are reported in Tables A2.1-A2.3. For consistency, all decimal numbers were rounded to two digits. "Value" and "uncertainty" refer to the quantitative result and uncertainty as calculated and reported by the laboratory; "z-score" is calculated by the EURL GMFF.

Table A2.1. Performance of "Category a" laboratories (NRL/882) for quantification of soybean event 40-3-2 in T1 of comparative testing ILC-EURL-GMFF-CT-02-14; data are in m/m % (/ = not available; data in italics were originally reported in cp/cp %, but have been converted into m/m % by the EURL GMFF).

Laboratory Code	Test Item 1		
	40-3-2 Soybean (μ_R = 1.11 m/m %)		
	Value m/m %	Uncertainty m/m %	z-score
L04	0.58	0.15	-1.3
L06	*0.62*	*0.07*	-1.2
L09	1.12	0.71	0.1
L10	1.81	0.69	1.2
L11	1.25	0.37	0.4
L14	0.79	0.24	-0.6
L20	1.00	0.26	-0.1
L21	*0.74*	*0.16*	-0.8
L23	1.21	0.23	0.3
L24	1.52	0.35	0.8
L27	*2.26*	*0.55*	1.7
L28	2.28	0.61	1.7
L30	0.82	0.38	-0.5
L38	1.50	0.3	0.8
L39	0.91	0.23	-0.3
L44	0.98	0.17	-0.2
L46	0.96	0.27	-0.2
L47	1.33	0.44	0.5
L57	0.96	0.27	-0.2
L60	2.90	0.34	2.2
L61	0.79	0.28	-0.6
L64	0.88	0.12	-0.4
L66	0.30	0.07	-2.7
L67	1.67	0.2	1.0
L70	0.64	0.19	-1.1
L73	0.45	22.54	-1.8
L74	1.55	0.71	0.8
L81	0.41	0.12	-2.0

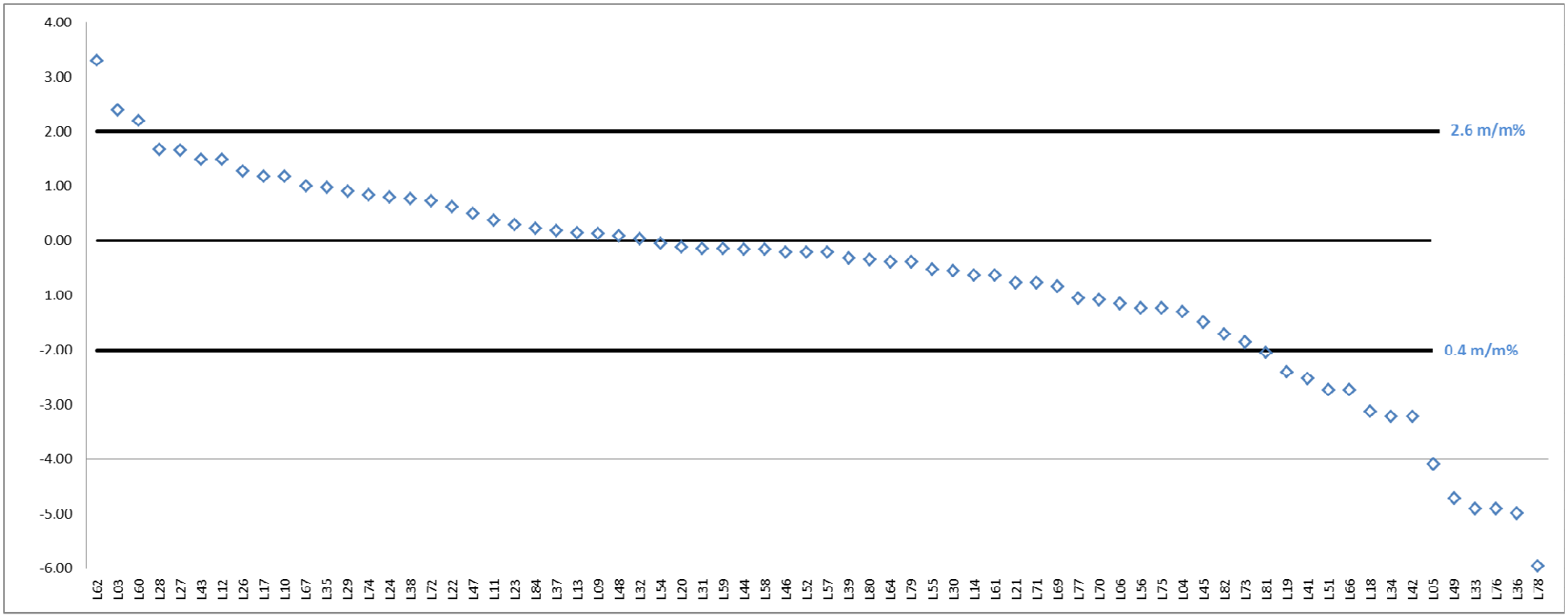
Table A2.2. Performance of "Category b" laboratories (NRL/120) for quantification of soybean event 40-3-2 in T1 of comparative testing ILC-EURL-GMFF-CT-02-14; data are in m/m % (/ = not available; data in italics were originally reported in cp/cp %, but have been converted into m/m % by the EURL GMFF).

Laboratory Code	Test Item 1		
	40-3-2 Soybean (μ_R = 1.11 m/m %)		
	Value m/m %	Uncertainty m/m %	z-score
L05	0.16	0.07	-4.1
L12	2.09	0.46	1.5
L13	1.13	0.7	0.2
L19	0.35	0.105	-2.4
L29	1.60	0.5	0.9
L32	1.07	0.15	0.0
L34	0.24	0.1	-3.2
L35	*1.65*	*0.18*	1.0
L41	0.33	0.1	-2.5
L45	0.53		-1.5
L51	0.30	0.01	-2.7
L52	0.96	0.34	-0.2
L54	1.03		0.0
L56	0.60	0.12	-1.2
L69	0.72	0.04	-0.8
L71	0.74	0.17	-0.8
L75	0.60	0.12	-1.2
L76	0.11	0.06	-4.9
L84	1.17	0.14	0.2

Table A2.3. Performance of "Category c" laboratories (non-NRL) for quantification of soybean event 40-3-2 in T1 of comparative testing ILC-EURL-GMFF-CT-02-14; data are in m/m % (/ = not available; data in italics were originally reported in cp/cp %, but have been converted into m/m % by the EURL GMFF).

Laboratory Code	Test Item 1		
	40-3-2 Soybean (μ_R = 1.11 m/m %)		
	Value m/m %	Uncertainty m/m %	z-score
L03	3.17	2.01	2.4
L17	1.82		1.2
L18	0.25		-3.1
L22	1.40	0.4	0.6
L26	1.90	0.5	1.3
L31	0.99	0.29	-0.1
L33	0.11	0.4	-4.9
L36	0.11	0.03	-5.0
L37	1.15		0.2
L42	*0.24*	0.06	-3.2
L43	2.10	1.33	1.5
L48	1.10	0.4	0.1
L49	0.12	0.04	-4.7
L55	0.83	0.16	-0.5
L58	0.98	0.15	-0.2
L59	*0.99*		-0.1
L62	*4.81*		3.3
L72	1.48		0.7
L77	0.65		-1.0
L78	0.07	0.017	-6.0
L79	0.88		-0.4
L80	0.90	0.57	-0.3
L82	*0.48*	0.95	-1.7

Figure A2. Z-scores for soybean event 40-3-2 in Test Item 1 on the basis of a robust mean of 1.11 m/m % (◊).



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