



JRC VALIDATED METHODS, REFERENCE METHODS AND MEASUREMENTS REPORT

Report on the Verification of the Performance of MON 87427, MON 89034, MIR162 and MON 87411 event-specific PCR-based Methods applied to DNA extracted from GM Stack MON 87427 x MON 89034 x MIR162 x MON 87411 maize

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European Union Reference Laboratory for GM Food and Feed

Executive Summary

An application was submitted by Monsanto Company, as represented by Monsanto Europe S.A./N.V. to request the authorisation of genetically modified stack (GM stack) MON 87427 x MON 89034 x MIR162 x MON 87411 maize (glyphosate tolerance, protection against certain lepidopteran insect pests and protection against certain coleopteran insect pests) 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 GM stack MON 87427 x MON 89034 x MIR162 x MON 87411 maize is MON-87427-7 x MON-89034-3 x SYN-IR162-4 x MON 87411-9.

The GM stack MON 87427 x MON 89034 x MIR162 x MON 87411 maize has been obtained by conventional crossing between the genetically modified maize events: MON 87427, MON 89034, MIR162 and MON 87411, without any new genetic modification.

The EURL GMFF has previously validated individually, and declared fit for purpose, the detection methods for the single events MON 87427, MON 89034, MIR162 and MON 87411 (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/MPR%20Report%20Application%202010_2015.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 MON 87427 x MON 89034 x MIR162 x MON 87411 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 MON 87427 x MON 89034 x MIR162 x MON 87411 maize.

This report is published at <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>.

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Quality assurance

The EURL GMFF is ISO 17025:2005 accredited [certificate number: Belac 268 TEST (Flexible Scope for DNA extraction, DNA identification and real Time PCR)] and ISO 17043:2010 accredited (certificate number: Belac 268 PT, proficiency test provider).

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/MPR%20Report%20Application%2020_10_2015.pdf) the EURL GMFF carries out an *in-house* verification of the performance of each event-specific methods 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 MON 87427 x MON 89034 x MIR162 x MON 87411 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. Step 1 (dossier reception and acceptance)

Monsanto Company 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 MON 87427 x MON 89034 x MIR162 x MON 87411 and from non GM maize.

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

3. Step 2 (dossier scientific assessment)

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.

One request of complementary information regarding adjustments to the buffer composition used for MIR162 method, complementary information on the origin of the material used for sequencing MIR162 trait, clarifications on linearity experiments and purity of the positive control DNA sample were addressed to the applicant. The EURL GMFF verified the data and the complementary information received and accepted the received clarifications as satisfactory.

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 MON 87427 x MON 89034 x MIR162 x MON 87411 maize genomic DNA. Means are the average of fifteen replicates obtained through four runs performed with ABI 7500 real-time PCR equipment. Percentages are expressed as GM DNA / total DNA x 100.

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 stated. 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 MON 87427, MON 89034, MIR162 and MON 87411 methods applied to GM stack MON 87427 x MON 89034 x MIR162 x MON 87411 maize.

MON 87427 *			
Sample GM %	Expected value (GMO %)		
	0.085	1	10
Mean	0.082	0.95	10.40
RSD _r (%)	12.57	8.81	4.94
Bias (%)	-3.93	-4.81	3.97
MON 89034 *			
Sample GM %	Expected value (GMO %)		
	0.085	1	10
Mean	0.079	0.98	10.75
RSD _r (%)	14.42	6.28	5.23
Bias (%)	-7.01	-1.82	7.51

MIR162*			
Sample GM %	Expected value (GMO %)		
	0.085	1	10
Mean	0.086	0.96	9.94
RSD_r (%)	12.81	8.75	9.51
Bias (%)	1.51	-3.92	-0.59
MON 87411 *			
Sample GM %	Expected value (GMO %)		
	0.085	1	10
Mean	0.10	1.11	10.56
RSD_r (%)	13.42	6.21	6.27
Bias (%)	21.74	10.96	5.55

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

The applicant applied the following modifications to the validated methods:

- the validated method for MON 89034 was verified in combination with the taxon-specific reference system *hmg* described in the validation protocol of MON 87460 (CRLVL04/09VP).
- The method for MIR162 was implemented using the reference dye Sigma R4526 instead of the sulforhodamine in the reaction buffer and without additional MgCl₂ supplemented to the mix.
- The applicant used a calibration standard made by serial dilution instead of the ΔC_q values of calibration samples.
- For the method for detection of the GM event MON87411 the reaction volume was reduced from 50 μ L to 25 μ L.
- The DNA extraction of the control samples was performed with three extractions with chloroform:isoamyl alcohol (CIA 24:1) [step 13] instead of two.

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. Step 3 (EURL GMFF experimental testing)

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 MON 87427 x MON 89034 x MIR162 x MON 87411 maize.

4.1 Materials

The following control samples were provided by the applicant:

- genomic DNA of GM stack MON 87427 x MON 89034 x MIR162 x MON 87411 maize, hemizygous for the loci, as positive control sample.
- genomic DNA of conventional (non-GM) maize whose genetic background is LH244 x LH287, as negative control sample.

The EURL GMFF prepared test samples of different GMO concentrations by mixing genomic DNA extracted from GM stack MON 87427 x MON 89034 x MIR162 x MON 87411 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 achieved. Table 2 shows the five GM concentrations used in the verification of the MON 87427, MON 89034, MIR162 and MON 87411 methods when applying them to genomic DNA extracted from the GM stack MON 87427 x MON 89034 x MIR162 x MON 87411 maize.

Table 2. Percentage (GM %) of MON 87427, MON 89034, MIR162 and MON 87411 in MON 87427 x MON 89034 x MIR162 x MON 87411 stack genomic DNA contained in the verification samples.

MON 87427 GM %* [[GM DNA / total maize DNA x 100]]	MON 89034 GM %* [[GM DNA / total maize DNA x 100]]	MIR162 GM %* [[GM DNA / total maize DNA x 100]]	MON 87411 GM %* [[GM DNA / total maize DNA x 100]]
0.06	0.09	0.10	0.06
0.20	0.40	0.40	0.55
0.90	0.90	0.90	0.90
3.0	3.0	2.0	2.0
8.0	8.0	5.0	5.0

* percentage expressed in copy number ratio.

The protocols submitted by the applicant were implemented in the EURL GMFF laboratory in accordance with the protocols already published for the individual MON 87427, MON 89034, MIR162 and MON 87411 GM events (available at <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>). The protocol for MON 89034 was implemented with the modification of the taxon-specific reference system as described in 4.4.1.

4.2 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 MON 87427, MON 89034, MIR162 and MON 87411 depends *"on the isolation of sufficient quantity and quality of purified DNA."*

"The provided DNA extraction method is intended for extraction of genomic DNA from seed which results in primarily high molecular weight DNA, indicating that the DNA is intact with limited fragmentation". The applicant also informed the EURL GMFF that during the processing of maize grains into food and feed ingredients *"a number of treatments may influence the quality and the intactness of the DNA contained in the final product ^{4,5}. In some cases, additional rounds of processing in order to clean up the DNA and eliminate PCR inhibitors may be required in order to achieve a quality of genomic DNA suitable for PCR ^{6,7}."*

The applicant also informed that, *"regardless of the DNA extraction method employed, studies have shown that the processing steps for maize result in the significant degradation of high molecular weight DNA and failure to PCR amplify products greater than a few 100 base pairs ^{4,5}; in addition, random DNA fragmentation is known to lead to variability in quantitating DNA by qPCR ⁸, thus affecting the ability to accurately quantify the presence of a GM event in processed fractions."*

On a general note 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 <http://gmo-crl.jrc.ec.europa.eu/summaries/CRL-VL-16-05-XP-Corrected-version-2.pdf>

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

4.3 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, or *adh1*, alcohol dehydrogenase 1. 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 MON 87427, MON 89034, MIR162 and MON 87411, 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 %.

4.4 *PCR methods*

During the verification study, the EURL GMFF carried out parallel tests on DNA extracted from GM stack MON 87427 x MON 89034 x MIR162 x MON 87411 maize using the single detection methods previously validated for the respective single GM events MON 87427, MON 89034, MIR162 and MON 87411.

For detection of GM maize events MON 87427, MON 89034, MIR162 and MON 87411, DNA fragments of 95-bp, 77-bp, 92-bp and 109-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 TAMRA (carboxytetramethylrhodamine) as a quencher dye at their 3'-end for three events (MON 87427, MIR162 and MON 87411) and a non-fluorescent quencher MGB-NFQ (minor groove binder 3' nonfluorescent Quencher) for MON 89034.

For quantification of GM maize events MON 87427, MON 89034 and MON 87411, a taxon-specific reference system amplifies a 79-bp fragment of high mobility group (*hmg*) a maize endogenous gene (GenBank AJ131373.1), using two *hmg* gene-specific primers and a gene-specific probe labelled with FAM (6-carboxyfluorescein) and TAMRA (carboxytetramethylrhodamine) at the 5' and 3' end respectively.

For quantification of GM maize events MIR162, a taxon-specific reference system amplifies a 135-bp fragment of alcohol dehydrogenase 1 (*adh1*) a maize endogenous gene (GenBank AY691949), using two *adh1* gene-specific primers and a gene-specific probe labelled with VIC and TAMRA (carboxytetramethylrhodamine) at the 5' and 3' end respectively.

For the relative quantification of GM maize events MON 87427, MON 89034 and MON 87411 standard curves are generated both for the MON 87427, MON 89034 and MON 87411 and for the high mobility group (*hmg*) 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 MON 87427, MON 89034 and MON 87411 DNA is estimated.

For relative quantification of GM maize event MIR162 DNA in a test sample, the ΔCq values of calibration samples are used to calculate, by linear regression, a standard curve (plotting ΔCq values against the logarithm of the relative amount of MIR162 event DNA). The ΔCq values of the unknown samples are measured and, by means of the regression formula, the relative amount of MIR162 event 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>.

4.4.1 Deviations from the validated methods

No deviations from the original validated methods were introduced for MON 87427, MON 87411 and MIR162.

The validated method for MON 89034 was verified in combination with the taxon-specific reference system *hmg* according to the reaction conditions described in the validation protocol of MON 87460 (CRLVL04/09VP http://gmo-crl.jrc.ec.europa.eu/summaries/2012-01-27_MON87460_validated_Method.pdf). The EURL GMFF had already approved the modification of the reaction conditions through the verification study EURL-VL-04/13 (http://gmo-crl.jrc.ec.europa.eu/summaries/JRC102290_EURL-VL-04-13-VR.pdf).

4.5 Results

Tables 3, 4, 5, 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 $[10^{(-1/\text{slope})} - 1] \times 100$, and of the coefficient of determination (R^2) reported for all PCR systems in the eight runs, for GM maize events MON 87427, MON 89034, MIR162 and MON 87411. Slope values were rounded to two digits.

Table 3. Values of standard curve slope, PCR efficiency and R^2 coefficient for the MON 87427 method on GM stack MON 87427 x MON 89034 x MIR162 x MON 87411 maize.

Run	MON 87427			hmg		
	Slope	PCR Efficiency (%)	R^2 coefficient	Slope	PCR Efficiency (%)	R^2 coefficient
1	-3.34	99	1.00	-3.29	101	1.00
2	-3.38	98	1.00	-3.30	101	1.00
3	-3.43	96	0.99	-3.24	104	1.00
4	-3.29	101	1.00	-3.31	100	1.00
5	-3.15	108	1.00	-3.28	102	1.00
6	-3.07	112	1.00	-3.33	100	1.00
7	-3.00	115	1.00	-3.23	104	1.00
8	-3.18	106	0.99	-3.31	100	1.00
Mean	-3.23	104	1.00	-3.29	102	1.00

Table 4. Values of standard curve slope, PCR efficiency and R^2 coefficient for the MON 89034 method on GM stack MON 87427 x MON 89034 x MIR162 x MON 87411 maize.

Run	MON 89034			hmg		
	Slope	PCR Efficiency (%)	R^2 coefficient	Slope	PCR Efficiency (%)	R^2 coefficient
1	-3.46	95	1.00	-3.26	103	1.00
2	-3.42	96	1.00	-3.26	103	1.00
3	-3.47	94	1.00	-3.28	102	1.00
4	-3.56	91	1.00	-3.27	102	1.00
5	-3.41	96	1.00	-3.31	100	1.00
6	-3.44	95	1.00	-3.31	100	1.00
7	-3.44	95	1.00	-3.29	101	1.00
8	-3.48	94	1.00	-3.30	101	1.00
Mean	-3.46	95	1.00	-3.28	102	1.00

Table 5. Values of standard curve slope, PCR efficiency and R^2 coefficient for the MIR162 method on GM stack MON 87427 x MON 89034 x MIR162 x MON 87411 maize.

Run	MIR162		
	Slope	PCR Efficiency (%)	R^2 coefficient
1	-3.49	93	1.00
2	-3.55	91	1.00
3	-3.43	96	1.00
4	-3.02	115	1.00
5	-3.24	104	1.00
6	-3.41	96	1.00
7	-2.98	116	0.99
8	-3.43	96	1.00
Mean	-3.32	101	1.00

Table 6. Values of standard curve slope, PCR efficiency and R^2 coefficient for the MON 87411 method on GM stack MON 87427 x MON 89034 x MIR162 x MON 87411 maize.

Run	MON 87411			hmg		
	Slope	PCR Efficiency (%)	R^2 coefficient	Slope	PCR Efficiency (%)	R^2 coefficient
1	-3.31	100	1.00	-3.31	100	1.00
2	-3.20	105	1.00	-3.42	96	1.00
3	-3.65	88	0.99	-3.34	99	1.00
4	-3.36	99	1.00	-3.36	98	1.00
5	-3.45	95	1.00	-3.32	100	1.00
6	-3.31	100	1.00	-3.35	99	1.00
7	-3.31	100	1.00	-3.32	100	1.00
8	-3.25	103	1.00	-3.35	99	1.00
Mean	-3.36	99	1.00	-3.35	99	1.00

The mean PCR efficiencies of the GM and species-specific systems were above or equal to 95% (104 % and 102 % for MON 87427 and *hmg* systems, 95 % and 102 % for MON 89034 and *hmg* systems, 101 % for MIR162, 99 % for both MON 87411 and *hmg* systems respectively). 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, 6 confirm the appropriate performance characteristics of the four methods when tested on GM stack MON 87427 x MON 89034 x MIR162 x MON 87411 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 MON 87427 x MON 89034 x MIR162 x MON 87411 maize see Tables 7, 8, 9, 10.

Table 7. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the MON 87427 method applied to genomic DNA extracted from GM stack MON 87427 x MON 89034 x MIR162 x MON 87411 maize.

MON 87427					
Unknown sample GM %	Expected value (GMO %)				
	0.06	0.20	0.90	3.0	8.0
Mean	0.05	0.17	0.81	2.9	8.4
SD	0.01	0.03	0.08	0.33	1.00
RSD_r (%)	19	16	10	12	12
Bias (%)	-17	-17	-10	-4.1	4.6

Table 8. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the MON 89034 method applied to genomic DNA extracted from GM stack MON 87427 x MON 89034 x MIR162 x MON 87411 maize.

MON 89034					
Unknown sample GM %	Expected value (GMO %)				
	0.09	0.40	0.90	3.0	8.0
Mean	0.08	0.36	0.84	2.8	7.4
SD	0.01	0.02	0.04	0.13	0.45
RSD_r (%)	11	7.0	4.3	4.6	6.1
Bias (%)	-9.8	-11	-6.9	-8.0	-6.9

Table 9. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the MIR162 method applied to genomic DNA extracted from GM stack MON 87427 x MON 89034 x MIR162 x MON 87411 maize.

MIR 162					
Unknown sample GM %	Expected value (GMO %)				
	0.10	0.40	0.90	2.0	5.0
Mean	0.10	0.40	0.92	2.0	5.0
SD	0.02	0.07	0.09	0.31	0.72
RSD_r (%)	20	17	10	16	14
Bias (%)	2.0	-0.70	2.5	0.62	0.31

Table 10. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the MON 87411 method applied to genomic DNA extracted from GM stack MON 87427 x MON 89034 x MIR162 x MON 87411 maize.

MON 87411					
Unknown sample GM %	Expected value (GMO %)				
	0.06	0.55	0.90	2.0	5.0
Mean	0.06	0.49	0.87	1.9	5.0
SD	0.01	0.07	0.11	0.14	0.44
RSD_r (%)	22	14	13	7.7	8.8
Bias (%)	-7.0	-11	-3.0	-7.3	0.73

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, 10, the values range from -17 % to 4.6 % for MON 87427, from -11 % to -6.9 % for MON 89034, from -0.70 % to 2.5 % for MIR162 and from -11 % to 0.73 % for MON 87411. Therefore, the four methods satisfy the above mentioned requirement

throughout their respective dynamic ranges, also when applied to DNA extracted from GM stack MON 87427 x MON 89034 x MIR162 x MON 87411 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 10 % and 19 % for MON 87427, between 4.3 % and 11 % for MON 89034, between 10 % and 20 % for MIR162 and between 7.7 % and 22 % for MON 87411, the four methods satisfy this requirement throughout their respective dynamic ranges when applied to DNA extracted from GM stack MON 87427 x MON 89034 x MIR162 x MON 87411 maize.

5. Conclusions

The performance of the four event-specific methods for the detection and quantification of maize single line events MON 87427, MON 89034, MIR162 and MON 87411, when applied to genomic DNA extracted from GM stack MON 87427 x MON 89034 x MIR162 x MON 87411 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 MON 87427, MON 89034, MIR162 and MON 87411, can be equally applied for the detection and quantification of the respective events in DNA extracted from the GM stack MON 87427 x MON 89034 x MIR162 x MON 87411 maize or any of its sub-combinations, supposed that sufficient genomic DNA of appropriate quality is available.

6. References

1. Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed (Text with EEA relevance). OJ L 268, 18.10.2003, p. 1–23.
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