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CERTIFICATION REPORT

Certification of a Soya 356043 Reference Material for its DNA Copy Number Ratio

Certified Reference Material ERM®-BF425c

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Certification of a Soya 356043 Reference Material for its DNA Copy Number Ratio

Certified Reference Material ERM®-BF425c

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ABSTRACT

This report describes the certification of the Certified Reference Material (CRM) ERM®-BF425c for its DNA copy number ratio. This CRM is composed of conventional and genetically modified soya 356043 seed powders.

ERM-BF425c is part of a set of soya CRMs containing different mass fractions of GM soya 356043. The CRM was processed and originally certified for its soya 356043 mass fraction by the European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE.

European Commission Recommendation (EC) No 787/2004 advises to express the content of GM food and feed products as the percentage of GM DNA copy numbers in relation to target taxon-specific DNA copy numbers calculated in terms of haploid genomes, further referred to as the DNA copy number ratio. An interlaboratory comparison was conducted in 2008 to certify the existing CRM for its DNA copy number ratio. The CRM is intended for quality control of measurements of DNA copy number ratios of soya event 356043 in GM food and feed. Hereby, the plasmid DNA (pDNA) ERM®-AD425 should be used for calibration together with the soya 356043 event-specific real-time Polymerase Chain Reaction method validated by the European Union Reference Laboratory for GM Food and Feed (EURL-GMFF) (available via: http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm). ERM-AD425 contains a 99 bp fragment of the 5' plant/insert junction specific for the soya event 356043. Additionally, the plasmid carries a 243 bp fragment of the species-specific *lectin* gene *le1*, specific for the soya taxon.

The CRM ERM-BF425c is available in glass bottles containing 1 g of soya seed powder, closed under argon atmosphere and is certified for its DNA copy number ratio. The minimum amount of sample to be used is 200 mg.

Certified value and related uncertainty

ERM-BF425c	Certified values	Uncertainty [3]
Soya 356043 mass fraction [1]	10.0 g/kg [2]	1.1 g/kg
Soya 356043 DNA copy number ratio [4]	0.85 % [5]	0.11 %

1) Mass fraction of soya 356043 (unique identifier code DP-356∅43-5) based on the masses of genetically modified 356043 soya seed powder and non-modified soya seed powder and their respective water content.

2) The certified value is traceable to the International System of Units (SI).

3) The certified uncertainty is the expanded uncertainty with a coverage factor $k = 2$, corresponding to a level of confidence of about 95 % estimated in accordance with ISO/IEC Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM:1995), ISO, 2008.

4) The copy number ratio of soya 356043 (unique identifier code DP-356∅43-5) is defined by the soya 356043 real-time Polymerase Chain Reaction quantification method validated by the European Union Reference Laboratory for GM Food & Feed (EURL-GMFF, available on http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm) and calibrated with the soya 356043 plasmid DNA Certified Reference Material ERM®-AD425.

5) The certified DNA copy number ratio is the unweighted mean of 15 accepted data sets. It is traceable to the International System of Units (SI).

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GLOSSARY

ALS[1]	acetolactate synthase protein
ANOVA	analysis of variance
bp	base pair
cp	copy number
CRM	Certified Reference Material
CTAB	cetyltrimethylammonium bromide
Ct-value	Cycle threshold, number of PCR cycles to pass a set threshold
DNA	deoxyribonucleic acid
dsDNA	double-stranded deoxyribonucleic acid
ε	PCR efficiency
EDTA	ethylenediaminetetraacetic acid
ERM®	trademark of European Reference Materials
EURL-GMFF	European Reference Laboratory for GM Food & Feed formerly known as Community Reference Laboratory for GM Food & Feed (CRL-GMFF)
g	standard gravity
gat4601[1]	gene encoding a glyphosate acetyltransferase (GAT) protein
gDNA	genomic DNA
gDNAℓ	genomic DNA extracted from leaves
gDNAs	genomic DNA extracted from seed powder ERM-BF425c
GM	genetically modified
gm-hra[1]	gene coding for a modified acetolactate synthase
GM-HRA[1]	modified acetolactate synthase enzyme
GMO	genetically modified organism
IRMM	Institute for Reference Materials and Measurements
k	coverage factor
MS_{bb}	mean sum of squares between bottles
MS_{wb}	mean sum of squares within bottles
n	number of samples analysed
N	number of bottles analysed
N_d	number of data sets
N_{ds}	number of data subsets
NTC	non template control
p	probability
PCR	polymerase chain reaction
pDNA	plasmid DNA
R^2	coefficient of determination
rel	relative (subscript used to describe relative expressed values)
RM	Reference Material
RSD	relative standard deviation
RT	room temperature
s	standard deviation
s_{bb}	standard deviation between bottles
TE buffer	10 mmol/L Tris-HCl, 1 mmol/L EDTA (pH 8.0)
TE_{low}	1 mmol/L Tris-HCl, 0.01 mmol/L EDTA (pH 8.0)
U	expanded uncertainty
u_{bb}	standard uncertainty related to the between-bottle inhomogeneity
$u_{bb,\ rel}$	relative standard uncertainty related to the between-bottle inhomogeneity
u^*_{bb}	inhomogeneity that can be hidden by the method repeatability; minimum uncertainty contribution related to the between-bottle inhomogeneity
u_{char}	standard uncertainty introduced by the characterisation
U_{CRM}	expanded uncertainty of the certified value
u_{lts}	standard uncertainty related to the stability
$\bar{x}$	mean
$\bar{x}_i$	mean obtained for data set i

[1] Following international nomenclature guidelines, three-letter non-italic capital letter codes refer to the protein, whereas lowercase italic letters are used for genes.

1 INTRODUCTION

Legislation in the European Union demands the labelling of food products consisting of or containing "more than 0.9 % genetically modified organisms" (GMOs), provided the GMO has been placed on the market in accordance with Community legislation [1]. This enforces the necessity to develop and validate reliable quantitative methods as well as the need to produce reference materials (RMs) to calibrate and control the application of these methods. This threshold of 0.9 % is commonly understood as a mass fraction and the Institute for Reference Materials and Measurements (IRMM) certifies gravimetric mixtures of genetically modified (GM) seed powder and non-GM seed powder for their GM mass fraction.

In October 2004, the European Commission recommended to express the content of GM food and feed as the percentage of GM DNA copy numbers in relation to target taxon-specific DNA copy numbers calculated in terms of haploid genomes [2]. As a consequence, it has been decided to additionally certify the existing CRMs for their GM DNA copy number ratio. The DNA copy number ratio is determined according to the following equation (1):

$$\text{DNA copy number ratio}\,[\%] = \frac{\text{GM DNA copy numbers [cp]}}{\text{Target taxon specific DNA copy numbers [cp]}} \cdot 100 \qquad (1)$$

The soya 356043 event is registered in the Organisation for Economic Co-operation and Development Unique Identification Registry (available via http://bch.cbd.int/database/lmo-registry/), as specified in the Regulation (EC) No 65/2004 of 14/01/2004 establishing a system for the development and assignment of unique identifiers for GMOs [3]. In accordance to this regulation, the soya event 356043 received the unique identifier DP-356∅43-5.

The GM *Glycine max* L. (Soya) line 356043 was developed by Pioneer Hi-Bred International Inc. (Johnston, IA, US) [4]. The soya 356043[2] was genetically modified by insertion of *gat4601* and *gm-hra* genes, along with the necessary regulatory elements for the expression in the soya plant. The GAT4601 protein is a glyphosate acetyltransferase (GAT), encoded by an optimised form of the *gat* gene from *Bacillus licheniformis*, that confers tolerance to the glyphosate. The GM-HRA protein is an acetolactate synthase (ALS), encoded by an optimised form of the endogenous *als* gene from *G. max*, that confers tolerance to ALS-inhibiting herbicides such as chlorimuron and thifensulfuron [4].

CRM ERM-BF425c, consisting of non-GM and GM soya 356043 seed powders, was certified in 2007 for its GM mass fraction [5]. In 2008, an interlaboratory comparison was conducted to certify ERM-BF425c additionally for its DNA copy number ratio. The event-specific soya 356043 real-time PCR detection method, validated by the European Union Reference Laboratory for GM Food and Feed (EURL-GMFF) on 11/02/2009, was used in this study [6]. The measurements were calibrated with the pDNA ERM-AD425, containing DNA sequences specific for the soya event 356043 and for the taxon in a 1 to 1 ratio [7]. The plasmid contains a 99 bp fragment of the 5' plant genome/insert junction as well as a 243 bp fragment of the soya endogenous lectin gene (*le1*) [6, 7]. Both fragments are specifically targeted by the event-specific soya 356043 real-time PCR quantification method used.

With respect to the DNA copy number ratio certification ERM-BF425c is intended to be used for quality control of measurements of the DNA copy number ratio of soya event 356043 in GM food and feed using the ERM-AD425 calibrant and the event-specific soya 356043 real-time PCR detection method. ERM-BF425c should not be used for calibration for copy number measurements.

[2] Soya 356043, DP-356043-5 soya and 356043 soya are used as synonyms and all refer to the unique identifier DP-356∅43-5. The commercial name assigned to genetically modified 356043 soybean in the US market is Optimum™ GAT™ Soybean.

2 PARTICIPANTS

2.1 CHARACTERISATION

The following laboratories participated in the characterisation study of ERM-BF425c for its DNA copy number ratio:

- Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit, Oberschleißheim, DE*, (SAL, SAL-BY-L 20-04-02)
- Bundesinstitut für Risikobewertung, Berlin, DE* (AKS, AKS-PL-22001)
- Danish Plant Directorate, Laboratory for Diagnoses in Plants, Food and Feed, Lyngby, DK* (DANAK, 330)
- Ente Nazionale Delle Sementi Elette ENSE, Tavazzano, IT
- Eurofins Analytik GmbH, WEJ Dept. Biology 135, Hamburg, DE* (DACH, DAC-PL-0526-07-04)
- European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE* (BELAC, 268-TEST)
- FOD Wetenschappelijk Instituut Volksgezondheid, Afdeling Bioveiligheid en Biotechnologie, Brussels, BE* (BELAC, 081-TEST)
- Groupe d'études et de contrôle des variétés et des semences GEVES (BioGEVES), Surgères, FR* (COFRAC, 1-1540)
- Hainaut Vigilance Sanitaire, Institut Provincial d'Information et d'Analyses Sanitaires, Mons, BE* (BELAC, 068-TEST)
- European Commission, Joint Research Centre, Institute for Health and Customer Protection (IHCP), Ispra, IT* (DACH, DAC-PL-0459-06-00)
- European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE* (BELAC, 268-TEST)
- Institut für Hygiene und Umwelt, Hamburger Landesinstitut für Lebensmittelsicherheit, Gesundheitsschutz und Umweltuntersuchungen, Hamburg, DE* (DACH, DAC-PL-0137-01-10)
- Instituut voor Landbouw- en Visserijonderzoek (ILVO), Merelbeke, BE* (BELAC, 033-TEST)
- Istituto Superiore di Sanità, Rome, IT
- Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana, Rome, IT* (SINAL, 0201)
- Korea Research Institute of Standards and Science (KRISS) - Organic and Bio Analysis Group, Daejeon, KR
- Landeslabor Schleswig-Holstein, Lebensmittel-, Veterinär- und Umweltuntersuchungsamt, Neumünster, DE* (AKS, AKS-PL-10101)
- Lifeprint GmbH DNA Analysis, Illertissen, DE* (DAP, DAP-PL-3515.00)
- Livsmedelsverket - National Food Administration, Biology Division, Uppsala, SE* (SWEDAC, 1457)
- National Food Research Institute, Molecular Engineering Lab, Tsukuba, JP* (International Accreditation Japan, ASNITE 0018R)
- Nacionalni Inštitut za Biologijo- National Institute of Biology (NIB), Ljubljana, SI* (Sovenska Akreditacija, LP-028)
- Nacionaliné Veterinarijos Laboratorija - National Veterinary Laboratory, Vilnius, LT* (DAP, DAP-PL-3328.99)
- NEOTRON, Modena, IT* (SINAL, 0026)
- Ontario Plant Laboratories - Canadian Food Inspection Agency, Ontario, CA* (Standards Council of Canada, 316)
- RIKILT, Wageningen, NL* (RvA, L014)
- Ministère Agriculture et Pêche, Laboratoire National de la Protection des Végégtaux, Fleury les Aubrais, FR
- Staatliches Gewerbeaufsichtsamt Hildesheim, Dez. 33 Gentechnik, Hildesheim, DE* (DACH, DAC-PL-0360-05-00)
- TNO quality of life - Food & Biotechnology Innovations - GMO foods, Zeist, NL* (RvA, L027)
- Tullilaboratorio - Finnish Customs Laboratory, Espoo, FI* (FINAS, T006)
- Umweltbundesamt Wien, Wien, AT* (BMWA, 200)
- USDA, Grain Inspection, Technical Service Division, Kansas City, MO, US
- Vysoka Skola Chemicko-technologicka v Praze - Institute of Chemical Technology, Prague, CZ* (Czech accreditation institute, No. 111/2006)
- Výzkumný ústav rostlinné výroby - Research Institute for Crop Production, Prague, CZ* (Czech accreditation institute, No. 8/2007)

2.2 CERTIFICATION

- European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE*♦ (BELAC, 268-TEST)

* Laboratory holds ISO/IEC17025 accreditation for DNA based GMO measurements. The accreditation body and registration number are mentioned between brackets.

♦ Laboratory is accredited for ISO Guide 34. The accreditation body and registration number are mentioned between brackets.

3 CRM PREPARATION

ERM-BF425c is a gravimetrically prepared dried soya seed powder containing a certified mass fraction of 10.0 g/kg of the GM soya 356043 with an expanded uncertainty (U, $k = 2$) of 1.1 g/kg. ERM-BF425c was processed in 2007 as part of a set of four CRMs certified for their mass fraction of soya 356043. For a detailed description of the processing of ERM-BF425c, the reader is referred to an earlier report [5].

4 HOMOGENEITY

4.1 HOMOGENEITY STUDY

The homogeneity and minimum sample intake of ERM-BF425c were investigated at the time of the processing and certification for the mass fraction of the CRM in 2007 [5].

The GM content determined in the frame of the certification of the matrix CRM is expressed as a mass fraction and not as a DNA copy number ratio. However, this has no impact on the estimation of the homogeneity at a given sample intake level.

The results of the homogeneity study are summarised in (Table 1). The value of s_{bb} (Table 1) was used as an estimate for the uncertainty contribution related to the between-bottle inhomogeneity (u_{bb}) and was included in the calculation of the overall uncertainty of the certified value (Section 7.3).

Table 1: Between bottle inhomogeneity

Mean mass fraction related to the between-bottle ($_{bb}$) inhomogeneity of ERM-BF425c, analysed by real-time PCR using a sample intake of 200 mg ($N = 25$, $n = 4$); s_{bb} is for standard deviation between bottles, $s_{bb,\,rel}$ is the relative standard deviation between bottles, u^*_{bb} is for the inhomogeneity that can be hidden by method repeatability. It is used as the minimum uncertainty contribution from homogeneity. $u^*_{bb,\,rel}$ is the relative minimum uncertainty from homogeneity.

Mean mass fraction [%]	s_{bb} [%]	$s_{bb,\,rel}$	u^*_{bb} [%]	$u^*_{bb,\,rel}$
0.960	0.043	0.045	0.034	0.036

4.2 MINIMUM SAMPLE INTAKE

A sample intake of 200 mg of powder was used for DNA extraction by the GENE*Spin* method in the homogeneity study. The assumption that this amount ensures a sufficient homogeneity was investigated and proved to be correct. For the validity of the certified value of ERM-BF425c, sample intakes not smaller than 200 mg have to be used [5].

5 STABILITY

The short- and long-term stability of ERM-BF425c was investigated at the time of the certification of the material for its mass fraction [5].

5.1 SHORT-TERM STABILITY

It could be concluded that ERM-BF425c can be shipped under ambient conditions [5].

5.2 LONG-TERM STABILITY

The mass fraction of the soya 356043 seed powder was unaffected by short-term incubation at elevated temperatures (Section 5.1). This was also observed for other soya seed powders evaluated at IRMM.

As there is no reason to expect a different behaviour of ERM-BF425c compared to other soya matrices when stored at 4 °C for long periods, the long term stability of the soya 356043 CRM powders relies on data from the post-certification monitoring of the soyas ERM-BF410, ERM-BF425 and ERM-BF426. These soya CRMs have been monitored for more than 6 years using ELISA and/or its event-specific real-time PCR detection methods. The relative standard uncertainty calculated ($u_{lts,\ rel}$) from the available data obtained by its event-specific real-time PCR detection method is 2.2 % estimated on the basis of a shelf life of 12 months [8].

Post-certification monitoring is being carried out at regular intervals in order to further check the stability of ERM-BF425c.

6 CHARACTERISATION

6.1 STUDY SET-UP

Thirty-two laboratories were selected on the basis of proven experience and quality management systems in place. For the characterisation of ERM-BF425c with respect to its DNA copy number ratio, a total of 42 analyses were requested. To detect a possible dependence of the real-time PCR detection method from the DNA extraction method, three different protocols were applied to extract DNA from the unknown soya seed powders. Fourteen independent analyses were allocated to each DNA extraction method.

Soya is a difficult matrix to extract DNA from as the powders absorb the extraction buffer leading to low yield and poor quality of the DNA. Therefore, three DNA extraction protocols were adapted to obtain DNA of good quality and of high enough concentration to be used in real-time PCR:

i) a modified protocol based on the DNA*Extractor* kit (Eurofins GeneScan GmbH, Freiburg, DE) with a Genomic-tip 20/G (Qiagen, Benelux B.V., Venlo, NL) purification step was utilised (Annex 1)

ii) the cetyltrimethylammonium bromide (CTAB) method combined with a Genomic-tip 20/G purification step , originally validated for cotton 3006-210-23/281-24-236 [9] was used. The protocol was adapted for a sample intake of 200 mg (Annex 2)

iii) a slightly modified protocol of the GENE*Spin* extraction kit (Eurofins GeneScan GmbH, Freiburg, DE) was employed (Annex 3).

Matrix CRM ERM-BF425c, DNA extraction kits, DNA calibrants, nuclease-free water, plasmid dilution buffer (TE_{low} containing 2 ng/µL ColE1 background plasmid DNA) and real-time PCR consumables were shipped to the participating laboratories on dry ice.

For each independent analysis two bottles of ERM-BF425c were provided, designated as unknown powder bottles. From each bottle, three samples were extracted and analysed ($N = 2$, $n = 3$), further referred to as "Unknowns" (U1-U3 for bottle 1, U4-U6 for bottle 2). U1-U3 were analysed on day 1 and U4-U6 were analysed on day 2, *i.e.* the complete analysis of one data set had to be spread over at least two days. The DNA concentration was estimated by UV spectrometry or fluorometry. The DNA concentration of each extract was adjusted to 20 ng/µL with nuclease-free water (referred to as "undiluted extracts"). Afterwards, all extracts were diluted 3, 6, 9 and 12 times. Dilution series were analysed in quadruplicate by the EURL-GMFF validated event-specific real-time PCR method [6] using primer pairs and labelled TaqMan® probes specific for the soya 356043 event and for the soya endogenous *lectin* gene (Figure 1). Two DNA calibrants were used for all real-time PCR experiments, namely pDNA calibrant ERM-AD425 [7] and gDNA extracted from leaves of soya 356043 plants, which were tested beforehand individually to contain the 356043 event. Dilution series were prepared by the participating laboratories for both calibrants. Genomic DNA from leaves was diluted in nuclease-free water with concentrations ranging from 10^5 to 5 cp/µL. Plasmid DNA ERM-AD425 was diluted in plasmid dilution buffer (TE_{low} containing 2 ng/µL ColE1 background plasmid DNA) with concentrations ranging from 5 cp/µL to $2 \cdot 10^5$ cp/µL. Therefore, one set of analysis generated two data sets, *i.e.* one that was calibrated with pDNA ERM-AD425 and one that was calibrated with gDNA from leaves.

Real-time PCR experiments using TaqMan Universal mastermix were carried out according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, US) on 96 well microtiter plates with all runs performed for 45 cycles. One laboratory adapted the set-up for the performance in 384 well plates.

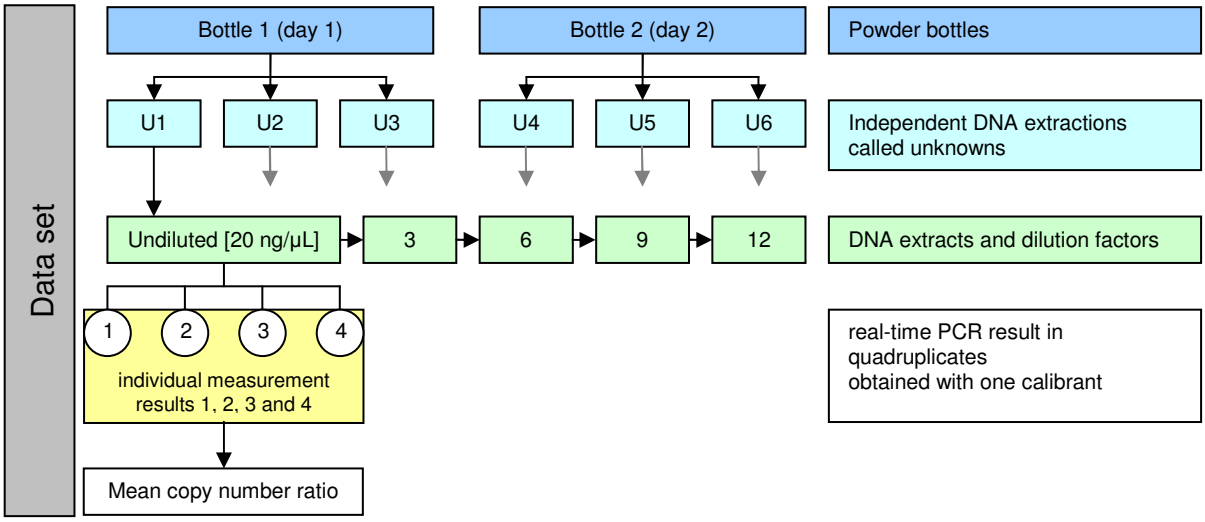

Figure 1: Analysis scheme for one data set
This scheme was followed for one calibrant by all participating laboratories during the copy number certification study. Undiluted U1 is given as an example. The same steps are also followed for all other samples and their dilutions. The same pipetted DNA extracts, *i.e.* the quadruplicates that are shown in the yellow box, are also analysed using the other calibrant. A data set comprises of six DNA extractions and their dilutions analysed in quadruplicates by real-time PCR by one calibrant.

6.2 ACCEPTANCE CRITERIA FOR DATA SETS

Data acceptance criteria for this study were established. Most of them are also described by Charels *et al.* [9] as outlined below. Data sets that did not fulfil all acceptance criteria were excluded. The number of data sets that failed particular acceptance criteria is given. A summary is given in Table 2.

As two independent analyses (days) were carried out per calibrant, one data set comprises a total of four calibration curves consisting of the detection of the endogeneous and transgenic targets using either the pDNA calibrant ERM-AD425 or gDNA calibrant extracted from leaves. The results obtained on the two days of analysis were considered separately for the two calibrants. This means that the rejection of a data set calibrated with pDNA ERM-AD425 did not trigger the exclusion of the data set that was calibrated with gDNA from leaves and vice versa.

6.2.1 Technical acceptance criteria

Only data sets with negative Non-template controls (NTC) were accepted. Six data sets were rejected for the gDNA calibrant and eight for the pDNA calibrant for this reason.

Data sets were rejected if any dilution inconsistency in the dilution series was noticeable in the trend of reported threshold cycles (Ct), *i.e.* increasing dilution is reflected by an increasing Ct. In this study no dilution inconsistencies were noticed. Therefore no data set was rejected on this basis.

Means of the triplicate or quadruplicate measurements were not accepted if the Ct variability between one triplicate or quadruplicate was higher than 1.5 of the mean Ct of the remaining replicates within the particular dilution point. Dilution points were also excluded if an amplification signal was absent. This did not trigger the rejection of the complete data set, unless the remaining number of acceptable dilution points within the dilution series was insufficient. On the basis of this criterion, one data set was rejected for the gDNA calibrant.

6.2.2 Calibration curves

The calibration curves for this copy number study needed to fulfil the minimal performance requirements which were set by the method validation guidelines of the EURL-GMFF [10].

In particular, these selection criteria apply to the slope and linearity of the calibration curves. Each data set consisted of 4 calibration curves. For the ERM-AD425 pDNA calibrant 136 calibration curves and 140 calibration curves for gDNA calibrant from leaves could still be calculated.

Calibration curves with a coefficient of determination (R^2) of minimum 0.98 were accepted. Eight calibration curves for the gDNA calibrant and five calibration curves for the pDNA calibrant were rejected that did not fulfil this requirement.

Control limits for PCR efficiencies (ε) were applied. For both calibrants, transgene and endogene mean PCR efficiencies and their standard deviations were calculated. Control limits were calculated per calibrant, transgene and endogene as the mean plus or minus one standard deviation, *i.e.* four intervals were generated. The minimum and maximum values were then applied as the lower and higher limits of the PCR efficiency. In this study the resulting control limits were 76 % and 110 %. These efficiencies correspond to a slope of -4.07 and -3.10, respectively. For data sets calibrated with gDNA calibrant from leaves 12 calibration curves were not accepted as their efficiencies were lower than 76 %. Two data sets were rejected due to calibration curves with efficiencies higher than 110 %. For data sets calibrated with pDNA calibrant ERM-AD425, one calibration curve was rejected due to efficiency below 76 % and 14 calibration curves due to efficiencies above 110 %.

In total, 13 data sets for the pDNA calibrant and 16 data sets gDNA calibrant were rejected due to not acceptable calibration curves.

6.2.3 Working interval

DNA extracts and dilutions exhibiting a Ct value falling outside the working interval of the calibration curve were excluded for further calculations. No complete data set was rejected due to this criterion.

6.2.4 Copy number ratios, DNA extracts and their dilutions

The mean Ct values for the undiluted DNA extracts and their dilutions for each unknown were converted into transgene and endogene copy numbers of which copy number ratios were calculated for each unknown for each day and each calibrant. Only the copy number ratios of the undiluted extracts were used for each accepted unknown to calculate the certified value.

Two criteria were applied for the complete acceptance of the data set: Firstly, the copy number ratio of an undiluted extract was considered for calculation if at least one more copy number ratio from within its dilution series could be calculated. Secondly, for a data set to be accepted a minimum of two DNA extracts per day needed to fulfil this criterion, *i.e.* maximum six copy number ratios and minimum four copy number ratios per data set and calibrant were accepted.

Two data sets for the gDNA and one data set for the pDNA calibrant were rejected due to insufficient data points per day.

6.2.5 Relative Standard Deviation

The relative standard deviation (RSD) was calculated for the copy number ratios per day and for both days per data set. Only data sets that had an RSD under repeatability conditions per day and for the whole data set below 25 % were accepted. This criterion led to the rejection of four data sets for the gDNA calibrant from leaves and five data sets for the pDNA calibrant ERM-AD425.

6.3 ACCEPTED DATA SETS

15 and 13 data sets were accepted for the pDNA calibrant ERM-AD425 and for the gDNA calibrant from leaves, respectively.

For one data set from the gDNA calibrant and for one data set from the pDNA calibrant only five of the six unknowns per data set were accepted. For all other data sets all 6 unknowns were used to calculate the mean copy number ratio.

The results of the copy number ratios per data set are summarised in Table 3. The mean copy number ratios are given in Table 4.

Table 2: Overview of rejected data sets and their reasons for exclusion
Blank cells indicate that data set was accepted. Data sets accepted for pDNA and gDNA calibration are omitted.

Data set	DNA extraction method	ERM-AD425 pDNA calibrant	gDNA calibrant from leaves
1	DNA*Extractor* kit[1]	Technical	Technical
2	DNA*Extractor* kit[1]	Calibration curve (R^2)	
3	DNA*Extractor* kit[1]	Calibration curves (ε < 76 %)	Calibration curves (ε > 110 %)
4	DNA*Extractor* kit[1]	Insufficient data points	Calibration curve (ε > 110 %)
5	DNA*Extractor* kit[1]		RSD
6	DNA*Extractor* kit[1]	Technical	Technical
7	DNA*Extractor* kit[1]	RSD	Calibration curves (ε > 110 %)
9	DNA*Extractor* kit[1]	Calibration curve (R^2)	Calibration curves (R^2)
10	DNA*Extractor* kit[1]	Calibration curves (ε >110 %, R^2)	
11	DNA*Extractor* kit[1]	Calibration curves (ε < 76 %, R^2)	
12	DNA*Extractor* kit[1]		Calibration curve (ε < 76 %)
13	DNA*Extractor* kit[1]	Calibration curve (ε < 76 %)	RSD
14	DNA*Extractor* kit[1]	Technical	Technical
15	CTAB[2]	Technical	Calibration curves (ε > 110 %)
16	CTAB[2]		Technical
17	CTAB[2]	Calibration curve (R^2)	Calibration curve (ε > 110 %)
19	CTAB[2]	Calibration curve (ε < 76 %)	Technical
20	CTAB[2]	Calibration curves (ε < 76 %)	
21	CTAB[2]	Calibration curve (ε < 76 %)	
22	CTAB[2]		RSD
23	CTAB[2]		Calibration curves (ε > 110 %)
24	CTAB[2]	RSD	
25	CTAB[2]	Calibration curves (ε < 76 %)	
26	CTAB[2]	Calibration curves (ε < 76 %, R^2)	
27	CTAB[2]	Technical	Technical
29	GENE*Spin*[3]	Technical	Technical
30	GENE*Spin*[3]		Calibration curve (ε > 110 %)
31	GENE*Spin*[3]		Calibration curve (ε > 110 %)
33	GENE*Spin*[3]	Insufficient data points	
34	GENE*Spin*[3]	Technical	Technical
35	GENE*Spin*[3]	Calibration curve (R^2)	Calibration curve (R^2)
36	GENE*Spin*[3]	Calibration curve (ε < 76%)	
38	GENE*Spin*[3]	Calibration curve (R^2)	Calibration curves (R^2)
39	GENE*Spin*[3]	RSD	RSD
41	GENE*Spin*[3]	RSD	RSD
42	GENE*Spin*[3]	Calibration curve (ε > 110 %)	Insufficient data points

[1] DNA was extracted using a modified protocol based on the DNA*Extractor* kit (Eurofins GeneScan GmbH, Freiburg, DE) with an additional Genomic-tip20 (Qiagen, Benelx, Venlo, NL) purification step (ANNEX 1)
[2] DNA was obtained by the CTAB DNA extraction with an additional Genomic-tip20 purification step (ANNEX 2)
[3] DNA was extracted with the modified protocol for the GENE*Spin* kit (Eurofins GeneScan GmbH, Freiburg, DE) (ANNEX 3)

Table 3: Results from Copy Number Certification Study

Mean DNA copy number ratio [%] and standard deviation (*s*) obtained for accepted data sets. Blank cells indicate that the data set was excluded. Data sets excluded for pDNA and gDNA calibrations are not given.

		DNA copy number ratio ± *s* [%]	
Data set	DNA extraction method	ERM-AD425 pDNA calibrant	gDNA calibrant from leaves
2	DNA*Extractor* kit[1]	0.97 ± 0.14	
5	DNA*Extractor* kit[1]		1.50 ± 0.30
8	DNA*Extractor* kit[1]	0.77 ± 0.07	0.90 ± 0.08
10	DNA*Extractor* kit[1]	0.63 ± 0.13	
11	DNA*Extractor* kit[1]	0.97 ± 0.10	
12	DNA*Extractor* kit[1]		1.18 ± 0.15
16	CTAB[2]		1.21 ± 0.14
18	CTAB[2]	1.00 ± 0.10	0.94 ± 0.09
20	CTAB[2]	0.79 ± 0.07	
21	CTAB[2]	0.87 ± 0.18	
22	CTAB[2]		1.33 ± 0.25
23	CTAB[2]		1.21 ± 0.13
24	CTAB[2]	0.67 ± 0.13	
25	CTAB[2]	0.67 ± 0.09	
26	CTAB[2]	0.95 ± 0.08	
28	CTAB[2]	0.73 ± 0.08	0.87 ± 0.06
30	GENE*Spin*[3]		1.27 ± 0.11
31	GENE*Spin*[3]		1.11 ± 0.08
32	GENE*Spin*[3]	0.96 ± 0.05	1.20 ± 0.07
33	GENE*Spin*[3]	0.91 ± 0.13	
36	GENE*Spin*[3]	0.95 ± 0.21	
37	GENE*Spin*[3]		1.16 ± 0.08
40	GENE*Spin*[3]	0.87 ± 0.09	1.18 ± 0.11

[1] DNA was extracted using a modified protocol based on the DNA*Extractor* kit (Eurofins GeneScan GmbH, Freiburg, DE) with an additional Genomic-tip20 (Qiagen, Benelx, Venlo, NL) purification step (ANNEX 1)

[2] DNA was obtained by the CTAB DNA extraction with an additional Genomic-tip20 purification step (ANNEX 2)

[3] DNA was extracted with the modified protocol for the GENE*Spin* kit (Eurofins GeneScan GmbH, Freiburg, DE) (ANNEX 3)

Table 4: Means of results from Copy Number Certification Study

Mean copy number ratios and standard deviations (*s*) obtained with pDNA calibrant ERM-AD425 and gDNA calibrant from leaves (gDNAℓ) are calculated as the unweighted mean of means. N_d is the number of accepted data sets under reproducibility conditions.

	DNA copy number ratio ± *s* [%]	
	pDNA ERM-AD425	gDNAℓ
ERM-BF425c	0.85 ± 0.13 (N_d = 15)	1.15 ± 0.18 (N_d = 13)

6.4 STATISTICAL EVALUATION OF DNA CALIBRANTS

The mean DNA copy number ratios of the accepted data sets were compared per extraction method and calibrant type (Figure 2). The number of accepted data sets per DNA extraction method and calibrant was low and a normality test was therefore not done [11]. DNA copy number ratios within the interval created by the subtraction and addition of the standard deviation were compared. The DNA copy number ratios per extraction method when calibrated with ERM-AD425 had overlapping $\bar{x} \pm s$. This is also true for DNA copy number ratios obtained when gDNA from leaves was used as a calibrant. Therefore the data of the three different DNA extraction methods were pooled per calibrant.

DNA copy number ratios obtained by real-time PCR calibrated with ERM-AD425 or gDNA from leaves followed a normal distribution. No outliers were detected for data calibrated with the pDNA calibrant ERM-AD425 (Dixon, Nalimov *t*-test, Grubbs test, 95 % confidence level). For copy number ratios obtained when calibrated with gDNA from leaves one outlier was detected after the Nalimov *t*-test (95 % confidence level).

The mean DNA copy number ratio obtained when experiments were calibrated with gDNA extracted from leaves is higher than the value obtained with ERM-AD425 as calibrant (Figure 2, Table 4). A subsequent single factor ANOVA confirmed that the calibrant has a significant influence on the estimated DNA copy number ratio ($p = 1.09 \cdot 10^{-5}$, 95 % confidence level).

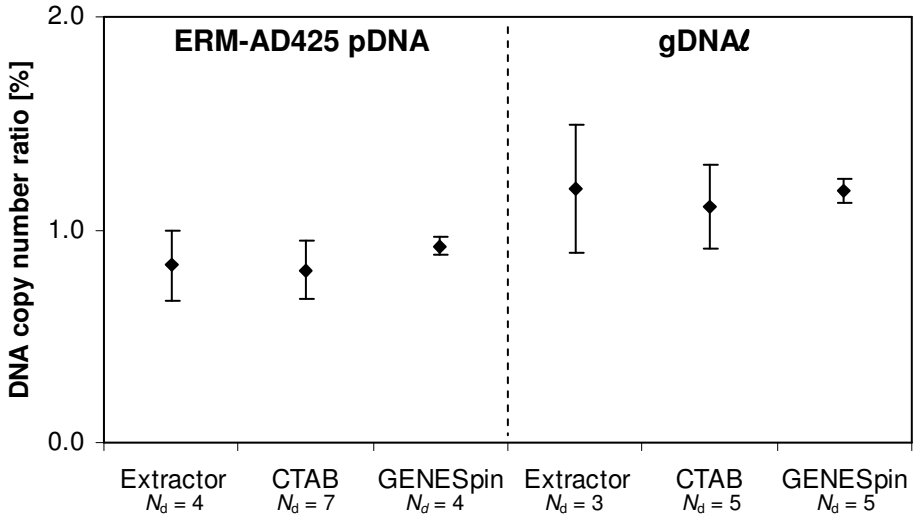

Figure 2: Influence of the DNA extraction method on the measured copy number ratio
Copy number ratio ± standard deviation *s* obtained with pDNA and gDNAℓ calibration and each extraction method are shown. N_d is the number of data sets used for evaluation, from each calibration and extraction method.

6.5 BEHAVIOUR OF PLASMID AND GENOMIC DNA

As there is a significant influence of the type of calibrant on the copy number ratio measured in ERM-BF425c, the data could not be pooled. Consequently, the DNA copy number ratio is defined by the calibrant. In this section, properties of the calibration curves, *i.e.* PCR efficiency and linearity of the regression line are compared to those of the unknowns in order to select the most appropriate calibrant.

The PCR efficiencies as well as the linearity of the regression lines were calculated on the basis of serial dilutions in plasmid dilution buffer of the pDNA calibrant ERM-AD425, dilutions of gDNA from leaves as well as dilutions of gDNA extracted from the ERM-BF425c

soya seed powder in nuclease-free water. To evaluate the behaviour of plasmid and genomic DNA, these two parameters have been compared and statistically analysed.

The first parameter was the PCR efficiency estimated for both transgenic and endogenous targets, using the three DNA types, *i.e.* pDNA ERM-AD425, gDNA extracted from leaves and gDNA extracted from ERM-BF425c seed powder. The PCR efficiencies were only compared if the respective data sets passed the selection criteria defined beforehand, namely the coefficient of determination (R^2) of the calibration curve, and PCR efficiency (see also Section 6.2). These selection criteria were applied in order to avoid interferences of technically weak results generated by the participating laboratories [10, 12]. Firstly, a R^2 value below 0.98 was not accepted within the study as it may reflect erroneous dilutions or inappropriate PCR amplification. Secondly, the PCR efficiencies interval was defined on the basis of pDNA ERM-AD425 and gDNA from leaves efficiencies (both materials used as calibrants in the study). The means of the PCR efficiencies were calculated for the endogeneous and transgenic targets using either the pDNA or gDNA from leaves calibrant. Four intervals were accordingly generated based on mean plus or minus one standard deviation. The minimum and maximum values of the resulting cut-off points were then used to define the lower and higher limits of the PCR efficiency of the study, *i.e.* 76 % and 110 % respectively. These values were consistent with the performance of the simplex real-time PCR method for quantification of the 356043 soya event observed with in-house performed studies and from results obtained by collaborations with external laboratories.

Mean PCR efficiencies ranged from 89.7 % to 99.6 % (Table 5). For both targets, PCR efficiencies were highest using the pDNA calibrant and lowest for the gDNA extracted from leaves. Comparing the targets, PCR efficiencies were higher for the endogenous gene *lectin* than for the transgene 356043 (Table 5).

Single factor ANOVA analysis showed that efficiencies obtained by pDNA calibrant and gDNA calibrant are significantly different for the endogenous gene *lectin* ($p = 8.14 \cdot 10^{-10}$, 95 % confidence level, Figure 3) and the transgene 356043 ($p = 4.57 \cdot 10^{-8}$, 95 % confidence level, Figure 3).

The distribution of the PCR efficiencies and their means for pDNA calibrant ERM-AD425, gDNA calibrant from leaves and gDNA extracted from ERM-BF425c seed powder was also compared and showed a large overlap for both targets for the three DNA types (Table 5, Figure 3). Statistical analysis proved that PCR efficiencies of pDNA, gDNA from leaves and gDNA from seed powder were significantly different for both *lectin* and 356043 target sequences.

A large number of outlier values were observed for the PCR efficiencies determined on the dilution series of the gDNA extracted from the ERM-BF425c seeds indicating some problems among the laboratories to work with a low number of DNA targets (Figure 3).

Table 5: Comparison of the real-time PCR efficiencies

Mean real-time PCR efficiencies of ERM-AD425 (pDNA) and gDNA extracted from leaves and seeds; N_{sd} stands for the accepted number of data subsets under repeatability conditions, s is the standard deviation.

Target sequence	**Mean real-time PCR efficiencies** ± s [%]		
	ERM-AD425 pDNA	ERM-BF425c gDNA$_s$	gDNA$_\ell$
lectin	99.6 ± 5.3 (N_{sd} = 65)	95.3 ± 5.5 (N_{sd} = 205)	92.6 ± 5.5 (N_{sd} = 79)
356043	95.7 ± 5.5 (N_{sd} = 73)	93.7 ± 6.5 (N_{sd} = 167)	89.7 ± 6.2 (N_{sd} = 56)

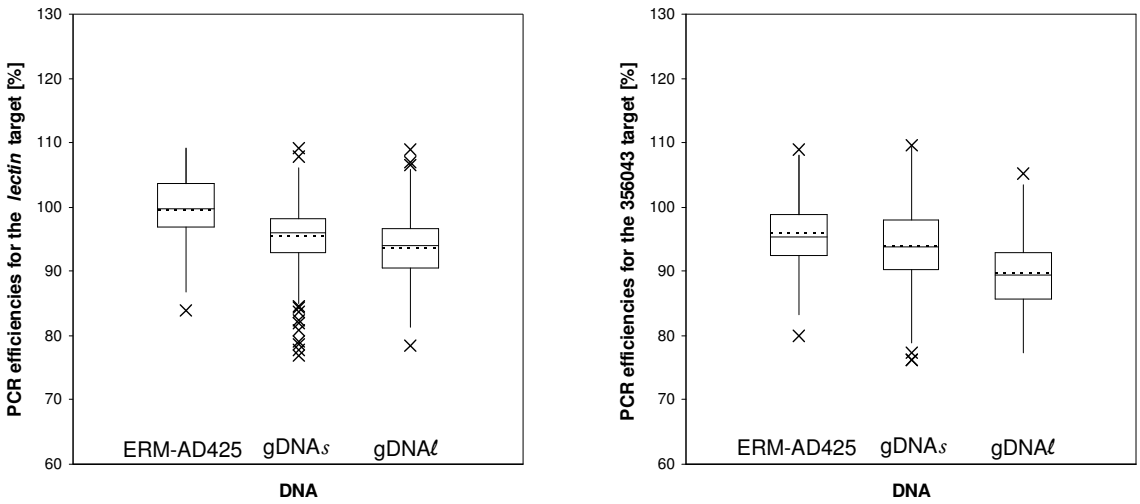

Figure 3: Box-and-whisker diagram illustrating PCR efficiencies for the *lectin* and 356043 target

Real-time PCR efficiencies for the *lectin* and 356043 target genes, based on the dilution series performed using either ERM-AD425 calibrant or gDNA extracted from ERM-BF425c seed powder (gDNA*s*) or from 356043 leaves (gDNA*ℓ*) are shown. The top and the bottom of the box are the lower and upper quartiles, respectively; the line near the middle of the box is the median. The ends of the whiskers are determined by substracting 1.5 times interquartile range (IQR) from the lower quartile, and adding 1.5 times IQR to the upper quartile, respectively. The dashed line corresponds to the mean value of each group, whereas extreme values (95 % confidence level) are marked with x.

The second parameter studied was the coefficient of determination (R^2) that provides information about the fitting of data to a linear regression line obtained for both targets and the three types of DNA (Table 6, Figure 4).

No major differences were observed between the three types of DNA for both targets. However, R^2 values were slightly higher for the calibrants than for the gDNA extracted from seed powder, revealing again the difficulties encountered by the laboratories to correctly quantify low number of DNA targets in the diluted samples (Table 6, Figure 4).

Table 6: Comparison of the coefficient of determination (R^2)

Comparison of coefficient of determination (R^2) of pDNA (ERM-AD425) and gDNA extracted from seeds (gDNA*s*) or leaves (gDNA*ℓ*); N_{sd} stands for the number of data subsets, *s* is the standard deviation.

	$R^2 \pm s$		
Target sequence	ERM-AD425 pDNA	ERM-BF425c gDNA*s*	gDNA*ℓ*
lectin	0.998 ± 0.002 (N_{sd} = 65)	0.997 ± 0.004 (N_{sd} = 205)	0.998 ± 0.003 (N_{sd} = 79)
356043	0.995 ± 0.003 (N_{sd} = 73)	0.994 ± 0.005 (N_{sd} = 167)	0.997 ± 0.002 (N_{sd} = 56)

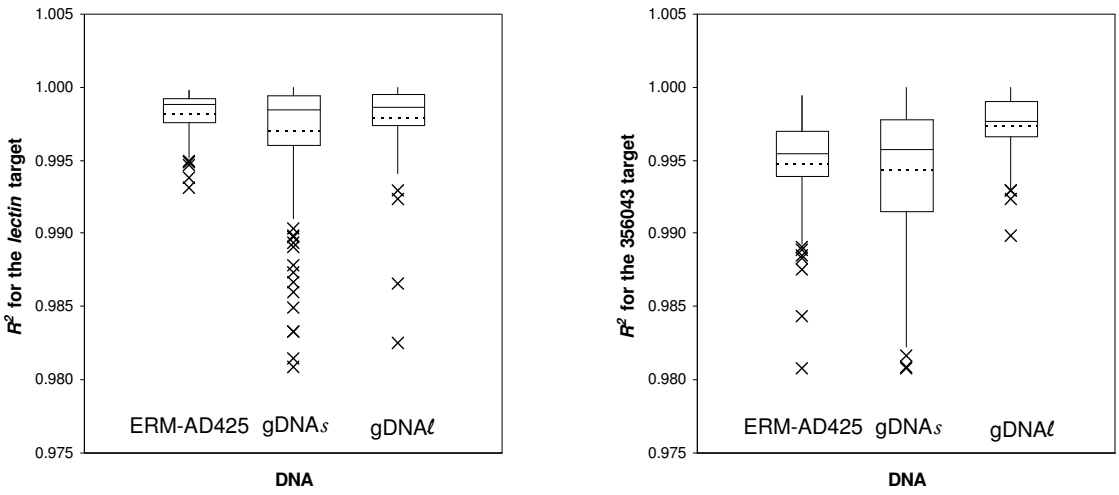

Figure 4: Box-and-whisker diagram representing the coefficient of determination (R^2) for the *lectin* and 356043 target

Coefficient of determination based on the dilution series performed using either ERM-AD425 calibrant, gDNA extracted from ERM-BF425c seed powder (gDNA*s*) or from 356043 leaves (gDNA*ℓ*) are shown. A description of a box-and-whisker diagram is given in the legend of Figure 3.

Finally, the 356043 DNA copy number ratio of the ERM-BF425c has been compared using either pDNA ERM-AD425 or the gDNA extracted from leaves as calibrants (Figure 5, Table 7). For this comparability study, copy number ratios obtained per day from accepted data sets were compared. The data sets from both calibrants follow a normal distribution. Though the DNA copy number ratios obtained for ERM-BF425c by the two different calibrants overlap, the mean DNA copy number ratios are very different. Single factor ANOVA analysis confirms that the data sets calibrated with ERM-AD425 are significantly different from the data sets calibrated with gDNA from leaves ($p = 1.36 \cdot 10^{-9}$, 95 % confidence level) (Figure 5). Therefore the DNA copy number ratios obtained when calibrated with ERM-AD425 or gDNA from leaves cannot be pooled.

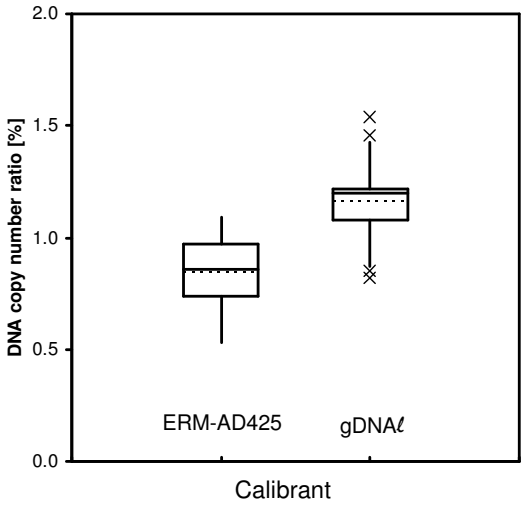

Figure 5: Box-and-whisker diagram and distribution graph of 356043 copy number ratios in ERM-BF425c

The left diagram shows the DNA copy number ratio using either pDNA ERM-AD425 or gDNA extracted from 356043 leaves (gDNA*ℓ*) as calibrant. A description of a box-and-whisker diagram is given in the legend of Figure 3.

Table 7: DNA copy number ratio in ERM-BF425c

Either pDNA ERM-AD425 or gDNA extracted from 356043 leaves (gDNAℓ) were used for calibration; N_{sd} stands for the number of accepted data subsets, *s* is the standard deviation.

	DNA copy number ratio ± *s* [%]	
	pDNA ERM-AD425	gDNAℓ
ERM-BF425c	0.85 ± 0.14 (N_{sd} = 30)	1.15 ± 0.18 (N_{sd} = 26)

In this study statistical analyses have shown that the two calibrants, *i.e.* pDNA ERM-AD425 and gDNA from leaves behave in a different way with respect to the PCR efficiencies of the transgenic and endogenous target sequences (Figure 3). The individual PCR efficiency of each target sequence has a significant impact on GM quantification by real-time PCR. Therefore, the effect of a low difference in PCR efficiencies of the transgenic and endogenous targets on GM quantification by real-time PCR can generate a large difference in copy number due to the exponential nature of the PCR amplification. Consequently, such significant differences in PCR efficiencies of the calibrants may explain the difference between the mean values (Table 7).

An additional measurement of gDNA extracted from ERM-BF425c was done by digital PCR [13, 14]. Digital PCR is a real-time PCR method that is independent of a calibrant but dependent on the same PCR chemistry, primers and probes as real-time PCR. Digital PCR involves distributing the PCR solution containing template nucleic acid molecules across a very large number of individual partitions prior to amplification. Following PCR amplification, a count of the proportion of partitions containing a detectable number of PCR amplicons can be used to estimate the total number of template DNA copies in the original DNA extract. The DNA copy number ratio was then calculated from the total number of template DNA copies. The DNA copy number ratio and its expanded uncertainty (k = 2) obtained by simplex digital PCR for gDNA extracted from ERM-BF425c was 1.02 ± 0.04 % (N = 3, n = 21, with each n measured in 5 replicate measurements) and is in line with the theoretical copy number ratio of 1 for ERM-BF425c. Thus, the copy number ratio obtained by the independent digital PCR is between the copy number ratios obtained by real-time PCR calibrated with pDNA ERM-AD425 and gDNA extracted with leaves.

Based on studying the analytical behaviour of pDNA and gDNA, it can be concluded that both calibrants, *i.e.* pDNA ERM-AD425 and gDNA extracted from soya 356043 leaves are suitable to calibrate quantitative PCR method applied here. However, the user should be aware that the choice of calibrant influences the measured copy number ratio and can lead to significant different results. The theoretical copy number ratio for ERM-BF425c is 1. Using the pDNA calibrant ERM-AD425 the measured DNA copy number ratio might be underestimated by about 15 %. On the other hand, when gDNA extracted from leaves is used as a calibrant the measured DNA copy number ratio might be overestimated by 15 %. Studies have indicated that the most suitable approach is to set a reference system based on pDNA as described in the certification report [6] as the primary calibrant for DNA copy number ratio measurements together with the approved GM quantification method published by the EURL-GMFF [1].

7 CERTIFIED VALUE AND UNCERTAINTY BUDGETS

7.1 METROLOGICAL TRACEABILITY

The certified DNA copy number ratio is defined as the soya 356043 DNA copy numbers divided by the soya-specific DNA copy numbers calculated in terms of haploid genomes [2]. It is expressed in percent. These DNA copy number ratios are determined using the soya 356043 event-specific real-time PCR method calibrated with the pDNA CRM ERM-AD425. Therefore, and given the interlaboratory comparison comprising 15 accepted data sets, the identity of the measurand is defined by the event-specific EURL-GMFF validated real-time PCR detection method [6] calibrated with the soya 356043 pDNA CRM ERM-AD425 [7]. The measurement results from the interlaboratory comparison were shown to be independent of the DNA extraction method applied (see Section 6.4).

Given the procedure and calibrant, the certified value is traceable to the International System of Units (SI).

7.2 CERTIFIED VALUE

The certified value is based on the soya 356043 DNA copy numbers and soya-specific DNA copy numbers. The DNA copy number ratio obtained for each data set, and expressed in %, is calculated as shown in equation 2:

$$\text{DNA copy number ratio [\%]} = \frac{\text{soybean 356043 DNA copy numbers [cp]}}{\text{soybean specific DNA copy numbers [cp]}} \cdot 100 \qquad (2)$$

Because the data are normally distributed (see Section 6.4), the mean ($\overline{x}$) is calculated as the unweighted mean of data set means (equation 3).

$$\overline{x} = \frac{\sum_{i=1}^{N_d} \overline{x}_i}{N_d} \qquad (3)$$

$\overline{x}$ = mean value
$\overline{x}_i$ = mean DNA copy number ratio obtained for data set *i*
N_d = number of data sets

7.3 UNCERTAINTY BUDGET

The expanded uncertainty of the certified value (U_{CRM}) comprises standard uncertainty contributions from the characterisation ($u_{char,\ rel}$), the homogeneity ($u_{bb,\ rel}$) and the stability ($u_{lts,\ rel}$) [8]. The uncertainty of the calibrant is negligible and therefore not considered [7]. The expanded uncertainty is estimated with equation 4, with k = coverage factor:

$$U_{CRM} = k \cdot \overline{x} \cdot \sqrt{u_{\text{char,rel}}^2 + u_{\text{bb,rel}}^2 + u_{\text{lts,rel}}^2} \qquad (4)$$

The uncertainty from the characterisation ($u_{char,rel}$) has been assessed during the interlaboratory comparison by calculating the standard deviation of the normally distributed data. It is calculated using equation 5:

$$u_{\text{char,rel}} = \frac{s}{\overline{x} \cdot \sqrt{N_d}} \qquad (5)$$

The uncertainty related to the homogeneity at 200 mg level has been estimated on the basis of a normally distributed population of real-time PCR measurement results [5]. The uncertainty contribution from the stability (u_{lts}) has been estimated on the basis of real-time PCR results following long-term monitoring of other soya materials [5]. A coverage factor

$k = 2$ was used to calculate the expanded uncertainty corresponding to a level of confidence of about 95 % (Table 8).
The standard uncertainty contribution introduced by the long-term stability is smaller than the standard uncertainty contributions from the characterisation and from the homogeneity of the soya powders (Table 8)

Table 8: **Uncertainty budget for the DNA copy number ratio of soya 356043 in ERM-BF425c.**

CRM certified value DNA copy number ratio	**Relative standard uncertainty contributions**			**Expanded uncertainty U_{CRM} ($k = 2$)**
[%]	$u_{bb,rel}$ [1]	$u_{lts,rel}$ [2]	$u_{char,rel}$ [3]	[%]
0.85	0.045	0.023	0.039	0.11

[1] Relative standard uncertainty introduced by the inhomogeneity at 200 mg level.
[2] Relative standard uncertainty related to the long-term stability, estimated for a shelf life of 12 months.
[3] Relative standard uncertainty introduced by the characterisation.

7.4 COMMUTABILITY

ERM-BF425c is prepared from non-GM and GM 356043 soya powders. The certified value is a DNA copy number ratio based on the 356043 DNA copy numbers and soya-specific DNA copy numbers expressed in percent; the DNA copy number ratio is defined by the real-time PCR detection method for 356043 soya [6] and calibrated with the plasmid DNA Certified Reference Material ERM-AD425 [7].

When using ERM-BF425c for the quality control of copy number ratio measurements of DNA extracted from food and feed samples commutability problems should to be considered [15].

The two calibrants tested during certification led to significant differences in the measured copy number ratio value and both calibrants, pDNA ERM-AD425 as well as gDNA extracted from leaves differed with either minus 15 or plus 15 % from the theoretical value of 1. It could not be proven during commutability study that one of the calibrants behaves more similar to the gDNA extracted from soya powder.

For practical reasons pDNA ERM-AD425 was selected to be the highest point of the calibration chain, ensuring for instance full characterisation and the reproduction of additional batches of calibrants. Values measured with the 356043 real-time PCR method and calibrant ERM-AD425 on ERM-BF425c and food and feed samples are reproducible and comparable. However, the user has to be aware that the values obtained with this measurement system as described in this report are likely to deviate with about 15 % from a true value.

8 INSTRUCTIONS FOR USE

8.1 INTENDED USE

The material ERM-BF425c is certified for both its mass fraction of the soya GM event 356043 [5] and the DNA copy number ratio when applying the soya 356043 event-specific real-time PCR and calibration with pDNA ERM-AD425 [this report]. The DNA copy number ratio is based on the 356043 copy numbers and soya-specific copy numbers, expressed in percent.

The material certified for its DNA copy number ratio is intended to be used for quality control of measurements of the soya 356043 DNA copy number ratios in GM food and feed. Genomic DNA may be extracted from the powder applying a validated method of choice for further use in real-time PCR measurements. However, the user should have in mind that soya powders are a difficult matrix to extract DNA from as the powders absorb the extraction buffer. This can result in a low yield and quality of DNA. Therefore, modifications might be necessary as described in ANNEX 1, ANNEX 2 and ANNEX 3. Experiments should be performed exclusively with the soya 356043 event-specific quantification method [6], calibrated with the pDNA calibrant ERM-AD425 [7] as the copy number ratio is defined by this method and calibrant (see Section 7.1).

8.2 HANDLING

The bottles containing the CRM should be allowed to reach the room temperature prior to opening. The dry soya powder is hygroscopic. Users are therefore advised to close bottles immediately after taking a sample.

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ANNEX 1

Modified protocol for DNA extraction from soya seed powder using the DNAExtractor kit (Eurofins GeneScan GmbH, Freiburg, DE)

1. Weigh 200 mg of plant powder in 15 mL falcon tubes
2. Add 750 µL Lysis Buffer (preheated at 50 °C) to each falcon tube
3. Mix thoroughly[3]
4. Add another 750 µL Lysis Buffer
5. Add 15 µL RNase A and 15 µL Proteinase K, mix
6. Incubate 2 h at 60 °C, shake every 15 min
7. Centrifuge 10 min at 10000 *g* at room temperature (RT)
8. Transfer supernatant (about 800 µL) to a new 15 mL falcon tube
9. Add 600 µL chloroform and mix
10. Centrifuge 15 min at 10000 *g* at RT
11. Transfer supernatant (about 650 µL) a new 15 mL falcon tube
12. Add 2 volumes of absolute ethanol, mix
13. Centrifuge 15 min at 10000 *g* at RT
14. Discard the supernatant, spin down again for 5 s, remove any remaining supernatant
15. Resuspend the pellet in 500 µL TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0), preheated to 50 °C by vortexing (at least 10 s at max speed)[4]
16. Add 2.5 mL G2 buffer and mix thoroughly
17. Incubate 2 h at 50 °C
18. Centrifuge 5 min at 10000 *g* at RT
19. Transfer supernatant to a 15 mL falcon tube
20. Equilibrate a QIAGEN Genomic-tip 20/G with 1 mL QBT
21. Apply the sample to the equilibrated Genomic-tip 20/G
22. Wash the genomic-tip 20/G three times with 1 mL buffer QC
23. Elute the genomic DNA with 1 mL QF buffer and collect the DNA in a 15 mL falcon tube
24. Add 2 volumes absolute ethanol to each tube, invert 10 times (if no DNA strands are appearing, incubate the tubes at -20 °C for at least 30 min)
25. Centrifuge 30 min at 10000 *g* at 4 °C, discard the supernatant
26. Wash the pellet with 1 mL 70 % ethanol
27. Centrifuge 10 min at 13000 *g* at 4 °C
28. Discard the supernatant and air-dry the pellet for 10 min
29. Dissolve the pellet in 100 µL nuclease-free water preheated at 50 °C and incubate at 50 °C for 10 min
30. Incubate overnight at 4 °C to dissolve the pellet completely

[3] Mixing can be done by vortexing although the powder is very sticky and difficult to mix. Alternatively, a glass Pasteur pipet, with closed tip obtained through melting by flame can be used for mixing.

[4] The pellet does not dissolve well. It will dissolve upon addition of G2 buffer and incubation at 50 °C.

ANNEX 2

Modified protocol for DNA extraction from soya seed powder using CTAB combined with a Genomic-tip 20/G purification step (Qiagen, Benelux B.V., Venlo, NL)

Reagent	Composition, specifications
H_20	Sterile nuclease-free water
RNase A	100 mg/mL
Proteinase K	20 mg/mL
Chloroform	p.a. quality
Ethanol	96 %, p.a. quality
NaCl solution	1.2 mol/L NaCl
CTAB extraction buffer	1.4 % mass concentration CTAB 1.0 mol/L NaCl 0.1 mol/L Tris-HCl pH 8.0 15.0 mmol/L Na_2EDTA
CTAB precipitation buffer	0.5 % mass concentration CTAB 40 mmol/L NaCl 50 mmo/L Tris-HCl pH 8.0
TE Buffer	1 mmol/L Tris pH 8.0 0.01 mmol/L EDTA

Procedure

1. Weigh 200 mg of plant powder in 15 mL falcon tubes
2. Add 4 mL CTAB Extraction Buffer to each falcon tube, mix[5]
3. Incubate 60 min at 60 °C, shake every 15 min
4. Centrifuge 10 min at 3000 *g* at RT
5. Transfer supernatant to a 15 mL falcon tube containing 4 mL chloroform
6. Mix thoroughly, incubate 5 min at RT
7. Centrifuge 10 min at 3000 *g* at RT (use a conical rotor)
8. Transfer 2 - 2.5 mL supernatant to a new 15 mL falcon tube and add 5.5 mL of CTAB Precipitation Buffer
9. Mix thoroughly, incubate 30 min at RT
10. Centrifuge 20 min at 10000 *g* at RT
11. Discard the supernatant, spin down again for 5 s, remove any remaining supernatant
12. Resuspend the pellet in 500 µL of TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0) preheated to 50 °C by vortexing (at least 10 s at max speed)[6]
13. Add 2.5 mL G2 buffer (containing 4 µL RNase A and 30 µL Proteinase K) and mix thoroughly
14. Incubate 2 h at 50 °C
15. Centrifuge 5 min at 10000 *g* m at RT
16. Transfer supernatant to a 15 mL falcon tube
17. Equilibrate a Qiagen Genomic-tip 20/G with 1 mL QBT

[5] Mixing can be done by vortexing although the powder is very sticky and difficult to mix. Alternatively, a glass Pasteur pipette, with closed tip obtained through melting by flame can be used for mixing.

[6] The pellet does not dissolve well. It will dissolve upon addition of G2 buffer and incubation at 50 °C.

18. Apply the sample to the equilibrated Genomic-tip 20/G
19. Wash the genomic-tip 20/G three times with 1 mL buffer QC
20. Elute the genomic DNA with 1 mL QF buffer and collect the DNA in a 2 mL eppendorf tube
21. Add 700 µL isopropanol, invert tube 10 times
22. Centrifuge 30 min at 10000 *g* at 4 °C, discard the supernatant
23. Wash the pellet with 1 mL 70 % (volume fraction) ethanol
24. Centrifuge 10 min at 13000 *g* at 4 °C
25. Discard the supernatant and air-dry the pellet for 10 min
26. Dissolve the pellet in 100 µL nuclease-free water preheated at 50 °C and incubate at 50 °C for 10 min
27. Incubate overnight at 4 °C to dissolve the pellet completely

ANNEX 3

Modified protocol for DNA extraction from soya seed powder using GENE*Spin*
(Eurofins GeneScan GmbH, Freiburg, DE)

1. Weigh 200 mg of plant powder in 2 mL tubes
2. Preheat Lysis Buffer CF to 65 °C immediately before use
3. Add 825 µL Lysis Buffer CF to the powder and mix carefully (15 s)
4. Add 15 µL Proteinase K and 15 µL RNase (20 mg/mL) and mix[7]
5. Incubate the mixture at 65 °C for 30 min
6. Centrifuge for 10 min at RT at 13000 *g*
7. Pipet 500 µL clear supernatant into a new 1.5 mL tube
8. Add 500 µL Buffer C4 and 333 µL Ethanol, vortex the mixture for 30 s
9. Place a GENE*Spin* column into a 2 mL collection tube and add 650 µL mixture onto the column
10. Centrifuge for 1 min at RT at 13000 *g* and discard flow-through
11. Place the GENE*Spin* column back into a 2 mL collection tube and add the remaining 650 µL mixture onto the column
12. Centrifuge for 1 min at 13000 *g* at RT and discard flow-through
13. Add 400 µL Buffer CQW onto the GENE*Spin* column, centrifuge for 1 min at 13000 *g* at RT and discard flow-through
14. Add 700 µL Buffer C5 onto the GENE*Spin* column, centrifuge for 1 min at 13000 *g* at RT and discard flow-through
15. Add another 200 µL Buffer C5 onto the GENE*Spin* column, centrifuge for 2 min at 13000 *g* at RT.
16. Preheat the Elution Buffer CE at 70 °C
17. Place the GENE*Spin* column into a new 1.5 mL tube.
18. Centrifuge 5 min at 10000 *g* at RT
19. Add 100 µL Elution Buffer CE onto the membrane, incubate for 5 min at RT, centrifuge for 1 min at 13000 *g* at RT

[7] Mixing can be done by vortexing although the powder is very sticky and difficult to mix. Alternatively, a glass Pasteur pipette, with closed tip obtained through melting by flame can be used for mixing.

European Commission

EUR 24695 EN – Joint Research Centre – Institute for Reference Materials and Measurements
Title: Certification of a Soya 356043 Reference Material for its DNA Copy Number Ratio - Certified Reference Material ERM®-BF425c
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Abstract

This report describes the certification of the Certified Reference Material (CRM) ERM®-BF425c for its DNA copy number ratio. This CRM is composed of conventional and genetically modified soya 356043 seed powders.

ERM-BF425c is part of a set of soya CRMs containing different mass fractions of GM soya 356043. The CRM was processed and originally certified for its soya 356043 mass fraction by the European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE.

European Commission Recommendation (EC) No 787/2004 advises to express the content of GM food and feed products as the percentage of GM DNA copy numbers in relation to target taxon-specific DNA copy numbers calculated in terms of haploid genomes, further referred to as the DNA copy number ratio. An interlaboratory comparison was conducted in 2008 to certify the existing CRM for its DNA copy number ratio. The CRM is intended for quality control of measurements of DNA copy number ratios of soya event 356043 in GM food and feed. Hereby, the plasmid DNA (pDNA) ERM®-AD425 should be used for calibration together with the soya 356043 event-specific real-time Polymerase Chain Reaction method validated by the European Union Reference Laboratory for GM Food and Feed (EURL-GMFF) (available via: http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm). ERM-AD425 contains a 99 bp fragment of the 5' plant/insert junction specific for the soya event 356043. Additionally, the plasmid carries a 243 bp fragment of the species-specific *lectin* gene *le1*, specific for the soya taxon.

The CRM ERM-BF425c is available in glass bottles containing 1 g of soya seed powder, closed under argon atmosphere and is certified for its DNA copy number ratio. The minimum amount of sample to be used is 200 mg.

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