Report on the Validation of a DNA Extraction Method for Soybean Seeds

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Joint Research Centre
Institute for Health and Consumer Protection
Biotechnology & GMOs Unit

Method development:

Pioneer Overseas Corporation

Method testing and single laboratory validation:

Community Reference Laboratory for GM Food and Feed (CRL-GMFF)
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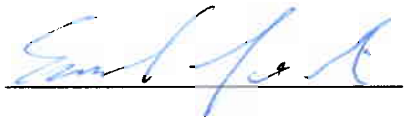

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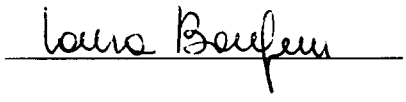

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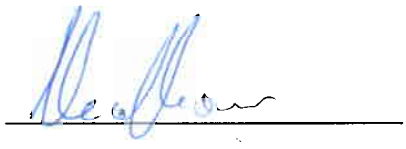

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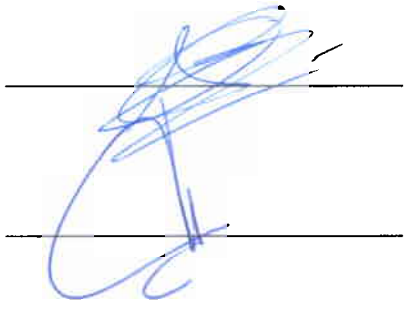

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Content

1. Introduction

This report describes the validation of a small-scale DNA extraction protocol to extract high quality genomic DNA from soybean for subsequent real-time PCR based detection methods and its applicability on the samples of food and feed provided by the applicant. This protocol can be used for the extraction of DNA from soybean seeds. It is a modified "CTAB-precipitation/Wizard" extraction method.

The purpose of the DNA extraction method described is to provide DNA with purity suitable for real-time PCR based detection methods.

This protocol is recommended to be executed by skilled laboratory personnel since hazardous chemicals and materials are used at some steps. It is strongly advised to take particular notice of all product safety recommendations and guidelines.

2. Materials (Equipment/Chemicals/Plasticware)

2.1. Equipment

The following equipment is used in the DNA extraction procedure described (equivalents may be used):

1. Pipettes with adjustable volume (e.g. Eppendorf Research, 2-20 µL, 20-200 µL, 100-1000 µL)
2. Incubator with shaker or shaking water bath (e.g. Heraeus Function Line B12 in combination with shaker GFL 3005 (Gesellschaft für Labortechnik mbH)
3. Balances for the preparation of buffers and solutions and for sample weigh in (e.g. Ohaus Scout II, Ohaus Adventurer)
4. Refrigerated centrifuge with rotors for 50 mL centrifuge tubes and microcentrifuge (e.g. Sigma 4 K 15C with suitable rotors)
5. Heating block for 2.0 mL microcentrifuge tubes (e.g. Bioblock Scientific 92333)
6. Vortex (e.g. NeoLab Vortex VM-300)
7. Vac-Man® Laboratory Vacuum Manifold, 20-sample capacity (Promega (A7231))
8. Diaphragm vacuum pump (e.g. KNF Neuberger Diaphragm vacuum pump N840 FT,18)

2.2. Chemicals

The following chemicals are used in the DNA extraction procedure described (equivalents may be used):

1. NaCl (p.a. quality or Molecular biology grade)

2. CTAB (p.a. quality or Molecular biology grade)
3. Tris base (p.a. quality or Molecular biology grade)
4. EDTA Na_2-salt (p.a. quality or Molecular biology grade)
5. HCl (p.a. quality)
6. Proteinase K (From Tritirachium album, DNases, RNases, Exonucleases not detectable, Molecular biology grade)
7. RNase A (From bovine pancreas, salt free, protease free and chromatographically homogeneous, ca. 90 Kunitz units/mg)
8. Sodium acetate (p.a. quality or Molecular biology grade)
9. Chloroform (p.a. quality)
10. Isopropanol (p.a. quality)
11. Ethanol (p.a. quality)
12. CTAB lysis buffer
13. Wizard® DNA Clean-Up system (100) (Promega, Cat. #A7280)
14. S-300 HR MicroSpin Column (50) (GE Healthcare, Cat. #27-5130-01)

2.3. Solutions

The following buffers and solutions are used in the DNA extraction procedure described:

1. **CTAB Lysis Buffer**
   - 2% w/v CTAB
   - 100 mM Tris HCl pH 8.0
   - 20 mM EDTA pH 8.0
   - 1.4 M NaCl

For 1 liter CTAB lysis buffer weigh out 81.8 g NaCl, 20 g CTAB, 12.1 g Tris-Base and 7.44 g Na_2EDTA in an appropriate beaker and add about 800 mL H_2O_{deion} Stir until all reagents are dissolved. Adjust pH with HCl to pH 8.0. Adjust volume to 1 liter with H_2O_{deion} Autoclave. Store at room temperature for up to 1 year.
(CTAB lysis buffer can alternatively be purchased from Applichem).

2. **CTAB precipitation solution**
   - 0.5 % w/v CTAB
   - 0.04 M NaCl

For 1 liter CTAB buffer weigh out 2.3 g NaCl and 5 g CTAB in an appropriate beaker and add about 800 mL H_2O_{deion} Stir until all reagents are dissolved. Adjust volume to 1 liter with H_2O_{deion} Autoclave.
Store at room temperature for up to 1 year.

3. **Proteinase K**
   - 20 mg/mL H_2O_{deion}

For 10 mL proteinase K solution dissolve 200 mg proteinase K in 10 ml H_2O_{deion}.
Store at -20 °C for up to 2 years.

4. **RNase A**
    - 91 mg/mL

Dissolve 0.5 g RNase in 5 mL 0.01 M sodium acetate (pH 5.2), aliquot in 1 mL portions, boil for 15 minutes to inactivate DNases, cool slowly to room temperature and add 100 µL 1 M Tris-HCl (pH 7.4) to each aliquot.
Store at -20 °C for up to 2 years.

5. **1.2 M NaCl**

Weigh out 70.1 g NaCl. Adjust volume to 1 liter with H_2O_{deion}. Autoclave.
Store at room temperature for up to 1 year.

6. **75% (v/v) Ethanol**

To prepare 200 mL, combine 150 ml 100% ethanol with 50 mL H_2O_{deion}
Store at room temperature for up to 5 years.

7. **80% (v/v) Isopropanol**

To prepare 200 mL, combine 160 ml isopropanol with 50 ml H_2O_{deion}
Store at room temperature for up to 5 years.

8. **1x TE buffer**
    - 10 mM Tris, pH 8.3
    - 1 mM EDTA

To prepare 100 ml 1 x TE buffer, combine 1 mL 1 M Tris (pH 8.3) and 200 µL 0.5 M EDTA (pH 8.0) and adjust the volume to 100 mL with H_2O_{deion} Autoclave.
Store at room temperature for up to 2 years.

9. **0.2x TE buffer**
    - 2 mM Tris, pH 8.3
    - 0.2 mM EDTA

To prepare 100 mL 1 x TE buffer, dilute 20 mL 1x TE buffer with with 80 ml H_2O_{deion} Autoclave.
Store at room temperature for up to 2 years.

2.4. Plasticware

1. 50 mL conical tubes (e.g. Greiner Bio One GmbH 6052080)
2. 1.5 mL and 2 mL microcentrifuge tubes (e.g. Roth, 4182.1, Eppendorf, 5409341)
3. filter tips (fitting the pipette models used)

Note: All plasticware has to be sterile and free of DNases, RNases and nucleic acids.

2.5. Precautions

- It is recommended to use clean containers for Waring blenders for grinding the seed bulk samples.

- All tubes and pipette tips have to be discarded as biological hazardous material.

2.6. Abbreviations

EDTA	ethylenediaminetetraacetic acid
PCR	polymerase chain reaction
RNase A	ribonuclease A
TE	Tris-EDTA
Tris	Tris(hydroxymethyl)aminomethane

3. Description of the methods

3.1. Sampling

For sampling of seeds of soybean, the applicant refers to the technical guidance documents and protocols described in Commission Recommendation 2004/787/EC on technical guidance for sampling and detection of genetically modified organisms and material produced from genetically modified organisms as or in products in the context of Regulation (EC) No 1830/2003.

3.2. Scope and applicability

The method for DNA extraction described below is suitable for the isolation of genomic DNA from a wide variety of matrices. However, validation data presented here are restricted to homogenized soybean seeds. Application of the method to other matrices may require adaptation.

3.3. Practicability

For DNA extraction using the method described below only standard equipment for molecular biological work is required, e.g. a centrifuge, an incubator and pipettes. Costs for reagents and consumables add up to about 9 € per DNA extraction. The whole procedure from sample weighing to the final purification step takes about 9 hours in total with 3 hours hands-on time.

3.3. Principle

The basic principle of the DNA extraction consists of first releasing the DNA present in the matrix into aqueous solution and further, concurrently or subsequently, purifying the DNA from PCR inhibitors. The present method starts with a thermal lysis step in CTAB lysis buffer followed by extraction with chloroform for removal of contaminants such as lipophilic molecules, proteins and CTAB/polysaccharide complexes. Subsequently a crude DNA extract is generated by precipitation with CTAB precipitation buffer. After dissolving the resulting precipitate, chloroform extraction and isopropanol precipitation, remaining inhibitors are removed by further purifying the crude DNA extract by silica resin purification system using the commercially

available “Wizard® DNA Clean-Up system” (Promega) and a size exclusion spin column method with a Sephadex matrix using the commercially available “S-300 HR MicroSpin Columns” (GE healthcare).

3.4. Extraction of genomic DNA from soybean seeds/grains

Samples should be processed to fine flour prior to extraction procedure. Possible methods of processing include a mortar and pestle with liquid nitrogen or a commercial blender.

Lysis / isopropanol precipitation of DNA

1. Transfer 30 mL CTAB lysis buffer and 60 µL proteinase K to 50 mL conical tube.
2. Weigh out 10 g of ground soybean into tube containing CTAB lysis buffer and Proteinase K, mix thoroughly.
3. Incubate for 2 - 4 hours at 60 °C with agitation, e.g. in an overhead shaker.
4. Spin down at room temperature for 5 minutes at 2700 – 3000x *g*.
5. Transfer 1 mL of the supernatant to a 2 mL microcentrifuge tube containing 5 µL RNase A.
6. Incubate for 15 minutes at 60 °C.
7. Centrifuge at room temperature for 1 minute at approximately 20000x *g*.
8. Transfer 900 µL of the supernatant to a 2 mL microcentrifuge tube containing 600 µL chloroform.
9. Vortex, centrifuge at room temperature for 10 minutes at approximately 20000x *g*.
10. Transfer 625 µL of the upper phase to a 2 mL microcentrifuge tube containing 1 mL CTAB precipitation solution.
11. Mix completely (invert tubes several times) and let stand at room temperature for 60 minutes to allow precipitate to form.
12. Centrifuge at room temperature for 10 minutes at approximately 20000x *g*.
13. Discard supernatant. Dissolve precipitate in 500 µL 1.2 M NaCl and pipette carefully up and down until the pellet is detached from the wall of the microcentrifuge tube. Take care that you don’t lose the pellet. It is very important that the pellet is completely dissolved. If you have problems dissolving the pellet, incubate for 15 minutes at 60 °C.
14. Add 500 µL chloroform and vortex for 30s.
15. Centrifuge at room temperature for 10 minutes at approximately 20000x *g*.
16. Transfer upper layer (aqueous phase) into a new microcentrifuge tube.
17. Add 0.6 volume parts of isopropanol.
18. Mix completely (inverting tubes several times) and let stand at room temperature for 30 minutes to allow precipitate to form.
19. Centrifuge at room temperature for 10 minutes at approximately 20000x *g*.
20. Discard supernatant, add 500 µL 75% ethanol and mix carefully.
21. Centrifuge at room temperature for 5 minutes at approximately 20000x *g*.
22. Carefully remove and discard the supernatant. Centrifuge again and remove all remaining ethanol. If any fluid remains, allow pellet to dry at room temperature. Be very careful, that you don’t lose the pellet.

23. Resuspend the pellet in 500 µL 0.2x TE buffer. Make sure that the pellet is dissolved. If you have problems dissolving the pellet, incubate for 15 minutes at 60 °C. Then centrifuge for 2 minutes at approximately 20000x *g* and transfer the supernatant into a fresh 2 mL microcentrifuge tube.

Purification using Wizard® DNA Clean-Up System (Promega)

(Based on manufacturer's instructions: technical Bulletin #TB141)

24. Agitate well the Wizard resin. If crystals or aggregates are present, dissolve by warming the resin to 37°C for 10 minutes. Cool to 25 – 30 °C before use. Add 900 µL of the resin to sample DNA. Mix carefully by repeatedly pipetting up and down. Incubate for 5 minutes at 60 °C.
25. Use one Wizard® minicolumn for each sample. Attach syringe barrels (included in the kit) to Wizard® minicolumns (label minicolumns!) and then attach the minicolumn/syringe barrel assembly to the valves of the vacuum manifold (Promega Vac-Man® Laboratory Vacuum Manifold).
26. Allow samples to cool to room temperature, then spin down briefly (pulse spin).
27. Mix sample carefully by repeatedly pipetting up and down, transfer to the Wizard minicolumn. Apply a vacuum to draw the solution through the minicolumn. Break the vacuum to the minicolumn.
28. Wash the DNA-resin mixture once by applying 2 mL 80% isopropanol to the syringe barrel and reapply a vacuum to draw the solution through the minicolumn.
29. Close the valve when all 80% isopropanol has run through the Wizard minicolumn. Do not let the Wizard minicolumn run dry.
30. Remove the syringe barrels from the vacuum manifold and place Wizard® minicolumns in 1.5 mL microcentrifuge tubes. Centrifuge at room temperature for 2 to 4 minutes at 10000x *g* to remove any residual isopropanol.
31. Place Wizard® minicolumns into clean 1.5 mL microcentrifuge tubes and add 100 µL 0.2x TE buffer (prewarmed to 65 °C). Let stand at room temperature for 1 minute.
32. Centrifuge at room temperature for 1 minute at approximately 10000x *g* to elute DNA. Remove the Wizard® minicolumn. Centrifuge the eluted DNA for 2 minutes at approximately 20000x *g*.
33. Transfer the supernatant into a fresh 1.5 mL microcentrifuge tube.

Purification using S-300 HR MicroSpin Columns (GE Healthcare)

34. Label fresh 1.5 mL microcentrifuge tube using permanent marker.
35. Vortex MicroSpin microcolumns to homogenize the gel filtration material.
36. Slightly unscrew the MicroSpin microcolumn caps (1/4 turn but do not remove caps). Break off the bottom end of the MicroSpin microcolumn using clean gloves, clean forceps or the tool supplied by GE Healthcare.

37. Place the MicroSpin microcolumn in a 2 mL microcentrifuge tube and centrifuge for 2 minutes at 735x *g*.
38. Place the MicroSpin microcolumn into a 1.5 mL microcentrifuge tube and pipette DNA solution onto the center of the MicroSpin microcolumn.
39. Centrifuge at room temperature for 2 minutes at 735x *g*.
40. Discard the MicroSpin microcolumn. The purified DNA solution is collected in the bottom of the 1.5 mL microcentrifuge tube.

4. Testing of the DNA extraction method by the Community Reference Laboratory for GM Food and Feed

The aim of the experimental testing was to verify that the DNA extraction method provides DNA of suitable quantity and quality for the intended purpose. The DNA extraction method should allow preparation of the analyte in quality and quantity appropriate for the analytical method used to quantify the event-specific analyte versus the reference analyte.

The CRL-GMFF tested the method proposed by the applicant on samples of food and feed consisting of ground soybean seeds provided by the applicant.

To assess the suitability of the DNA extraction method for real-time PCR analysis, the extracted DNA was tested on a real-time PCR equipment.

4.1. Preparation of samples

About 200 g of soybean seed material were ground using a GRINDOMIX GM 200 (Retsch GmbH) mixer.

4.2. DNA extraction

DNA was extracted following the method described above (see paragraph 3. “Description of the methods”); the DNA extraction was carried out on 6 test portions (replicates) and repeated over three different days, giving a total of 18 DNA extractions.

4.3. DNA concentration, yield and repeatability

Concentration of the DNA extracted was determined by fluorescence detection using the PicoGreen dsDNA Quantitation Kit (Molecular Probes).

Each DNA extract was measured twice, and the two values were averaged. DNA concentration was determined on the basis of a five point standard curve ranging from 1 to 500 ng/µL using a Biorad VersaFluor fluorometer.

The DNA concentration for all samples is reported in Table 1 below.

Table 1. DNA concentration (ng/μL) of eighteen samples extracted in three days: yellow boxes for samples extracted on day 1, green boxes for samples extracted on day 2 and blue boxes for samples extracted on day 3.

Sample	Concentration (ng/μL)
1	35.6
2	60.2
3	71.9
4	73.9
5	62.5
6	63.1
1	64.3
2	89.5
3	53.6
4	96.9
5	55.8
6	66.0
1	93.0
2	73.8
3	80.0
4	111
5	114
6	117

- ✓ DNA concentration (ng/μL)

Overall average	76.8 ng/μL
Standard deviation	22.6 ng/μL
Coefficient of variation	29%

- ✓ Yield (total volume of DNA solution: approx. 100 μL)

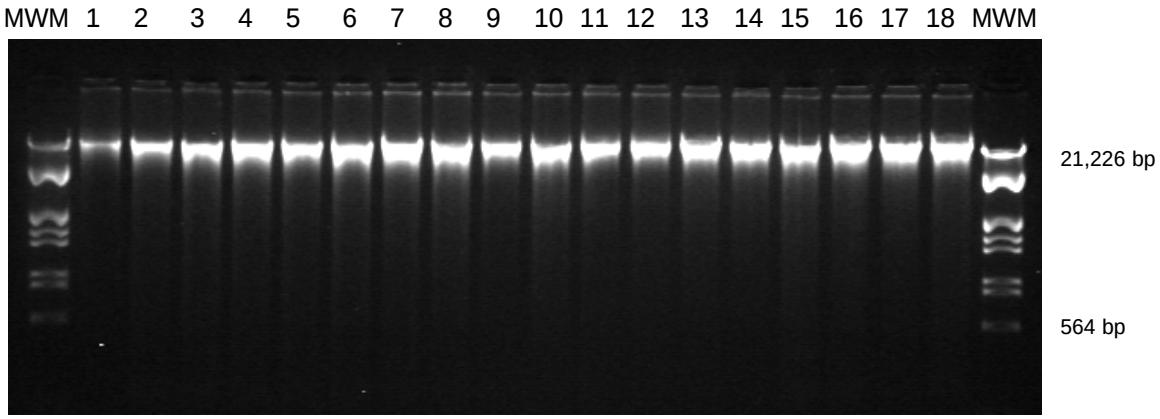
Overall average	7.68 μg
Standard deviation	2.26 μg
Coefficient of variation	29%

4.4. Fragmentation of DNA

The size of the extracted DNA was evaluated by agarose gel electrophoresis; 3 μL of the DNA solution were analysed on a 1.0% agarose gel (Figure 1).

The eighteen genomic DNA samples extracted as described above appeared as distinct fluorescent banding patterns migrating through the gel corresponding to high molecular weight DNA. None of the DNA samples showed indications of significant degradation ('smearing').

Figure 1. Agarose gel electrophoresis of eighteen genomic DNA samples extracted from soybean seeds. Lanes 1-6: samples extracted on day 1; lanes 7-12 samples extracted on day 2; lanes 13-18 samples extracted on day 3; lane MWM: 1 kb DNA molecular weight marker.



4.5. Purity / Absence of PCR inhibitors

In order to assess the purity and to confirm the absence of PCR inhibitors, the extracted DNA solutions were adjusted to a concentration of 30 ng/µL (hereafter referred as "undiluted" samples).

Subsequently fourfold serial dilutions of each extract were prepared with water (1:4, 1:16, 1:64, 1:256) and analysed using a real-time PCR system detecting the target sequence of the soybean endogenous control gene *Lectin* (*Le1*). The Ct values obtained for "undiluted" and diluted DNA samples are reported in Table 2.

Table 3 below reports the comparison of extrapolated Ct values versus measured Ct values for all samples and the values of linearity (R^2) and slope of all measurements.

To measure inhibition, the Ct values of the four diluted samples were plotted against the logarithm of the dilution and the Ct value for the "undiluted" sample (30 ng/µL) was extrapolated from the equation calculated by linear regression.

Table 2. Ct values of undiluted and fourfold serially diluted DNA extracts after amplification of the soybean endogenous gene, *Le1*. Yellow boxes for samples extracted on day 1, green boxes for samples extracted on day 2 and blue boxes for samples extracted on day 3.

	Undiluted (30 ng/µL)	Diluted			
DNA extract		1:4	1:16	1:64	1:256
1	19.16	21.29	23.43	25.50	27.62
2	19.77	22.03	24.10	26.20	28.21
3	20.93	23.04	25.18	27.33	29.40
4	19.99	22.07	23.95	26.24	28.51
5	19.90	21.80	24.02	26.20	28.27
6	20.70	23.04	25.10	27.13	29.03
1	20.18	22.25	24.34	26.36	28.30
2	20.88	22.86	24.89	27.01	29.00
3	19.37	21.69	23.70	25.78	27.96
4	19.90	21.99	23.89	26.05	28.48
5	20.03	22.00	24.13	26.23	28.22
6	20.10	22.18	24.34	26.30	28.24
1	20.94	23.02	25.08	27.06	29.25
2	20.90	22.93	25.04	27.02	29.13
3	21.08	23.06	25.14	27.21	29.22
4	20.88	22.96	24.94	27.03	29.21
5	20.93	23.05	25.07	27.07	28.99
6	21.06	22.94	24.97	27.06	29.27

Table 3. Comparison of extrapolated Ct values versus measured Ct values (amplification of soybean *Le1* gene). Yellow boxes for samples extracted on day 1, green boxes for samples extracted on day 2 and blue boxes for samples extracted on day 3.

DNA extraction	R^2	Slope*	Ct extrapolated	mean Ct measured	ΔCt**
1	0.999	-3.49	19.20	19.16	0.04
2	1.000	-3.43	19.97	19.77	0.20
3	1.000	-3.53	20.93	20.93	0.00
4	0.997	-3.59	19.79	19.99	0.21
5	0.999	-3.59	19.67	19.90	0.22
6	0.999	-3.32	21.07	20.70	0.37
1	0.999	-3.35	20.27	20.18	0.09
2	0.999	-3.41	20.80	20.88	0.08
3	0.998	-3.47	19.56	19.37	0.19
4	0.996	-3.59	19.69	19.90	0.20
5	0.999	-3.45	19.96	20.03	0.07
6	0.999	-3.35	20.22	20.10	0.12
1	0.999	-3.43	20.94	20.94	0.00
2	0.999	-3.42	20.88	20.90	0.02
3	0.999	-3.41	21.02	21.08	0.06
4	0.998	-3.47	20.82	20.88	0.06
5	0.998	-3.29	21.09	20.93	0.16
6	0.997	-3.50	20.79	21.06	0.27

*The expected slope for a PCR with 100% efficiency is -3.32; **delta Ct = abs (Ct extrapolated - Ct measured)

Subsequently the extrapolated Ct for the “undiluted” sample was compared with the measured Ct. The evaluation is carried out assuming that PCR inhibitors are present if the measured Ct value for the “undiluted” sample is > 0.5 cycles compared the calculated Ct value (ΔCt > 0.5). In addition, the slope of the curve should be between -3.6 and -3.1.

All ΔCt values of extrapolated versus measured Ct are < 0.5.

R^2 of linear regression is > 0.99 for all DNA samples. The slopes of the curves are all between -3.1 and -3.6.

5. Conclusion

The data reported confirm that the extraction method, applied to samples of food and feed provided by the applicant, produces DNA of suitable quantity and quality for subsequent PCR based detection applications.

The method is consequently applicable to samples of soybean seeds provided as samples of food and feed in accordance with the requirements of Annex I-2.C.2 of Commission Regulation (EC) No 641/2004.

6. Quality assurance

The CRL-GMFF carries out all operations according to ISO 9001:2000 (certificate number: 32231) and ISO 17025:2005 (certificate number: DAC-PL-0459-06-00) [DNA extraction, qualitative and quantitative PCR in the area of Biology (DNA extraction and PCR method validation for the detection and identification of GMOs in food and feed materials)]