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Molecular Biology and Genomics Unit

EURL
European Union Reference Laboratory
for GM Food & Feed

Report on the In-house Validation of a DNA Extraction Method from Oilseed rape Grains and Validated Method

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European Union Reference Laboratory for GM Food and Feed

Method development:

Pioneer Overseas Corporation

Method validation:

European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF)

Executive Summary

In accordance with relevant EU legislation[a], Pioneer Overseas Corporation provided to the European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF) a DNA extraction method for oilseed rape grains and the relevant samples (ground oilseed rape grains).

In line with its mandate[b], the EU-RL GMFF has conducted an in-house validation of this DNA extraction method. To this end it tested the DNA extraction method on the samples provided and evaluated its performance in terms of DNA yield, integrity and quality.

The in-house validation study confirmed that the method meets the method performance requirements as established by the EU-RL GMFF and the ENGL[c], and that it satisfies the provisions of Annex I-2.C.2 to Regulation (EC) No 641/2004.

The method is therefore fit for the purpose of producing rapeseed DNA of suitable quantity and quality for subsequent PCR-based analysis.

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

[a] Regulation (EC) No 641/2004 of 6 April 2004 "on detailed rules for the implementation of Regulation (EC) No 1829/2003".

[b] Regulation (EC) No 1829/2003 of 22 September 2003 "on genetically modified food and feed".

[c] Definition of minimum performance requirements for analytical methods of GMO testing. http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf

Quality assurance

The EU-RL GMFF is ISO 17025:2005 accredited [certificate number: ACCREDIA 1172, (Flexible Scope for DNA extraction and qualitative/quantitative PCR) - Accredited tests are available at http://www.accredia.it/accredia_labsearch.jsp?ID_LINK=293&area=7].

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

The EU-RL GMFF is also ISO 17043:2010 accredited (proficiency test provider) and applies the corresponding procedures and processes for the management of ring trials during the method validation.

The EU-RL GMFF conducts its activities under the certification ISO 9001:2008 of the Institute for Health and Consumer Protection IHCP provided by CERMET.

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1. Introduction

This report describes the validation of a DNA extraction method derived from the publicly available "CTAB" method [(1)] followed by an anion exchange chromatography with commercially available Qiagen columns "Genomic Tip 20/G", "Genomic Tip 100/G" or "Genomic Tip 500/G" 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 ground rapeseed.

This DNA extraction method is applicable to a variable amount of starting material, and three extraction scales are described, a small, a medium and a large scale. The data provided by the applicant are derived from a large scale DNA extraction, while the EU-RL GMFF performed the DNA extractions with the small scale version of the protocol.

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

This protocol is recommended to be executed only by skilled laboratory personnel as the procedures comprise the use of hazardous chemicals and materials. It is strongly advised to take particular notice of product safety recommendations and guidelines.

2. Materials (Equipment/Chemicals/Plastic ware)

2.1 Equipment

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

1. Pipettes with adjustable volume (Gilson)
2. Incubator (Eppendorf Thermo-mixer Comfort 5355)
3. Balances (Mettler Toledo XS2002S)
4. Centrifuges (Eppendorf 5810R and 5415D)
5. Vortex (MS1 Minishaker IKA)
6. Rotating wheel (PBI International)

2.2 Chemicals

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

1. "Genomic DNA Buffer Set" Kit (Qiagen 19060)
2. CTAB (Sigma No. H6269)
3. Tris-HCl (Sigma T3038)
4. Sodium chloride (Sigma S5150)
5. Proteinase K (Sigma P2308)
6. RNase A (M-Medical FC1740505)
7. 2-Mercaptoethanol (Sigma M3148)
8. Chloroform (Sigma C2432)
9. 1-Octanol (Sigma 293245)
10. Isopropanol (Sigma I9516)
11. Ethanol (Fluka 02860)
12. EDTA (Sigma E7889)
13. Tris-EDTA Buffer Solution (Fluka 93283)
14. Genomic-tip 500/G, 100/G or 20/G (Qiagen 10262, 10243 or 10223)

2.3 Solutions

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

CTAB extraction buffer

- 1 % (w/v) CTAB
- 10 mM EDTA
- 0.7 M NaCl
- 100 mM Tris-HCl, pH 8.0

For 1 litre CTAB extraction buffer, measure 10 g CTAB, 20.0 mL 0.5 M EDTA (pH 8.0), 40.9 g NaCl and 100 mL 1 M Tris-HCl (pH 8.0) into an appropriate beaker. Add about 800 mL H_2O_{deion}, and stir until all the CTAB and NaCl are dissolved. Adjust volume to 1 litre with H_2O_{deion}. Autoclave.

Store at room temperature for up to 1 year.

CTAB precipitation buffer

- 1 % (w/v) CTAB
- 10 mM EDTA
- 50 mM Tris-HCl, pH 8.0

For 1 litre CTAB precipitation buffer, measure 10 g CTAB, 20.0 mL 0.5 M EDTA (pH 8.0) and 50 mL 1 M Tris-HCl (pH 8.0) into an appropriate beaker and add about 800 mL H_2O_{deion}, and

stir until the CTAB is dissolved. Adjust volume to 1 litre with H_2O_{deion}. Autoclave.
Store at room temperature for up to 1 year.

Chloroform/1-Octanol (24:1)
- 1-Octanol
- Chloroform

For 1 litre, mix 40 mL of 1-octanol with 960 mL of chloroform.
Store at room temperature for up to 1 year.

Proteinase K (20 mg/mL)
For 10 mL proteinase K solution dissolve 200 mg proteinase K in 10 mL H_2O_{deion}.
Aliquot and store at -20°C for up to 2 years.

RNase A (100 mg/mL)
Add 2.5 mL H_2O_{deion} to 250 mg RNase A. Mix well.
Aliquot and store at -20 °C for up to 2 years.

Tris-EDTA buffer (1X TE) (e.g. from Fluka, 93283)
- 10 mM Tris-HCl, pH ?
- 1 mM EDTA

To prepare 100 mL 1x TE buffer combine 1 mL 1 M Tris (pH 7.5 to 8.0) 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.

70% (v/v) Ethanol
For 200 mL combine 140 mL 100% ethanol with 60 mL H_2O_{deion}.
Store at room temperature for up to 5 years.

2.4 Plasticware

1. "Genomic Tip 20/G", "Genomic Tip 100/G" or "Genomic Tip 500/G" columns (Qiagen 10223, 10243 or 10262)
2. 15 mL conical tubes (BD 352097)
3. Polystyrene graduated pipettes (BD)
4. 2.0 microcentrifuge tubes (Eppendorf 0030 120.094)
5. filter tips for pipettes

Note: all plastic ware should be sterile and free of DNases, RNases and nucleic acids.

2.5 Precautions

- Chloroform, octanol, and isopropanol are hazardous chemicals; therefore, all manipulations must be performed according to safety guidelines, under a fume hood.
- 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
CTAB	cetyltrimethylammonium bromide
HCl	hydrogen chloride
NaCl	sodium chloride

3. Description of the method

3.1 Scope and applicability

The method for DNA extraction described below is suitable for the isolation of high quality genomic DNA from ground rapeseed grains. Application of the method to other matrices may require adaptation and specific validation.

3.2 Practicability

The "CTAB-Anion-Exchange" DNA extraction method described below requires only standard molecular biology equipment, *e.g.* a centrifuge, an incubator and pipettes. The procedure takes about 30 minutes the first day and about 6 hours the second day of hands-on time.

3.3 Principle

The basic principle of DNA extraction consists of first releasing the DNA present in the sample into an aqueous solution and further, concurrently or subsequently, purifying the DNA from PCR inhibitors.
The "CTAB-Anion-Exchange" method starts with an overnight thermal lysis step using 16 g, 4 g or 1 g ground rapeseeds (depending on the scale of DNA extraction needed), in the CTAB extraction buffer (containing RNase A and Proteinase K). During lysis, the CTAB binds polysaccharides, cell wall debris and denatured proteins. After lysis and a chloroform extraction, a crude DNA extract is

generated by precipitation with CTAB precipitation buffer. This crude precipitate is resuspended and incubated in a lysis buffer with RNase A and Proteinase K. This incubation digests any remaining RNA, and strips the DNA from all bound proteins, which facilitates their removal during the purification process.
The final step involves loading the resulting lysate on an anion-exchange resin under appropriate low-salt and pH conditions using the commercially available gravity-flow Qiagen columns "Genomic-tip 20/G", "Genomic-tip 100/G" or "Genomic-tip 500/G". RNA, proteins, and low-molecular-weight impurities are removed by a medium-salt wash. Genomic DNA is eluted in a high-salt buffer and then concentrated and desalted by isopropanol precipitation

3.4 Lysis and CTAB precipitation of DNA

The DNA extraction method described can be used in three different scales: large, medium and small. In the medium scale protocol (hereafter referred to as the 100/G prep), prepare only one tube of 4 g of seed powder in step 1, transfer the supernatant to a 50 mL conical tube (at step 14), and use the volumes and tips shown in brackets for all subsequent steps. In the small scale protocol (hereafter referred to as the 20/G prep), utilise one tube of 0.1-1.0 g powder in step 1 and employ the smaller volumes shown in brackets throughout the protocol.

1. In each of four 50 mL conical tubes, mix the following: 4 g seed powder, 18 mL CTAB extraction buffer, 17.3 µL RNase A (100mg/mL) and 60 µL Proteinase K (20mg/mL) [for a 20/G prep, mix 0.1-1 g ground seed, 3.6 mL CTAB extraction buffer, 3.5 µL RNase A and 12 µL Proteinase K in a 15mL conical tube].
2. Add 200 µL of 2-Mercaptoethanol to each tube [for a 20/G prep, add 40 µL 2-Mercaptoethanol].
3. Securely cap the tubes and incubate overnight at ~65 °C with agitation.
4. Allow samples to cool down to room temperature for at least 15 min.
5. Add 10 mL Chloroform/1-Octanol (24:1) per tube and mix the tubes ~5 min in a rotating wheel [for a 20/G prep, add 2 mL Chloroform/1-Octanol (24:1)]. Note: Chloroform can dissolve many types of plastic, use appropriate containers and glass pipettes while working with chloroform.
6. Centrifuge at ~7000g for 10 min at room temperature to separate phases.
7. Carefully transfer each supernatant to a fresh 50 mL conical tube containing 22 mL CTAB precipitation buffer [for a 20/G prep, transfer the supernatant to a 15 mL conical tube containing 4.4 mL CTAB precipitation buffer]. Note: The starch and protein will form a thick layer separating the aqueous phase from the Chlorofrom/1-Octanol. Some of the Chloroform/1-Octanol may seep through the layer (and appear slightly yellow in contrast to the clear, DNA-rich aqueous solution), and should be avoided when transferring the upper aqueous phase.
8. Gently mix by inversion and incubate for~30 min at room temperature.
9. Centrifuge at ≥9200g for 20 min at room temperature to pellet DNA.

10. Carefully pour off and discard supernatant. Dissolve the pellet in 2 mL of pre-warmed (~50 °C) 1X TE buffer [for a 20/G prep, use 0.4 mL 1X TE]. Pipette carefully up and down or tap gently until the pellet is detached from the wall of the tube.

3.5 DNA purification using Genomic-tip 20/G, 100/G or 500/G and isopropanol precipitation

11. Add 9.5 mL Qiagen buffer G2, 17.3 µL RNase A and 50 µL Proteinase K to each tube [for a 20/G prep, add 1.9 mL G2, 3.5 µL RNase A and 10 µL Proteinase K to the tube].
12. Securely cap the tubes, and incubate at least 1 hour at ~50 °C with gentle shaking.
13. Centrifuge at ≥9200g for 5 min at room temperature to remove any particulate matter and prevent clogging of the Qiagen Genomic-tip.
14. Pool supernatant of the four tubes containing the replicate samples into one fresh 50 mL tube [do not pool tubes for the scaled down 100/G prep or 20/G prep but transfer the supernatant to clean tubes if a pellet is observed].
15. Saturate one Qiagen Genomic-Tip 500/G [100/G or 20/G, respectively] with 10 mL QBT [for the 100/G prep, use 4 mL QBT; for the 20/G, use 2 mL QBT]. Allow the buffer to run through the column before proceeding.
16. Apply combined sample [or single sample] to the saturated Qiagen Genomic tip. Allow it entering the resin by gravity flow.
17. Rinse the tip 2 times with 15 mL QC buffer [for the 100/G prep, use 7.5 mL QC buffer; for the 20/G prep, use three 1 mL rinses of QC buffer]. Discard the flow-through.
18. Elute DNA with 15 mL QF buffer (pre-warmed to ~50 °C) into a fresh 50 mL tube [for the 100/G prep, use 5 mL QF buffer to elute DNA into a fresh 15-mL tube; for the 20/G prep, combine two 1 mL elutions with QF buffer into a fresh 15-mL tube].
19. Precipitate DNA by adding 0.7 volumes of isopropanol at room temperature. Invert 10 to 20 times.
20. Centrifuge at ≥9200g for ~30 min at ~4 °C using a fixed angle rotor to pellet DNA.
21. Discard supernatant. Wash the pellet with 4 mL 70% ethanol (v/v) [for the 20/G preps, use 1 mL 70% ethanol (v/v)]. Vortex briefly to break up the pellet.
22. Centrifuge again the tubes at ≥9200g for ~10 min at ~4 °C.
23. Remove as much supernatant as possible and air dry the pellet for 5 to 10 min.
24. Add 2 mL of pre-warmed (~50 °C) 1X TE solution [for the 100/G prep, use 500 µL 1X TE or, for the 20/G prep, use 100 µL 1X TE].
25. Re-suspend the DNA by shaking for ~30 min at ~50 °C in a water bath. If desired, incubate overnight at ~4° C to further re-suspend the pellet and increase yield.
26. Transfer the DNA solution into a fresh 2 mL microcentrifuge tube.
27. Spin samples in a microfuge for ~2 min at max speed and transfer the supernatant to a fresh 2 mL tube.
28. Store the samples at ~ 4 °C until ready to use.

4. Testing of the DNA extraction method by the EU-RL GMFF

The EU-RL GMFF tested the method proposed by the applicant and described above, on samples consisting of ground rapeseed grains provided by the applicant. DNA extraction procedures should result in repeatable recovery, fragmentation profile, concentration and PCR quality of DNA extracts. The extracted DNA should be of suitable quantity and quality for the intended purpose[d].

4.1 DNA extraction

DNA was extracted from six test portions (replicates) following the small scale DNA extraction method (20/G prep) described in paragraph 3.4 ("Principle"). The procedure was repeated over three days, for a total of 18 DNA extractions.

4.2 DNA concentration, yield and repeatability

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

Each DNA extract was measured three times, and the three 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 average DNA concentration and the yield are summarised below.

- ✓ DNA concentration (ng/µL)

Overall average	303.3 ng/µL
Standard deviation of all samples	103.4 ng/µL
Coefficient of variation	34.11%

- ✓ Yield (µg)

Overall average	30.33 µg
Standard deviation	10.35 µg
Coefficient of variation	34.11%

[d] EURL/ENGL guidance document "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm)

Table 1 reports the data of concentration and yield for the 18 extracted samples.

Table 1. DNA concentration and yield of extracted samples

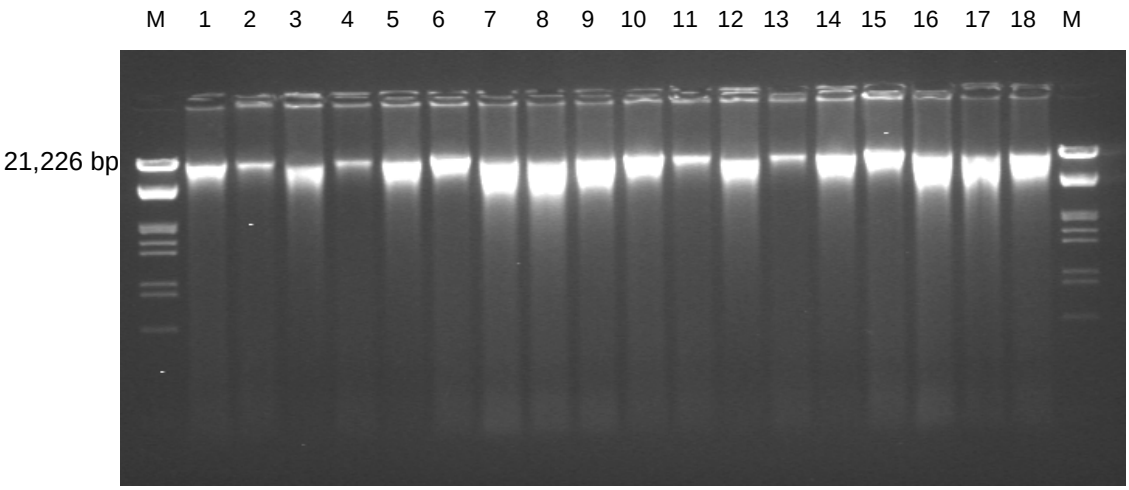
Sample	Concentration (ng/µL)	Yield (µg)
1	286.3	28.63
2	145.0	14.50
3	229.5	22.95
4	96.4	9.64
5	266.4	26.64
6	316.7	31.67
7	323.1	32.31
8	241.0	24.10
9	366.2	36.62
10	335.1	33.51
11	286.7	28.67
12	172.0	17.20
13	450.7	45.07
14	349.6	34.96
15	476.2	47.62
16	436.4	43.64
17	394.6	39.46
18	288.0	28.80

Note: In yellow boxes samples extracted on day 1; in green boxes samples extracted on day 2; in blue boxes samples extracted on day 3.

4.3 DNA Fragmentation

The size of the extracted DNA was evaluated by analysing it on a 1.0% agarose gel electrophoresis, to check that the DNA is not excessively fragmented for subsequent analyses. On the agarose gel, 4 µL of the DNA solutions are loaded (Figure 1).

Figure 1. Agarose gel electrophoresis of eighteen genomic DNA samples extracted from oilseed rape.



Lanes labelled 1-6: samples extracted on day 1, lanes labelled 7-12 samples extracted on day 2, lanes labelled 13-18 samples extracted on day 3; lanes labelled M: Lambda DNA/EcoRI+HindIII molecular weight markers.

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

4.4 Purity/Absence of PCR inhibitors

In order to assess the purity and to conduct a test for the presence of PCR inhibitors, the extracted DNA solutions were adjusted to a concentration of 20 ng/µL (hereafter referred to as "undiluted" samples).

Subsequently fourfold serial dilutions (1:4, 1:16, 1:64, 1:256) of each extract were prepared with TE low buffer (10mM Tris, 0.1mM EDTA, pH 8.0) and analysed in triplicate using a real-time PCR system detecting the target sequence of the endogenous gene acyl-ACP-thioesterase (*FatA(A)*).

The Ct values obtained for "undiluted" and diluted DNA samples are reported in Table 2.

Table 2. Ct values of undiluted and fourfold serially diluted DNA extracts after amplification of rapeseed gene *FatA(A)*.

Ct values					
	Undiluted	Diluted extracts			
DNA extract	(20 ng/µL)	1:4	1:16	1:64	1:256
1	23.01	24.91	26.98	29.22	31.33
2	22.95	24.97	27.04	29.14	31.49
3	22.89	24.94	27.16	29.55	31.81
4	22.90	24.87	26.94	29.30	31.29
5	22.87	24.90	26.93	29.18	31.59
6	22.98	24.97	26.99	29.37	31.36
7	22.84	24.75	26.81	28.97	31.17
8	22.09	24.15	26.15	28.47	30.58
9	22.96	24.89	27.08	29.15	31.13
10	23.06	24.97	26.99	29.03	31.04
11	22.72	24.81	26.84	28.96	30.81
12	22.48	24.50	26.56	28.58	30.46
13	23.09	25.01	27.08	29.28	31.27
14	22.98	24.96	27.00	29.09	31.29
15	22.96	24.98	26.95	29.22	31.27
16	23.29	25.14	27.15	29.42	31.46
17	22.97	25.00	27.04	29.26	31.36
18	23.16	25.11	27.10	29.87	31.89

Note: In yellow boxes samples extracted on day 1; in green boxes samples extracted on day 2; in blue boxes samples extracted on day 3.

To measure inhibition, the Ct values of the four diluted samples were plotted against the logarithm of the dilution and the Ct values for the "undiluted" samples (20 ng/µL) were extrapolated from the equation calculated by linear regression.

Subsequently the extrapolated Ct values for the "undiluted" samples were compared with the measured Ct data. It is assumed that PCR inhibitors are present if the measured Ct value for the "undiluted" sample is ≥ 0.5 cycles from the calculated Ct value. 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.

Table 3. Comparison of extrapolated Ct values versus measured Ct values (amplification of rapeseed gene *FatA(A).*

DNA extraction	R^2	Slope	Ct extrapolated	mean Ct measured	ΔCt**
1	1.00	-3.58	22.73	23.01	0.28
2	1.00	-3.59	22.75	22.95	0.20
3	1.00	-3.82	22.61	22.89	0.28
4	1.00	-3.59	22.69	22.90	0.21
5	1.00	-3.71	22.57	22.87	0.30
6	1.00	-3.58	22.79	22.98	0.20
7	1.00	-3.56	22.57	22.84	0.27
8	1.00	-3.59	21.94	22.09	0.16
9	1.00	-3.45	22.86	22.96	0.10
10	1.00	-3.36	22.95	23.06	0.11
11	1.00	-3.34	22.83	22.72	0.12
12	1.00	-3.30	22.55	22.48	0.07
13	1.00	-3.48	22.91	23.09	0.18
14	1.00	-3.50	22.82	22.98	0.17
15	1.00	-3.51	22.82	22.96	0.14
16	1.00	-3.53	22.98	23.29	0.31
17	1.00	-3.54	22.84	22.97	0.13
18	1.00	-3.84	22.71	23.16	0.45

Note: In yellow boxes samples extracted on day 1; in green boxes samples extracted on day 2; in blue boxes samples extracted on day 3.

**delta Ct = abs (Ct extrapolated - Ct measured)

According to the ENGL definition of minimum performance requirements for analytical methods of GMO testing[e] the expected slope for a PCR with 100% efficiency is -3.3; the accepted average value should be in the range of -3.6 and -3.1. In addition the average value of R^{2f} should be ≥ 0.98.

The table indicates that all ΔCt values of extrapolated versus measured Ct are < 0.5. The R^2 coefficient of linear regression is close to 1.0 for all DNA samples and the slopes of the curves are between -3.1 and -3.6 for most samples, except for samples 3, 5 and 18. This small deviation does not affect the performance of the DNA extraction method because it is limited to three extractions over eighteen, and the average value of the slope of all samples is between -3.1 and -3.6.

[e] EURL/ENGL guidance document "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm)

[f] R^2 is the correlation coefficient of a standard curve obtained by linear regression analysis.

5. Conclusion

The results confirm that the extraction method from oilseed rape grains provided by the applicant produces DNA of suitable quantity and quality for subsequent PCR-based analysis.

The method is applicable to oilseed rape samples provided by the applicant in accordance with the requirements of Annex I-2.C.2 to Commission Regulation (EC) No 641/2004.

If applied to complex food or feed products containing oilseed rape, because of the known difficulties in extracting high quality and quantity of DNA from such materials, particular care must be taken with regard to verifying the suitability of the extracted DNA for subsequent analyses.

6. References

1. Murray M.G., and Thompson W.F., 1980. Rapid Isolation of High Molecular Weight Plant DNA. Nucleic Acids Res. 8, 4321-4325.