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Report on the In-house Validation of a DNA Extraction Method from Ground Oilseed rape Seeds

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Report on the In-house Validation of a DNA Extraction Method from Ground Oilseed rape Seeds

27 April 2022

European Union Reference Laboratory for GM Food and Feed

Executive Summary

In accordance with relevant EU legislation[a], BASF Plant Science Company GmbH provided to the European Union Reference Laboratory for GM Food and Feed (EURL GMFF) a DNA extraction method for oilseed rape and the relevant samples (heat-devitalized intact seeds).

In line with its mandate[b], the EURL 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 ENGL[c], and that it satisfies the provisions of Annex III-3.C.2 to Regulation (EU) No 503/2013.

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

This report is published at https://gmo-crl.jrc.ec.europa.eu/method-validations.

[a] Regulation (EC) No 503/2013 of 3 April 2013 "on applications for authorisation of genetically modified food and feed in accordance with Regulation (EC) No 1829/2003 of the European Parliament and of the Council and amending Commission Regulations (EC) No 641/2004 and (EC) No 1981/2006".

[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/guidancedocs.htm

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Quality assurance

The EURL GMFF is ISO 17025:2017 accredited [certificate number: Belac 268 TEST (Flexible Scope for determination of Genetically Modified content in % (m/m) and % (cp/cp) in food and feed by DNA extraction, DNA identification and Real-time PCR and for determination of Genetically Modified content in % (cp/cp) in food and feed by DNA extraction and digital PCR)].

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

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Content

1. Introduction

This report describes the validation of a small-scale genomic DNA extraction method based on a "CTAB" (cetyltrimethylammonium bromide) lysis and chloroform extraction of protein and lipids, isopropanol precipitation of DNA followed by an anion exchange chromatography with commercially available columns (Promega Wizard® DNA Clean-Up System) and a gel filtration-based column (MoBiTec MobiSpin S-300) to remove PCR inhibitors. This protocol can be used for the extraction of DNA from ground oilseed rape seeds or grains.

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

It is recommended that this method is carried out only by skilled laboratory personnel as the procedures comprise the use of hazardous chemicals and materials. It is advised to take particular notice of products 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 (Rainin-Mettler Toledo)
2. Balances (Mettler Toledo XS2002S)
3. Centrifuges (Eppendorf 5810R and 5415D)
4. Vortex (MS1 Minishaker IKA)
5. Incubator (for 50 mL tubes, 65°C, with agitation) (Hybridization Incubator Combi-SV12-FINEPCR)
6. Freezer -20°C and Fridge 4°C (any model appropriate)
7. Fume hood (any model appropriate)

2.2 Chemicals

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

1. CTAB lysis buffer (Applichem A4150)
2. 1 X TE buffer pH 8.0 (SIGMA 93283)
3. Proteinase K (Sigma P2308)
4. RNase A (Sigma R6513)
5. Chloroform (Sigma C2432)

6. Isopropanol (Sigma I9516)
7. Ethanol (Fluka 02860)
8. Nuclease free water (AMBION AM9937)
9. Wizard® DNA Clean-Up System (Promega, Cat. No. A7280)
10. MobiSpin S-300 gel-filtration columns (MoBiTec, Cat. No. SCO310)

2.3 Solutions

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

1. **Tris-EDTA buffer (TE 0.1 X, pH 8.0)**

For 100 mL solution, add 10 mL of 1x TE pH 8.0 to 90 mL of deionized water. The solution can be stored at room temperature for up to 2 years.

2. **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.

3. **RNase A (100 mg/mL)**

- Dissolve 500 mg RNase A in 5 mL of $H_2O_{deion.}$
- Aliquot and store at -20 °C for up to 2 years.

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

To prepare 100 ml, add 80 ml of 100% isopropanol to 20 ml of H_2O_{deion}.
Store at room temperature for up to 1 year.

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

For 200 mL combine 150 mL 100% ethanol and fill up to 200 mL with H_2O_{deion}.
Store at room temperature for up to 1 year.

2.4 Plasticware

1. 25 mL Sterilin pipettes (ThermoFisher 47525)
2. 50 mL conical tubes (BD 352098)
3. 2.0 mL microcentrifuge DNA LoBind tubes (Eppendorf 0030108078)
4. 1.5 mL microcentrifuge DNA LoBind tubes (Eppendorf 0030108051)
5. Rainin filter tips for pipettes, aerosol-free

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

2.5 Precautions

- The protocol is recommended for skilled personnel because of the use of hazardous chemicals and materials
- Consideration of notice of products and safety recommendations and guidelines is strongly recommended.
- Chloroform, and isopropanol are hazardous chemicals; therefore, all manipulations have to be performed according to safety guidelines, under a fume hood.
- Strictly separate working areas for DNA extraction, PCR set-up and amplifications are advised.
- All equipment and lab benches should be free of DNA residues.
- 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

3. Description of the method

3.1 Scope and applicability

The method for DNA extraction described below is suitable for the isolation of small-scale high quality genomic DNA from ground oilseed rape seeds or grains. Application of the method to other matrices may require adaptation and possible further validation.

3.2 Practicability

The primary downstream use of genomic DNA from seed material is for real-time PCR-based detection methods. Therefore, the preparation of DNA from seed material should take place in an area dedicated for this purpose. All equipment (e.g. pipettes, centrifuges), lab ware and reagents used in this process should be stored and used only in the dedicated area. The entire procedure takes about 30 minutes for the first day, and about 6 hours for the second day.

3.3 Principle

The principle of the DNA extraction consists of first releasing the DNA present in the matrix into aqueous solution and further purifying the DNA from PCR inhibitors.

This method makes use of 5 g ground oilseed rape seed material, a CTAB buffer to lyse cells, and chloroform to precipitate proteins and lipids. The DNA in the aqueous phase is precipitated by isopropanol and purified using the commercially available Promega Wizard® DNA Clean-Up System and the MoBiTec MobiSpin S-300 gel-filtration columns. The DNA is eluted in a low salt buffer.

3.4 Grinding

Grinding of oilseed rape seeds not only facilitates the lysis by mechanically disrupting cellular structures and increasing the surface area, but is also indispensable for the generation of representative test portions by reducing the particle size.

3.5 DNA extraction protocol

1. For each ground seed pool, fill one 50 mL conical tube with 30 mL CTAB buffer and 50 µL Proteinase K (20 mg/mL).
2. Add 5 g of ground seeds into each tube containing CTAB buffer and Proteinase K and mix thoroughly.
3. Incubate overnight (10–20 hours) at 60°C with agitation.
4. Centrifuge at room temperature for 5 minutes at 2,700–3,000 x g.
5. Transfer approximately 1 mL of the supernatant to a 2 mL microcentrifuge tube containing 5 µL RNase A (100 mg/mL).
6. Incubate for 15 minutes at 60°C.
7. Centrifuge at room temperature for 1 minute at 20,000 x g.
8. Transfer 900 µL of the supernatant to a 2 mL microcentrifuge tube containing 600 µL chloroform and mix thoroughly by vortexing.
9. Centrifuge at room temperature for 10 minutes at 20,000 x g.
10. Transfer 625 µL of the upper phase to a 1.5 mL microcentrifuge tube containing 500 µL pre-cooled isopropanol.
11. Mix completely (invert tubes several times) and let stand at room temperature for 30 minutes to allow precipitate to form.
12. Centrifuge at room temperature for 10 minutes at 20,000 x g.
13. Discard supernatant. Add 500 µL 75% (v/v) ethanol.
14. Centrifuge at room temperature for 5 minutes at 20,000 x g.
15. Carefully remove and discard the supernatant. Centrifuge again and remove all remaining ethanol. Allow pellet to dry at room temperature.
16. Resuspend the pellet in 100 µL 0.1 X TE buffer pH 8.0 (pre-warmed to 65°C) by breaking up the pellet with a clean pipette tip.
17. Let stand for 30 minutes at 60°C. Please note: make sure the pellet is dissolved completely by carefully pipetting the solution up and down several times.
18. Let the sample cool down to room temperature for about 30 minutes, centrifuge for 1 minute at 20,000 x g and transfer the supernatant into a clean 1.5 mL microcentrifuge tube.

Purification using the Promega Wizard® DNA Clean-Up System

(following manufacturer's instructions)

1. Agitate well the Wizard resin. Add 900 µL to the extracted DNA (~100 µL). Mix carefully by repeated pipetting up and down.
2. Use one Wizard minicolumn for each DNA sample. Attach syringe barrels (included in the kit) to Wizard minicolumns (label minicolumns), and then attach the minicolumn or syringe barrel assembly to the valves of the vacuum manifold.
3. Transfer the sample to the Wizard minicolumn. Apply a vacuum to draw the solution through the minicolumn. Break the vacuum to the minicolumn.
4. Wash the DNA-resin mixture once by applying 0.8 mL 80% (v/v) isopropanol to the syringe barrel and reapply a vacuum to draw the solution through the minicolumn.
5. Close the valve when all 80% isopropanol has run through the Wizard minicolumn. Do not let the Wizard minicolumn run dry.
6. Remove the syringe barrels from the vacuum manifold and place Wizard minicolumns in 1.5 mL microcentrifuge tubes. Centrifuge at room temperature for 2–4 minutes at 10,000 x g to remove any residual isopropanol.
7. Place Wizard minicolumns into clean 1.5 mL microcentrifuge tubes and add 100 µL 0.1x TE buffer pH 8.0 (pre-warmed to 65°C). Let stand at room temperature for 1 minute.
8. Centrifuge at room temperature for 1 minute at 10,000 x g to elute DNA.

Further Purification using the MoBiTec MobiSpin S-300 gel-filtration columns

1. Label clean 1.5 mL microcentrifuge tubes using permanent marker.
2. Vortex MobiSpin microcolumns to homogenize the gel filtration material.
3. Slightly unscrew the MobiSpin microcolumn caps (1/4 turn, but do not remove caps). Unplug the bottom end of the MobiSpin microcolumn with clean gloves.
4. Place the MobiSpin microcolumn in a 2 mL microcentrifuge tube and centrifuge for 2 minutes at 735 x g.
5. Place the MobiSpin microcolumn into a 1.5 mL microcentrifuge tube and pipette DNA solution onto the center of the MobiSpin microcolumn.
6. Centrifuge at room temperature for 2 minutes at 735 x g.
7. Discard the MobiSpin 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 EURL GMFF

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

4.1 Preparation of samples

Two samples of heat-devitalised intact seeds (respectively GM and non-GM seeds) were received from the applicant and used for DNA extraction. The extraction method was validated *in-house* on non-GM oilseed rape ground seeds and further verified on oilseed rape LBFLFK ground seeds.

4.2 DNA extraction

The in-house validation of the DNA extraction method was carried out on six test portions (replicates) of non-GM oilseed rape seeds variety Kumily following the method described in paragraph 3.5 "DNA extraction protocol". The procedure was repeated in three days, for a total of 18 DNA extractions. The performance of the procedure was also verified on six test portions of 100 % LBFLFK oilseed rape seeds.

4.3 DNA concentration, yield and repeatability

The concentration of the extracted DNA solutions was determined by fluorescence detection using the PicoGreen dsDNA Quantitation Kit (Invitrogen) and a Biorad VersaFluor fluorometer. DNA concentrations were established on the basis of a five point standard curve ranging from 1 to 500 ng/µL. Each DNA extract was measured three times and the three values were averaged.

Table 1 reports the data of DNA concentration and yield for the 18 extracted samples from non GM seeds and the 6 extracted samples from the GM seeds.

[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. DNA concentration (ng/µL) and yield (µg DNA / g sample intake) of extracted samples

	Sample	Concentration (ng/µL)	Yield (µg)
A	1	131.30	2.63
	2	116.46	2.33
	3	136.83	2.74
	4	87.28	1.75
	5	121.81	2.44
	6	83.49	1.67
	7	128.88	2.58
	8	110.93	2.22
	9	111.28	2.23
	10	116.28	2.33
	11	109.72	2.19
	12	116.28	2.33
	13	108.00	2.16
	14	110.07	2.20
	15	107.83	2.16
	16	118.53	2.37
	17	118.01	2.36
	18	126.81	2.54
B	19	99.67	1.99
	20	73.18	1.46
	21	113.62	2.27
	22	97.93	1.96
	23	76.49	1.53
	24	102.63	2.05

A: samples from the non-GM oilseed rape seeds. Yellow boxes: DNA samples extracted on day 1; green boxes: DNA samples extracted on day 2; blue boxes: DNA samples extracted on day 3.
B: pink boxes, DNA samples extracted from GM oilseed rape seeds.

The average DNA concentration and yield for all extracted samples are reported in Table 2A and Table 2B below.

Table 2A. Concentration and yield of the DNA extraction procedure on non-GM oilseed rape seeds

Non-GM oilseed rape seeds*	
Average (ng/µL)	114.43
Standard deviation of all samples	13.47
Coefficient of variation (%)	11.77
Yield (µg DNA / g sample intake)	2.29

* in-house validation based on eighteen extracted samples

Table 2B. Concentration and yield of the DNA extraction procedure on GM oilseed rape seeds

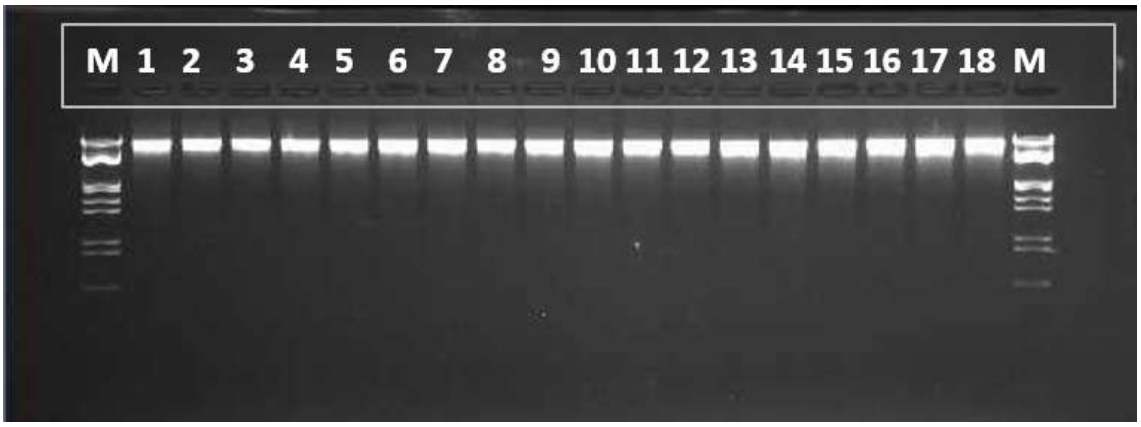
GM oilseed rape seeds**	
Average (ng/µL)	93.92
Standard deviation of all samples	15.79
Coefficient of variation (%)	16.82
Yield (µg DNA / g sample intake)	1.88

** verification based on six extracted samples

4.4 DNA Fragmentation

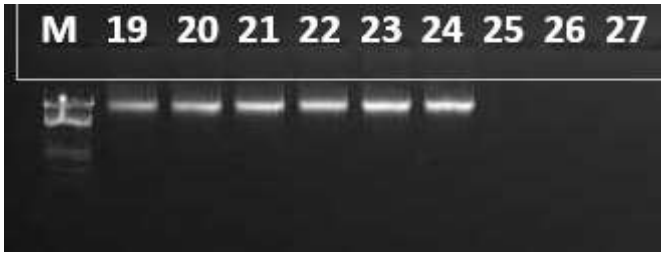
The size of the extracted DNA was evaluated by analysis on a 1.0% agarose gel electrophoresis, to check that the DNA is not excessively fragmented for subsequent analyses. One µL of the DNA solution was loaded per lane (Figure 1A and 1B).

Figure 1A. Agarose gel electrophoresis of eighteen genomic DNA samples extracted from ground non-GM oilseed rape seeds (samples 1-18).



Lanes 1-6: samples extracted on day 1, lanes 7-12: samples extracted on day 2, lanes 13-18: samples extracted on day 3; M: Lambda DNA/*Eco*RI+*Hind*III marker 3 (Thermofisher, SM0193).

Figure 1B. Agarose gel electrophoresis of six genomic DNA samples extracted from ground GM oilseed rape seeds (samples 19-24).



Lanes 19-24: samples extracted on day 3 from oilseed rape GM seeds; Lanes 25-27: blank DNA extraction controls; M: Lambda DNA/*Eco*RI+*Hind*III marker 3 (Thermofisher, SM0193).

The extracted genomic DNA samples appeared as distinct high molecular weight DNA bands on the gel. None of the DNA samples showed indication of significant degradation.

4.5 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 25 ng/µL (hereafter referred as "undiluted" samples).

Subsequently, fourfold serial dilutions (1:4, 1:16, 1:64, 1:256) of each extract were prepared with TE low buffer (1 mM Tris, 10 µM EDTA, pH 8.0) and analysed in triplicate using a real-time PCR method detecting the target sequence of the endogenous gene *FatA(A)* (QT-TAX-BN-001, GMOmethods database, https://gmo-crl.jrc.ec.europa.eu/gmomethods/). Five µL were used in each real-time PCR reaction.

The Cq values obtained for "undiluted" and diluted DNA samples are reported in Table 3.

Table 3. Cq values of undiluted and fourfold serially diluted DNA extracts after amplification of oilseed rape gene *FatA(A)*.

	Cq values					
		Undiluted	Diluted extracts			
	DNA extract	(25 ng/µL)	1:4	1:16	1:64	1:256
A	1	21.27	23.29	25.31	27.33	29.42
	2	21.23	23.33	25.35	27.37	29.32
	3	21.39	23.47	25.47	27.52	29.52
	4	21.38	23.46	25.41	27.45	29.50
	5	21.48	23.46	25.51	27.52	29.37
	6	21.34	23.33	25.23	27.20	29.15
	7	21.33	23.24	25.28	27.29	29.10
	8	21.46	23.38	25.38	27.33	29.37
	9	21.57	23.54	25.38	27.33	29.35
	10	21.70	23.61	25.38	27.29	29.31
	11	21.54	23.52	25.43	27.37	29.32
	12	21.57	23.49	25.43	27.34	29.30
	13	21.39	23.36	25.22	27.20	29.09
	14	21.37	23.35	25.32	27.20	29.28
	15	21.33	23.29	25.31	27.27	29.21
	16	21.38	23.31	25.30	27.24	29.18
	17	21.39	23.38	25.27	27.28	29.12
	18	21.22	23.16	25.18	27.09	29.12
B	19	21.21	23.25	25.11	27.06	29.03
	20	21.12	23.15	25.08	27.09	29.08
	21	21.46	23.56	25.40	27.38	29.33
	22	21.14	23.23	25.21	27.19	29.30
	23	21.21	23.20	25.15	27.14	29.09
	24	21.28	23.31	25.18	27.19	29.20

A: samples from the non-GM oilseed rape seeds. Yellow boxes: DNA samples extracted on day 1; green boxes: DNA samples extracted on day 2; blue boxes: DNA samples extracted on day 3.
B: pink boxes, DNA samples extracted from GM oilseed rape seeds.

To check for inhibition the Cq values of the four diluted samples were plotted against the logarithm of the dilution and the Cq values for the "undiluted" samples (25 ng /µL) were extrapolated from the equation calculated by linear regression.

Subsequently, the extrapolated Cq values for the "undiluted" samples were compared with the measured Cq data. It is assumed that PCR inhibitors are present if the measured Cq value for the "undiluted" sample is > 0.5 cycles from the calculated Cq value. Table 4 below reports the

comparison of extrapolated Cq values versus measured Cq values for all samples and the values of linearity (R^2) and slope of all measurements.

Table 4. Comparison of extrapolated Cq values versus measured Cq values (amplification of oilseed rape gene FatA(A).

	DNA extraction	R^2	Slope	Cq extrapolated	mean Cq measured	ΔCq*
A	1	1.00	-3.39	21.24	21.27	0.03
	2	1.00	-3.32	21.34	21.23	0.12
	3	1.00	-3.35	21.45	21.39	0.05
	4	1.00	-3.35	21.41	21.38	0.04
	5	1.00	-3.28	21.53	21.48	0.05
	6	1.00	-3.22	21.38	21.34	0.04
	7	1.00	-3.25	21.33	21.33	0.00
	8	1.00	-3.31	21.38	21.46	0.08
	9	1.00	-3.22	21.56	21.57	0.01
	10	1.00	-3.16	21.65	21.70	0.05
	11	1.00	-3.21	21.58	21.54	0.04
	12	1.00	-3.21	21.56	21.57	0.01
	13	1.00	-3.18	21.42	21.39	0.04
	14	1.00	-3.27	21.37	21.37	0.01
	15	1.00	-3.28	21.34	21.33	0.01
	16	1.00	-3.25	21.37	21.38	0.01
	17	1.00	-3.19	21.46	21.39	0.07
	18	1.00	-3.29	21.19	21.22	0.04
B	19	1.00	-3.20	21.29	21.21	0.09
	20	1.00	-3.29	21.15	21.12	0.03
	21	1.00	-3.21	21.59	21.46	0.13
	22	1.00	-3.36	21.18	21.14	0.04
	23	1.00	-3.27	21.23	21.21	0.02
	24	1.00	-3.27	21.30	21.28	0.02

A: samples from the non-GM oilseed rape seeds. Yellow boxes: DNA samples extracted on day 1; green boxes: DNA samples extracted on day 2; blue boxes: DNA samples extracted on day 3.

B: pink boxes: DNA samples extracted from GM oilseed rape seeds.

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

According to 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

[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)

value should be in the range of -3.6 and -3.1. In addition the average value of R^{2f} shall be ≥0.98.

The table indicates that all ΔCq values of extrapolated versus measured Cq are < 0.5. The R^2 coefficient of linear regression is > 0.99 for all DNA samples and the slopes of the curves are between -3.1 and -3.6 for all samples.

5. Conclusions

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

When 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.

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

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