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Verification Report on the Extraction and Analysis of GM Pollen DNA in Honey

Validation Report

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REPORT

Verification Report on the extraction and analysis of GM pollen DNA in honey

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Joint Research Centre
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Executive Summary

Following the judgment of 06 September 2011 on GM honey by the European Court of Justice (legal case C-442-09), the European Union Reference Laboratory for Genetically Modified Food and Feed (EU-RL GMFF) established by Regulation (EC) No 1829/2003, performed an in-house study to test the extraction and PCR analysis of genomic DNA from genetically modified pollen in honey.

The present report documents on an extraction method for isolation and analysis of pollen DNA present in honey, including the isolation and analysis of isolated genomic pollen DNA using real-time PCR on commercial honey samples and honey samples spiked with various levels of GM MON 810 pollen.

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Content

1. Introduction

Following the judgment of 06 September 2011 on GM honey by the European Court of Justice (legal case C-442-09), the European Union Reference Laboratory for Genetically Modified Food and Feed (EU-RL GMFF), established by Regulation (EC) No 1829/2003, was requested to carry out an in-house study to assess the extraction and PCR analysis of genomic DNA from genetically modified pollen in honey.

For this purpose, the EU-RL GMFF established an extraction procedure for pollen DNA from honey, including the isolation and analysis of isolated genomic pollen DNA using real-time PCR. The EU-RL GMFF tested herein honey samples and samples spiked with various levels of GM MON 810 pollen.

The present report documents the results of this EU-RL GMFF in-house study and documents an extraction method for isolation and analysis of pollen DNA present in honey.

2. Materials and methods

2.1 Materials

The EU-RL GMFF tested the following materials in this study:

- GM pollen samples hand-collected from commercial MON 810 fields during the summer of 2010 in the region of Gerona (Spain). Collected pollen were cleaned and stored dry at room temperature at the EU-RL GMFF. GM pollen sample was verified by real-time PCR and digital PCR (Folloni *et al*, 2012).
- Commercial honey samples as listed in Table 1.

The following control materials were used in this study:

- genomic DNA extracted from EU-RL GMFF control sample for GM maize MON 810;
- genomic DNA extracted from leaf tissue of in-house grown soy;
- genomic DNA extracted from rapeseed sp. Drakkar (RRM0138) control material for rapeseed;
- genomic DNA extracted from cotton sp. Coker (RRM0391) for cotton;

- genomic DNA extracted from EU-RL GMFF control material for sugar beet.

-Preparation of honey samples spiked with different levels of GM MON 810 pollen

Honey samples spiked with amounts of GM MON 810 pollen were prepared. Ten or fifty grams of solid honey material were heated up to 40-50ºC to allow the matrix to become fluid. MON 810 GM pollen test portions (as listed below) were weighted on a Mettler Toledo precision balance and transferred to a suitable container (e.g. a Falcon tube). The desired amount of fluid honey was added to each of the tube and pollen was mixed into the honey by gentle reversion of the tube. Two additional tubes filled with only honey sample served as negative controls (Figure 2 and Annex 1).

The following honey/pollen mixtures were prepared:

a) 10 g of commercial honey spiked with:
- 100 mg 5% GM maize pollen
- 100 mg 0.5 % GM maize pollen
- 100 mg 0.1% GM maize pollen
- 8 mg 50% GM maize pollen
- 6 mg 50% GM maize pollen
- 3 mg 50% GM maize pollen
- 5 mg GM pollen 1% control sample

b) GM pollen 1% and 0.1% control samples:
- 10 mg GM maize pollen in 1 g WT maize pollen (=GM pollen 1% control sample)
- 10 mg GM maize pollen in 10 g WT maize pollen (=GM pollen 0.1% control sample)

c) 50 g of farm-collected honey spiked with
- 450 mg 50% GM maize pollen
- 50 mg 50% GM maize pollen

2.2 Methods

DNA Quantification

Total DNA concentration was determined by fluorescence detection using the PicoGreen® dsDNA Quantification kit (Catalogue Number P7589, Molecular Probes). Suitable dilutions of each genomic DNA sample were prepared in 2 replicates and mixed with the PicoGreen® reagent. The DNA concentration was determined on the

basis of a five-point standard curve ranging from 0 to 500 ng/mL using a Bio-Rad VersaFluor Fluorometer as fluorescence detector.

Agarose Gel-electrophoresis

Intactness of the genomic DNA was assessed by 1.0% agarose gel electrophoresis and the DNA content was visualised by Ethidium Bromide staining.

PCR primers and reagents

All primers and probes were purchased from Microsynth at desalted purification grade for primers and PAGE purification grade for probes (see Table 2 for specifications). In the real-time PCR reactions, *Power* SYBR®Green PCR Master Mix (Part Number 4367659; Applied Biosystems) or TaqMan® PCR Core Reagents (Part Number 4304439; Applied Biosystems) and 10X TaqMan buffer A (Part Number P000089; Applied Biosystems) was used.

Real-time PCR analysis

The TaqMan® real-time PCR method CRL-VL-25/04VR validated by the EU-RL GMFF was applied for the detection of material derived from genetically MON 810 maize. A 25 µL reaction volume was applied containing 5 µL of template DNA, 10X TaqMan buffer A (Part Number P000089; Applied Biosystems), 200 µM dNTPs, 1.25 U/rx AmpliTaq Gold (Applied Biosystems), 0.5 U/rnx Amperase and 300 nM of each primer. The thermal program consisted of a single cycle of Amperase activation for 2 min at 50°C followed by DNA polymerase activation for 10 min at 95°C, and by 45 amplification cycles of 15 sec at 95°C (denaturing step) and 1 min at 60°C (annealing-extension step).

SYBR®Green PCR analyses were performed according to Mbongolo Mbella *et al*. (2011) with minor adaptations. For all PCR analyses, a standard 25 µL reaction volume was applied containing 1 µL of template DNA, 1X *Power* SYBR®Green PCR Master Mix (Part Number 4367659; Applied Biosystems), and 250 nM of each primer (Table 2), except 1600 nM for VPRCP1 and 1200 nM VPRBCP2. The thermal program consisted of a single cycle of DNA polymerase activation for 10 min at 95°C, followed by 50 amplification cycles of 15 sec at 95°C (denaturing step) and 1 min at 60°C

(annealing-extension step). After completion of the run, a melting curve analysis was performed by stepwise temperature increase (± 1.75°C/min) from 60°C to 95°C.

In both the SYBR®Green and the TaqMan® PCR analyses, the threshold cycle (C_t) for each sample was calculated in automatic mode according to manufacturer's specifications. If considered necessary (e.g. due to highly diverging baseline values close to the exponential phase), the threshold and baseline were adjusted manually.

Inhibition/Interference of real-time PCR analysis in DNA extracts

Four series of 1:4, 1:16, 1:64, and 1:256 dilutions of total DNA extracted from MON 810 control material and MON 810 pollen-spiked honey were prepared and analysed by real-time PCR using the above indicated *hmg* and MON 810 real-time PCR methods. The slope of the linear correlation between log copy-numbers versus C_t-value is a measure of the amplification efficiency of a PCR method. The relative ratio of the slopes of the above correlation for two PCR methods on the same DNA sample or the same PCR method on different DNA samples is then a measure of the inhibition/interference encountered by the method(s) in the amplification of their respective target(s).

3. Experimental design

The EU-RL GMFF pursued two approaches in the assessment of an extraction procedure for isolation of genomic DNA from pollen present in honey. On the one hand, honey samples were prepared by spiking commercially available honey with various levels of pollen collected from GM maize MON 810. On the other hand, commercial honey samples with an indicated pollen content description were tested for the presence of pollen from several major crops, in particular maize, soybean, rapeseed, sugar beet and cotton.

The extraction of genomic DNA of pollen in honey consists essentially of a two-step procedure: firstly, the pollen fraction of liquefied honey is enriched and collected by filtration and, secondly, total DNA from the collected pollen fraction is isolated by a

CTAB-based DNA extraction (eventually followed by a column-purification step in case inhibiting/interfering substances would still be present).

4. Method

A detailed description of the extraction method for genomic DNA of pollen in honey samples is given in Annex I.

The criteria set for the extraction protocol to be satisfactory were as follows:

- detect GM MON 810 pollen at spiking levels of 0.9% GM MON 810 material in a 0.1% pollen mass fraction (meaning 90 µg or ± 450 GM MON 810 pollen in 10 mg or 50.000 total maize pollen spiked in a 10 g honey sample);
- the extracted genomic DNA should contain no or only low levels of PCR inhibiting activity (<20% deviation in PCR efficiency, calculated as the ratio of their respective efficiencies using sample or reference DNA as template DNA)
- detect GM crop-specific markers by validated real-time PCR methods in commercial honey samples**.**

Notes:
- Mass pollen amount in honey is variable but in general reported to be very low (less than 0.1% of the total mass thus less than 10 mg total pollen per 10 g of honey). The quantity of pollen of a particular species is dependent on various factors, e.g. the season, the flora in the neighbourhood of the beehive(s).
- For the quantification of GM material in honey, the criteria set for GMO quantification by the ENGL can be applied. No specific assessment was made for GMO quantification in this study.

5. Results

5.1. Extraction and analysis of genomic DNA from honey spiked with pollen from genetically modified maize MON 810

Extraction of genomic DNA from GM pollen spiked into various honey samples was performed using the procedure described in Annex 1.
The measured quantities of extracted total DNA reflects the amounts of spiked material (see Table 3.a). The inhibition was verified for the SP0311_HM-BIH-01 sample and estimated lower than 10% for both samples (see Table 3.b). Also the experiments with the 1% GM pollen control samples demonstrated that the approach allowed detecting very low levels of GM material in honey (125 GM maize pollen grains were detected 4/4 in spiked honey samples with an estimation of the GM% at 0.79% assuming maximal efficiency for both PCR methods (see Table 4 a & b).

Thus, in all spiked honey samples, the presence of GM MON 810 DNA could be demonstrated. The relative GM % in the recovered total DNA was estimated based on the ratio of the average C_t-values obtained for the MON 810 target and the *hmg* maize reference gene. In most cases, the calculated relative ratio correlated well with the levels of GM material spiked in the honey samples. No further analyses were performed for quantifying the GM pollen in the materials.

Finally, the occurrence of non-plant DNA in the DNA extracts was evaluated by the presence of honey bee DNA in the extracts. Using a qualitative PCR method specific for a honey bee marker, Cytochrome c oxidase subunit I (COI) (Bærholm *et al*, 2010), positive signals could be obtained in several samples (see Figure 1).

5.2. DNA extraction and analysis of DNA extracts from pollen fractions from commercial honey for the presence of soy, maize, rapeseed, cotton and sugar beet

Total genomic DNA extraction from the pollen fraction isolated from honey samples obtained from various sources was performed using the same procedure described in Annex 1.

The respective DNA yields obtained from these honeys varied considerably ranging from 5 ng/uL to 35 ng/uL (with a total extraction volume of 60 uL).

From most honey samples purchased from the retailer market, information on the expected pollen content was available (see Table 1). Real-time PCR analyses for the presence of crop-specific markers for soy, maize, rapeseed, cotton and sugar beet were performed. These analyses confirmed the presence of pollen DNA from these crops in the respective extracts of the pollen fractions (see Table 5). In general, rapeseed honey contained high amounts of rapeseed pollen: only low to very low levels of soy or maize pollen, though at PCR detectable levels, were found in a number of samples. No trace of cotton or sugar beet DNA was found (data not shown).

6. Conclusions

- The honey-pollen extraction protocol developed at the EU-RL GMFF is suitable to reproducibly extract PCR-grade DNA from genetically modified maize pollen present in honey samples. A schematic presentation of the different steps in the protocol is shown in Figure 2.
- The lowest spiked amounts tested in this study are 5 mg 1% GM pollen in 10 g honey corresponding to 119 GM maize pollen grains (taking 0.21 ųg as the average weight of a maize pollen grain). Such level was reproducibly detected using the EU-RL GMFF protocol and the EU-RL GMFF validated real-time PCR methods.
- In honey samples purchased from the retailer market, the presence of maize, rapeseed and soy pollen could be demonstrated when applying the EU-RL GMFF protocol combined with EU-RL GMFF validated/verified real-time PCR methods.
- Taking into account the average content of total pollen and maize pollen in real honey samples (Stawiarz and Wrolewska, 2010), it is foreseeable that the protocol presented in this report is suitable for the detection of GM pollen in honey.

7. Quality assurance

The EU-RL GMFF carries out all operations according to ISO 9001:2008 (certificate number: CH-32232) and all technical activities regarding method validation under Regulation (EC) 1981/2006 according to ISO 17025:2005 (certificate number: ACCREDIA 1172, [Flexible Scope for DNA extraction and qualitative /quantitative PCR] - Reference Methods available at the Accredia WebSite under: http://www.accredia.it/accredia_labsearch.jsp?ID_LINK=293&area=7).

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Table 1: Overview of the honey samples applied in this study (including the country of origin of sample (as stated by manufacturer), the period of harvest and the organoleptic characteristics)

Sample name	Country of origin	Produced by	Period of Harvest	Honey type	Colour	Physical state
SP0311_HM-ROM-01	Romania	Private production	June 2011	Polyfloral	pale yellow	Liquid
SP0311_HM-HU-01	Hungary	Private production	September 2011	Polyfloral	amber	Liquid
SP0311_HM-BIH-01	Bosnia and Herzegovina	Private production	May-August 2011	Polyfloral	light beige	Crystallized
SP0311_HM-ROM-02	Romania	Private production	June 2011	Monofloral (Tilia)	bright yellow	Liquid
SP0311_HM-ROM-03	Romania	Private production	September 2011	Monofloral (Rape)	white	Crystallized
SP0311_HM-HU-02	Hungary	Private production	September 2011	Monofloral (Rape)	white	Crystallized
SP0311_HM-ITHU-01	Italy, Hungary	G.B. Ambrosoli S.p.A , Ronago (CO) IT	Not indicated	Polyfloral	light brown	Liquid
SP0311_HM-ITHUARG-01	Argentina, Italy, Hungary	Carrefour, Milano IT	Not indicated	Polyfloral	light brown	Liquid
SP0311_HM-ITHUARG-02	Argentina, Italy, Hungary	Cavallo srl, Zafferana Etnea (CT) IT	Not indicated	Polyfloral	light beige	Crystallized
SP0311_HM-FR-01	France	Famille Michaud for Luna di Miele srl, Milano, IT	Not indicated	Monofloral (Acacia)	very pale yellow	Liquid

Table 2: Description of the respective target genetic elements, their corresponding primer sequences, and the molecular weight of the amplicons generated with the different PCR methods used in this study.

Species	Targeted genetic element	Detection Chemistry	Primer Name	Primer sequence	Amplicon size [bp]	Specificity	Reference
Maize	High mobility gene	TaqMan	ZM1-F	TTGGACTAGAAATCTCGTGCTGA	79	Maize	EU-RL GMFF
			ZM1-R	GCTACATAGGGAGCCTTGTCCT			
			Probe ZM1	FAM-CAATCCACACAA ACGCACGCGTA-TAMRA			
Maize	MON 810	TaqMan	Mail-F1	TCGAAGGACGAAGGACTCTAACGT	92	Mon 810	EU-RL GMFF
			Mail-R1	GCCACCTTCCTTTTCCACTATCTT			
			Probe Mail-S2	FAM-AACATCCTTTGCCATTGCCCAGC-TAMRA			
Soybean	Lectin	SYBR®Green	sltm 1	AACCGGTAGCGTTGCCAG	81	Soy	Vaïtilingom, 1999
			sltm2	AGCCCATCTGCAAGCCTTT			
Maize	Alcohol dehydrogenase 1	SYBR®Green	ADH_alt Fwd	TCTCTTCCTCCTTTAGAGCTACCACTA	83	Maize specific	Mbongolo Mbella et al, 2011
			ADH_alt Rev	AATCGATCCAAAGCGAGATGA			
OSR	Cruciferin	SYBR®Green	Cru770 F	CAGCTCAACAGTTTCCAAACGA	85	OSR specific	Mbongolo Mbella et al, 2011
			Cru770 R	CGACCAGCCTCAGCCTTAAG			
Cotton	Stearoyl-ACP desaturase	SYBR®Green	S1F	CCAAAGGAGGTGCCTGTTCA	107	Cotton specific	Yang et al, 2005
			S2R	TTGAGGTGAGTCAGAATGTTGTTC			
Sugar beet	Glutamine synthetase	SYBR®Green	GluA3-F	GACCTCCATATTACTGAAAGGAAG	118	Sugar beet specific	H7-1 CRL validation, 2006
			GluA3-R	GAGTAATTGCTCCATCCTGTTCA			
Plant Kingdom	Ribulose-1,5-bisphosphate carboxylase oxygenase	SYBR®Green	VPRBCP1	AGGTCTAADGGRTAAGCTAC	95	general plant primer	Debode et al, 2004
			VPRBCP2	AGYCTTGATCGTTACAAAGG			
Apis spp	Cytochrome c oxidase subunit I	Agarose	XSF	CCCCAGGATCATGAATTAGC	116	*Apis* spp specific	Bærholm et al, 2010

Table 3: Assessment of the extraction of genomic DNA from GM pollen spiked in honey.

3.A: Overview of the results on the extraction of genomic DNA from different levels of GM MON 810 pollen spiked in different honeys.

Honey	Spike	# extractions	DNA yield ng/ul	% PCR inhibition	Measured Ct-value HMG	Measured Ct-value MON 810	Conclusion
SP0311_HM-BIH-01	450 mg maize pollen (50% GM) in 50g honey	4	202,2 ± 66	acceptable	26,72±0,4	28,57±0,4	GMO detected at lower estimated GM ratio
SP0311_HM-BIH-01	50 mg maize pollen (50% GM) in 50g honey	4	9,45 ±1,73	not tested	26,19±0,4	27,79±0,28	GMO detected at correct estimated GM ratio
SP0311_HM-FR-01	100 mg maize pollen (0.5% GM) in 10g honey	4	12,12± 3,15	not tested	30,55±0,62	37,95±0,72	GMO detected at lower estimated GM ratio
SP0311_HM-FR-01	100 mg maize pollen (5% GM) in 10g honey	1	34,7	not tested	24,58±0,008	29,32±0,09	GMO detected at correct estimated GM ratio
SP0311_HM-FR-01	10 mg maize pollen (5% GM) in 10g honey	1	3,4	not tested	29,86± 0,08	34,24± 0,22	GMO detected at correct estimated GM ratio
SP0311_HM-ITHU-01	100 mg maize pollen (0,1%GM) in 10g honey	1	7,3	not tested	27,94± 0,4	36,82±0,09	GMO detected at correct estimated GM ratio
SP0311_HM-FR-01	8 mg maize pollen (50% GM) in 10g honey	1	3,5	not tested	36,2±0,69	37,68±1,01	GMO detected at correct estimated GM ratio
SP0311_HM-FR-01	6 mg maize pollen (50% GM) in 10g honey	1	3,5	not tested	37,01±1,77	38,67±0,8	GMO detected at correct estimated GM ratio
SP0311_HM-FR-01	3 mg maize pollen (50% GM) in 10g honey	1	2,6	not tested	32,9±0,4	35,46±0,28	GMO detected at lower estimated GM ratio

3.B: Efficiency comparison and inhibition/interference assessment of MON 810 PCR methods applied on total DNA extracted from control material and the pollen-spiked honey sample SP0311_HM-BIH-01.

SP0311_HM-BIH-01 0,9% E1	**Pollen**	**Control**	**% Inhibition or interference**
HMG	-3,4606	-3,4985	**1,09%**
MON 810	-3,7162	-3,5301	**5,01%**
Efficiency deviation	**7,38%**	**0,90%**	

Table 4: Overview of the analysis of honey sample spiked with GM maize pollen sample. In Table 4.A the results of the extraction and real-time PCR analysis of the respective GM pollen samples are summarized. In Table 4.B 5 mg 1.0% GM maize pollen sample were spiked in 10g honey and analysed in a similar way.

A: Analysis of 10 mg the 0.1% and 1.0 %GM pollen samples.

Pollen	# extractions	DNA yield ng/ul	Measured Ct-value HMG	Measured Ct-value MON 810	Positives	Conclusion
SP0311_PollenMix 0,1%	4	4,7 ± 1,68	27,25±0,5	-	0/3	GMO detected at lower estimated GM ratio
				38,76	1/3	
				39±0,5	2/3	
				36,64±0,41	3/3	
SP0311_PollenMix 1%	4	5,7 ±1,03	27,13±0,2	34,57±1	3/3	GMO detected at correct estimated GM ratio
					3/3	
					3/3	
					3/3	

B: SP0311_HM-ROM 02 honey (10 g) spiked with 5 mg SP0311_PollenMix 1% GM pollen sample.

Sample	# extractions	DNA yield ng/ul	Measured Ct-value HMG	Measured Ct-value MON 810	Positives	Conclusion
SP0311_HM-ROM 1%	4	2,42 ± 0,6	30,36±0,3	37,9±1,55	3/3	GMO detected at an acceptable estimated GM ratio
				36,1±0,3	3/3	
				38,5±1,05	2/3	
				36,73±0,21	3/3	

Table 5: Analysis of natural and commercial honey samples for the presence of pollen from field crops. Extracted DNA was dissolved in a final volume of 60 µl. In all samples, 1 µl of extract were analysed in duplicate.

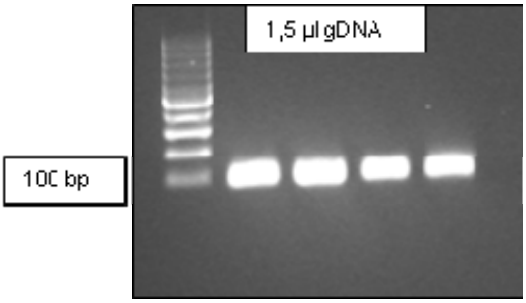
Honey	Detection Method	Tested Species	# extractions	DNA yield ng/ul	Measured Ct-value	Measured T_m-value*	Remarks**
SP0311_HM-BIH-01	Alcohol dehydrogenase 1 SYBR®Green	Maize	4	4,73±1,91	36,09 ±0,29	75,8	Low amounts of maize pollen detected
	Cruciferin SYBR®Green	Rapeseed			32,52±0,2	81,1	Low amounts of rapeseed pollen detected
SP0311_HM-HU-02	Cruciferin SYBR®Green	Rapeseed	2	23,3±2,5	22,63±0,06	80,7	High amounts of rapeseed pollen detected
SP0311_HM-HU-01	Cruciferin SYBR®Green	Rapeseed	2	15,5±0,5	27,68±0,72	80,3	Moderate amounts of rapeseed pollen detected
SP0311_HM-ROM 03	Cruciferin SYBR®Green	Rapeseed	2	36,5±2,8	22,69±0,1	80,6	High amounts of rapeseed pollen detected
SP0311_HM-ROM 01	Alcohol dehydrogenase 1 SYBR®Green	Maize	2	1,1	37,19±0,24	75,9	Low amounts of maize pollen detected
	Cruciferin SYBR®Green	Rapeseed			36,62±2,08	80,3	Low amounts of rapeseed pollen detected
SP0311_HM-ROM 02	Cruciferin SYBR®Green	Rapeseed	2	2,3±1,7	29,7±0,2	81	Moderate amounts of rapeseed pollen detected
SP311_HM-FR-01	Cruciferin SYBR®Green	Rapeseed	2	5,45±3	30,44±0,2	81	Moderate amounts of rapeseed pollen detected
SP0311_HM-ITHUARG-01	Alcohol dehydrogenase 1 SYBR®Green	Maize	2	15,4±0,1	39,32 ±4,28	75,9	Trace amounts of maize pollen detected
	Cruciferin SYBR®Green	Rapeseed			29,44±0,21	80,6	Moderate amounts of rapeseed pollen detected
	Lectin SYBR®Green	Soy			35,06±0,74	80,6	Low amounts of soy pollen detected
SP0311_HM-ITHUARG-02	Alcohol dehydrogenase 1 SYBR®Green	Maize	2	8,25±1,25	38,30±1,69	76	Trace amounts of maize pollen detected
	Cruciferin SYBR®Green	Rapeseed			27,70±0,4	80,6	Moderate amounts of rapeseed pollen detected
SP0311_HM-ITHU-01	Cruciferin SYBR®Green	Rapeseed	2	7,05±1,48	32,40±0,33	80,7	Moderate amounts of rapeseed pollen detected

* The T_m-values for the respective targets in Reference Materials were as follows: "Alcohol dehydrogenase 1" in maize leaf material: T_m= 75,9; "Cruciferin" in rapeseed reference material T_m= 80,8; "Lectin" in soy reference material: T_m= 80,6.

** The quantitative estimation were set according to the measured average C_t-values as follows: "High"=C_t <25; Moderate: C_t-value between 25 but below 31; Low: C_t -value between 31 and 38; Trace: C_t -value > 38.

Figure 1: Agarose electrophoresis of rbcL and COI-*Apis* spp amplification products obtained with genomic DNA of honey samples as template. (Outer left column: Molecular size marker (Gene Ruler™ 100bp Plus DNA ladder); next lanes show the amplification products with template DNA extracted from honeys SP0311_HM-BIH-01, SP0311_HM-HU-02, SP0311_HM-ROM-01 and SP0311_HM-ITHUARG-01, respectively)

rbcL-plant



COI-*Apis* spp

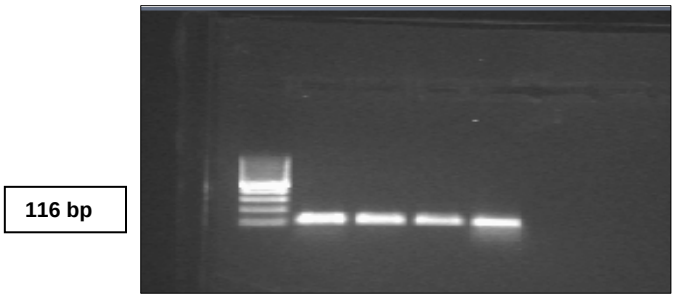
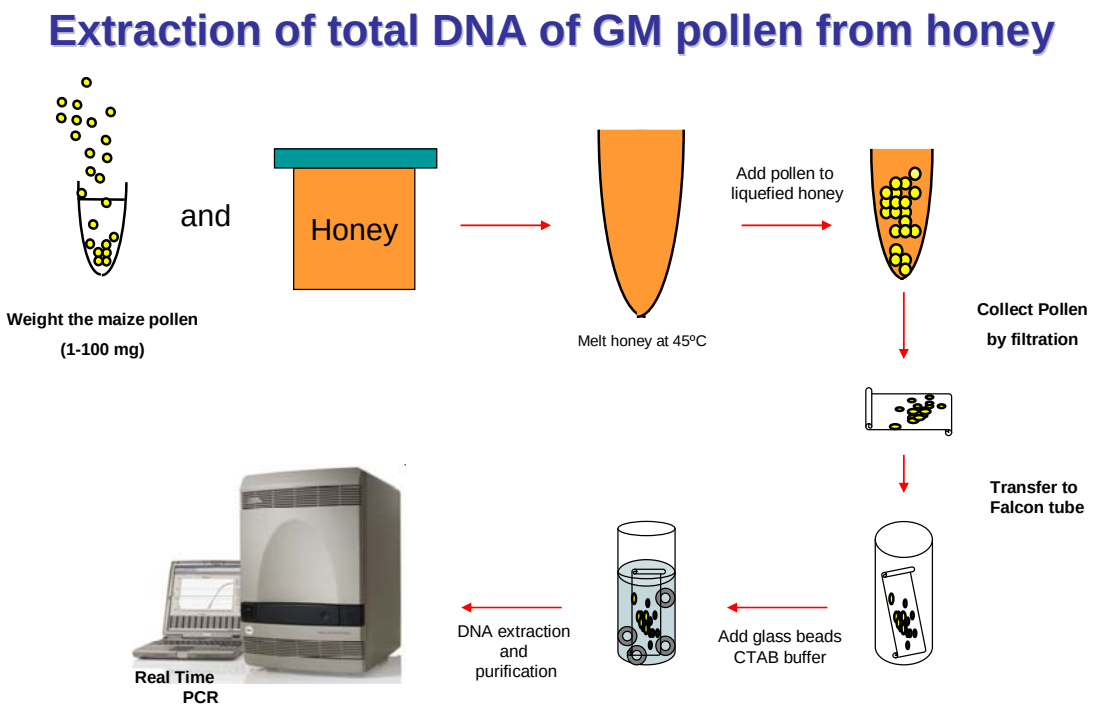


Figure 2: Overview of the extraction procedure of genomic DNA of spiked pollen in honey.



European Commission - Directorate General Joint Research Centre

Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit

Standard Operating Procedure
DNA Extraction from honey and pollen – CTAB

Revision	Author	Reviewer	QS Agreement	Approver
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Document history			
Revision	Date	Drafted by	Comments
	05/07/2012	I. Matetovici	Creation of the document

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1 Object

This method is used to extract genomic DNA from maize, soy, rapeseed pollen present in honey.

2 Applicability domains

The method for DNA extraction described below is suitable for the isolation of genomic DNA from maize, soy, rapeseed pollen in honey. Application of the method to other substances may require adaptation and possible further specific optimatisation.

3 Reference documents

Folloni S., Kagkli D.M., Rajcevic B. Guimarães N., Valicente F., Van Droogenbroeck B., Van den Eede G. Van den Bulcke M. Detection of airborne genetically modified maize pollen by real-time PCR (*in press*)

4. Abbreviations used

CTAB**:** Cetyltrimethylammonium Bromide
DNA: Deoxyribonucleic acid
EDTA: Ethylenediaminetetraacetic acid
RNase: Ribonuclease
Tris: Tris[hydroxymethyl] aminomethane

5. Equipment

Water bath or heating block
12µM membrane filter (cellulose nitrate)
Vacuum filtration system (Sartorius)
Microcentrifuge (Eppendorf or equivalent)
Micropipettes
Vortexer
Microcentrifuge tubes
Rack for reaction tube
Vinyl or later gloves
Vacuum device
Mini-shaker IKA (or equivalent)
1 mm glass beads

NOTE:
Plastic ware has to be sterile and free of DNAses, RNases and nucleic acids. Filter pipette tips protected against aerosol should be used.

6. Reagents

2% CTAB- buffer
Chloroform (99% at least)
Isopropanol (99, 7 % at least)
Ethanol (96% at least)
Ethanol-solution 70% (v/v)
Proteinase K solution 20 mg/ml
RNase A solution 0.5 mg/ml
Sterile molecular biology grade water

7. Procedure

PRELIMINARY REMARKS:

- The procedure requires sterile conditions. Contamination may be avoided during sample preparation by using single-use equipment, decontamination solutions and by avoiding the formation of dust.

- During all steps of DNA preparation, wear gloves and change gloves regularly.

- Avoid extensive vortexing of genomic DNA.

Step 1: Honey solubilisation and pollen fraction collection

1) Heat the honey jar at 40-50 °C until the matrix becomes fluid.

2) Weight the desired amount of honey to a proper recipient (e.g. a 50 ml Falcon tube when analysing 10g of honey)

3) Add 4 volumes of preheated (40-50 °C) sterile deionised water.

3) Put the samples on an agitator and rotate them until the honey is completely dissolved.

4) After the honey is completely dissolved filter the wet solution on a 12µM filter using a Vacuum filtration system (Sartorius) or equivalent.

5) Transfer the filters into an appropriate recipient (e.g. a 15 ml Falcon tube).

Notes:

- When using large amounts of honey, the 12µM filter may become saturated with material and filtering hampered. Take the filter our of the filter unit, transfer to the Falcon tube and replace by a new 12µM filter and continue the filtration. This handling can be repeated until the complete honey solution has been filtered.

- Honey contains in general low amounts of pollen (in the range of 1000 pollen grains/gram honey). A suitable amount for analysis may be 10-50g, although larger amounts may be required (e.g. 500g).

- Filters with pollen material can be stored for short periods at 4ºC. Storage for longer periods is to be avoided but if the case, air-dried filters can be stored in a Falcon tube at minus 20 ºC

Step II: Isolation and Purification of genomic DNA of collected pollen fraction

1) Take the filter(s) in the Falcon tube

2) Add 1 ml of molecular biology grade water

3) Add 1 volume of glass beads (1 mm glass beads)

4) Add 2 ml of 2% CTAB-buffer

5) Shake at maximum strength using a mini-shaker IKA (or equivalent) to disrupt the pollen grains; first shake for about 1 min, and then allow settling for about 30 sec on ice and finally shake again for 2 min

6) Add 50 µl Proteinase K and incubate with agitation at least 2 hours (or over night) at 60° C

7) Add 1 µl of RNase and incubate 30 min at room temperature

8) Centrifuge for 15 min at 5000 *x* g

9) Transfer the supernatant in 1 ml fractions to a 2 ml Eppendorf tube and add 900 µl of chloroform (or repeat in different tubes until the supernatant is completely collected)

10) Mix for 30 sec and centrifuge at 12000 *x* g for 10 min

11) Transfer the aqueous phase to a new tube and add 0.8 volume of Isopropanol

12) Incubate for 30 min at room temp

13) Centrifuge at 12000 *x* g for 10 min

14) Discard the supernatant

15) Wash the pellet with 1 ml of ethanol 70%

16) Mix by inverting, centrifuge at 12000 *x* g for 5 min

17) Discard the supernatant and repeat the washing step

18) Dry the pellet (room temp or concentrator)

20) Re-dissolve the DNA in 60 µl sterile molecular biology grade water. Add the water in the first tube and resuspend the DNA, then take the obtained solution and added to the next tube until all the tubes are pooled.

Optional
21) Purify the DNA samples using the Genomic DNA Clean& Concentrator™ kit (Zymo Research) according with manufacturers' instructions.

8. Procedure Buffer preparation and required reagents

The following reagents/buffers/solutions are required for the DNA extraction procedure:

8.1 Equipment

Weighting equipment
pH meter
Heating stirrer
Autoclave
Pipettes and micropipettes

8.2 Reagents

CTAB**:** Cetyltrimethylammonium Bromide (Ultrapure grade)
Tris: Tris[hydroxymethyl] aminomethane (Molecular Biology grade) or 1 M Tris-HCl pH 8.0
EDTA: Ethylenediaminetetraacetic acid (titration 99.0%)
Ethanol (98% at least)
NaCl (99% at least)
NaOH (98% at least, anhydrous)
Sterile molecular biology grade water

8.3 Formulations

2% CTAB-buffer

20 g/l CTAB……...4 g
1.4 M NaCl……16.4 g
0.1 M Tris*……3.15 g
20 mM Na_2EDTA……1.5 g

* Alternatively 20 ml of 1M ready-to-use solution of Tris-HCl pH 8.0 can be used.
Preparation:

a. add 100 ml distilled water
b. adjust pH to a value of 8.0 with 1M NaOH
c. fill up to 200 ml and filter sterilise
d. store buffer at 4°C for max. 6 months.

NaOH 1 M

Dissolve 2 g of NaOH in 50 ml sterile water

Ethanol-solution 70% (v/v)

70 ml of pure ethanol are mixed with 30 ml of distilled sterile water.

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Authors: Marc Van den Bulcke, Irina Matetovici, Marco Mazzara, Guy Van den Eede, Joachim Kreysa

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Abstract

Following the judgment of 06 September 2011 on GM honey by the European Court of Justice (legal case C-442-09), the European Union Reference Laboratory for Genetically Modified Food and Feed (EU-RL GMFF) established by Regulation (EC) No 1829/2003, performed an in-house study to test the extraction and PCR analysis of genomic DNA from genetically modified pollen in honey.

The present report documents on an extraction method for isolation and analysis of pollen DNA present in honey, including the isolation and analysis of isolated genomic pollen DNA using real-time PCR on commercial honey samples and honey samples spiked with various levels of GM MON 810 pollen.

LB-NA-25524-EN-N

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