



Digital PCR for analysis of peanut and soybean allergens in foods

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ARTICLE INFO

Article history:

Received 19 February 2018

Received in revised form

19 April 2018

Accepted 20 April 2018

Available online 23 April 2018

1. Introduction

The food allergies are a relevant problem of public health for severity and prevalence affecting over 220 million people worldwide (Manea, Ailenei, & Deleanu, 2016). International recognition of food allergy, needs to provide information to protect sensitive consumers resulted in different regulatory frameworks around the world for labeling of priority allergens. Peanut and soybean are members of the leguminous plant family and may elicit severe reactions in sensitized person. Peanut is one of the four foods common to all list of priority allergens present in International Regulatory Chart (FARPP, 2018; Gendel, 2012). On the other hand, the soybean is widely used in the food industry for its multiple functional properties. In Europe, according to Regulation (EU) No 1169/2011, labeling is mandatory for soybean and peanut, among others, when used as ingredients in the recipe of non- and packaged foods. Furthermore, the allergen can be present in food as hidden allergens (undeclared allergens) as the result of an unintentional cross-contact contamination. In Europe the precautionary allergen labelling (PAL) is voluntary and used to limit risk of allergy to the possible unintended allergen presence (UAP) (Bucchini, Guzzon, Poms, & Senyuva, 2016; DunnGalvin et al., 2015; Remington et al., 2015). To assure consumer safety is needed the control of labeling compliance as well as of industrial management with specific and sensitive analytical methods. The available approaches for detection and quantification of food allergens (FA) are proteomics, immunological and DNA-based tests,

such as mass spectrometry (MS), enzyme-linked immunosorbent assay (ELISA) and lateral flow devices, polymerase chain reaction (PCR), respectively (Poms, Klein, & Anklam, 2004; Monaci and Visconti, 2010; Koeberl, Clarke, & Lopata, 2014; De la Cruz et al., 2017). The mentioned methods are most commonly used in allergen analysis, but each of these exhibits merits and drawbacks. Mass spectrometry (MS) has received much attention as a potential confirmatory method because it is a direct specific quantitative method and it can detect multiple allergens in the same analysis (Heick, Fischer, & Pöpping, 2011; Johnson et al., 2011; Pilolli, De Angelis, & Monaci, 2017; Planque et al., 2017). However, quantification of allergens using MS requires a high level of expertise and costly equipment, unavailable in many laboratories. ELISA is the most widely used technique in routine analysis, because it presents a high sensitivity, low costs, ease of use and speed but it shows disadvantages in complex or processed food matrices. Indeed, many processing conditions such as the length of time of heat treatment, pH and sugar reduction may affect the structure of the proteins and reduce their solubility, with impacts on the extraction efficiency and specificity (Iqbal et al., 2016; Pilolli et al., 2017; Török et al., 2015). The PCR, especially real-time PCR, is a very specific and sensitive tool, available in many laboratories for several applications, but it is an indirect method in allergens analysis, as it detects DNA of allergenic proteins or other genes, instead of proteins. However, the European legislation imposes labeling indications for the presence of certain substances or products causing allergies and intolerances (Regulation (EU) No 1169/2011) but not necessarily the allergenic proteins, except for possibly gluten content (Commission Implementing Regulation (EU) No 828/2014). Several PCR protocols have been published and are available (De la Cruz et al., 2017) for many food allergens, while the method does not distinguish the animal food product, such as egg and milk, from the animal species, as chicken and beef, and so the PCR method is not suitable for the detection of these two allergens. Nevertheless, PCR has some benefits that allowed its introduction as an official analytical method in Germany and Japan (BVL L44.00-11, 2013; Sakai, Adachi, Akiyama, & Teshima, 2013). The conventional or classical PCR provides qualitative results of end-point amplification product by gel electrophoresis. With the advent of the second-generation PCR or real-time PCR

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(qPCR), monitoring amplification progression and using fluorescence probes, the method begins more expensive, but is less laborious and more specific and sensitive, and enables to quantify nucleic acids using a calibration curve (Raso & Biassoni, 2014). In recent years, last generation of PCR, or digital PCR (dPCR) has been applied in many fields (Dreo et al., 2014; Hall Sedlak & Jerome, 2014; Huggett et al., 2013; Košir, Spilberg, Holst-Jensen, Zel, & Dobnik, 2017; Sanders, Mason, Foy, & Huggett, 2013), even though the concept was defined in the 1990s (Sykes et al., 1992; Vogelstein & Kinzler, 1999). The working principle of digital PCR is based on fractionated PCR by dispersal of reaction volume with sample in many droplets or cavity as separate reactions and counting positive and negative partitions. Therefore, this new technology combines the advantages of real-time PCR with a partitioned counting, providing precise and accurate quantification of nucleic acids without the need of a standard curve, especially for low concentration samples and complex samples, reducing susceptibility to inhibitors (Huggett et al., 2013). Moreover digital PCR is suitable for multiplex analysis because the systems do not compete against the others (Köppel & Bucher, 2015). As mentioned, many studies about detection of food allergens were focused on qPCR results but there are no available data on dPCR. This work was directed mainly to verify the suitability and applicability of droplet digital PCR (ddPCR) to food allergens detection, focusing on peanut and soybean allergens. The first step of our study was the evaluation of optimal test portion (1 g and 2 g) and then the assessment and validation of the best extraction and purification methods from food matrices that is one of the most critical issue to improve sensitivity of DNA-based methods. Starting from published results (Hird, Lloyd, Goodier, Brown, & Reece, 2003; Scaravelli, Brohée, Marchelli, & Van Hengel, 2008), and from our experience on official control analysis for GMO, two different DNA extraction methods based on CTAB and guanidine hydrochloride buffers were combined with four commercial kits for DNA isolation and/or purification. Then, the target sequences and the qPCR methods were selected. Four recently published and unpublished qPCR methods for both peanut and soybean were tested for specificity, limit of detection, robustness, efficiency and linearity in fast mode (JRC95544, 2015, p. 24; Pierboni, Curcio, Tovo, Torricelli, & Rondini, 2016). One qPCR method for peanut and two for soybean were selected and applied in ddPCR, given that a qPCR assay validated can be transferred to dPCR for GMO analysis (Dobnik et al., 2018). To check the reliability of selected methods for detection and quantification of peanut and soybean allergens, market samples were tested by ELISA kits, in-house qPCR, qPCR kits and ddPCR.

This study reports on the first time an evaluation of digital PCR use for the detection of allergens in foods, with the aim to produce a greater contribution for the food safety of allergic populations. The objectives of the paper were to evaluate the applicability of digital PCR to detect and quantify the amount of allergens, and to improve analytical sensitivity to allergens detection, to develop in future a multi-screening analysis for detecting allergens in food.

2. Material and methods

2.1. Samples

Soybean flour, roasted peanuts and other market samples were purchased in a local supermarket.

Leaves of fruits and vegetables were collected and ground under liquid nitrogen, using mortar and pestle. If necessary, the other samples were milled with Grindomix GM200 (Retsch, Haan, Germany); to high fat products, such as peanut, tree nut and snack, dry ice was added during grinding to prevent melting and clumping.

The ground samples were homogenized with plastic bag technique (EUR 27021 EN, 2014). DNA of animals were supplied from other laboratory of our Institute.

2.2. Reference materials

Maize (AOCS 0406-A), rice (AOCS 0306-D2), canola (AOCS 0306-B2), sugar beet (AOCS 1206-A) and cotton (AOCS 0306-A) were purchased from AOCS (American Oil Chemists' Society), and potato (ERM-BF421a), was obtained from IRMM (Institute for Reference Materials and Measurements).

SureFood® QUANTARD Allergen 40 (R-Biopharm, CONGEN Biotechnologie GmbH, Germany), Allergen RM 800 (BIOTECON Diagnostics, GmbH Hermannswerder, Germany) and standard material IRMM-481 Peanut Test Material (IRMM, Belgium) were also used.

2.3. DNA extraction and purification

Eight DNA extraction methods were performed combining CTAB and guanidine hydrochloride based extraction buffer (ISO 21571:2005/Amd.1:2013; Germini et al., 2005) with four commercial kits for DNA purification, following the manufacturer instructions for the isolation of peanut and soybean DNA: (I) NucleoSpin® gDNA Clean-up (Macherey-Nagel); (II) SureFood® Prep Advanced (R-Biopharm); (III) Wizard® DNA Clean up System (Promega); (IV) Foodproof® Sample Preparation Kit III (Biotecon Diagnostics). Each method was tested against two different test portions of 1 g and 2 g for soybean and roasted peanut. Two hundred mg of sample were extracted, for the products grinded with liquid nitrogen and for the reference materials, and 2 g, for all the other products. Samples were extracted in duplicate and sterile distilled water instead of sample, was used as a negative extraction control (ISO 24276:2006/Amd.1:2013). The selected method among those tested above, was called method a. The method a was modified in method b by removing isopropanol and ethanol 70% steps from ISO 21571:2005/Amd.1:2013 protocol. The method b was validated in three different days, performing six replicated extractions of roasted peanut and soybean flour each day. All DNA extracts were serially diluted to 1:4, 1:6, 1:64, 1:256 (JRC95544, 2015, p. 24). The reference materials were also extracted with commercial kit II and IV in agreement with instructions. The concentration of extracted nucleic acids was determined photometrically or fluorimetrically using BioSpectrometer® fluorescence (Eppendorf®, Hamburg, Germany) and Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific) for fluorimetry. The DNA quantity was also indicated by the Cq value; a maximum Cq difference of 0.5 between replicates was used as a limit of acceptability of data repeatability (De Ronde et al., 2017). The quality of DNA samples was verified by an inhibition test (Waublinger & Grohmann, 2014; EUR 29015 EN, 2017, p. 38) using fast real-time PCR assay targeting the *actin* gene (Torricelli, Pierboni, Tovo, & Rondini, 2016). The test consisted in analyzing two fast real-time PCR replicates of undiluted DNA and its dilution 1:4 for each extracted sample. In absence of inhibitors, the difference between the measured mean cycle threshold (Cq) of undiluted and diluted DNA (ΔCq) should be of 2, with an acceptability range of 1.5–2.5.

2.4. Real-time PCR

Real-time PCRs were run in fast mode by 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific), using the following cycling conditions: 95 °C for 20 s, 40 cycles with 95 °C for 3 s and 60 °C for 30 s. The reaction volume was 20 µl, with 1X of TaqMan® Fast Universal PCR Master Mix (2X) No AmpErase UNG (Thermo Fisher

Scientific), 2 µL of DNA and primers/probes at concentrations achieved by optimization test. Data analysis was carried out by SDS 2.4 software. Each PCR run included not template control (NTC) and positive amplification control.

2.4.1. Oligonucleotides

The used primers and probes were synthesized by Invitrogen (Thermo Fisher Scientific) and details are reported in Table 1. The new oligonucleotides were designed using Primer Express software v 3.0 (Applied Biosystems®, Foster City, USA).

2.4.2. Optimization of primer and probe concentrations

For the optimization of primer and probe concentrations, about 130 hge (haploid genome equivalents) of peanut and soybean, corresponding to about 0.4 ng and 0.2 ng of respective DNA, were tested. The number of the target sequence copies was calculated by assuming that the haploid genome size (1C) of soybean and peanut were 1.15 pg and 2.92 pg respectively (Arumuganathan & Earle, 1991). Three concentrations of 150, 300 and 900 nM, carrying out nine combinations tested in four replicates, were performed for each set of oligonucleotides.

Primers and probe concentrations were chosen considering the lowest mean quantification cycle (Cq) as an indicator for the best sensitivity, together with the lowest repeatability standard deviation (rSD) as indicator of repeatability and a higher ΔRn value, that represents the normalized reporter fluorescence signal.

2.4.3. Specificity

The specificity of the primer and probe sets was assessed in silico by applying the Blast program on the Nucleotide Collection (nr/nt) sequence database BLAST.

The specificity was checked experimentally testing inclusivity and exclusivity panel of about sixty samples, in duplicate (Supplementary Table 1), using concentrations adjusted on Cq 28–30 when necessary, obtained by actin fast real-time PCR inhibition assay (subsection 2.3).

2.4.4. LOD, amplification efficiency and linearity

The evaluation of limit of detection (LOD) was performed testing 12 dilutions series each in six replicates, repeated in three separated runs (A, B, C) under repeatability conditions, starting from peanut and soybean DNAs extracted in validation test and evaluated with fluorimeter. Last dilution, where all 18 results give amplification, was defined LOD₆ and verified in 60 replicates together upstream or downstream dilution to define LOD₉₅ or LOD of method. The amplification efficiency and linearity were defined respectively by slope and correlation coefficient R² of the regression line obtained with the first five dilutions used in LOD₆ test (Broeders et al., 2014; JRC95544, 2015, p. 24).

2.4.5. Robustness

The target sequence copy numbers tested for robustness corresponds to the LOD value of method multiplied by three (JRC95544, 2015, p. 24).

A multi-factorial experiment design with two PCR runs was performed in triplicate: run 1 for reference method and run 2 carried out on a different thermal cycler, that is 7500 Fast Real-Time PCR System (Applied Biosystems®, Foster City, USA), with different Master mix concentration (−10%), different primer and probe concentrations (−30%), different reaction volume (+1 µL and −1 µL) and with annealing temperature farther from Tm (melting temperature) of primers (Pierboni et al., 2016).

2.4.6. Commercial PCR kits

The designed PCR systems were compared to following two PCR kits for the detection and quantification of peanut and soybean: foodproof® Peanut Detection Kit, foodproof® Soya Detection Kit (both of BIOTECON Diagnostics), SureFood® ALLERGEN ID Peanut, SureFood® ALLERGEN ID Soya (both of R-Biopharm). The commercial assay runs were performed as described in the enclosed protocols.

2.5. Droplet digital PCR

Droplet Digital PCR was performed by mixing 10 µL of 2X

Table 1
Description of primers and probes.

	System Name	Name	Sequence (5'-3')	Amplicon lenght (bp)	Reference	GenBank number
peanut	Arah1-MGB	Arah1-pgF1	TCCCGTCAAGCGGTTTA	54	this work	AF432231
		Arah1-pgR1	CCCCGATCCTACCGTTTTG			
		Arah1-pgP1	FAM-CACCCGCTACGGGAA-MGBNFQ			
	Arah1-TAMRA	Arah1-pgF2	GGGAAGAAACATCTCGGAACAA	86	this work	AF432231
		Arah1-pgR2	CCGGATCCTACCGTTTTGGT			
		Arah1-pgP2	FAM-CCCGTCAAGCGGTTTAGCACCC-TAMRA			
	Arah2-MGB	Arah2-pgF4	CAATGGCCAAGCTCACCATA	54	this work	AY007229
		Arah2-pgR4	GGGCAGCGAGGAGGAAA			
		Arah2-pgP4	FAM-TAGTAGCCCTCGCCC-MGBNFQ			
	Arah2-TAMRA	Arah2-F3	GCTCGAGAGGGCGAACCT	66	Hird et al., 2003	AY007229
Arah2-R3		TCCTCGTCACGTTGGATCTTC				
Arah2-P3		FAM-AGGCCCTGCGAGCAACATCTCATG-TAMRA				
soybean	Glym5-MGB	MGlym5-pgF	CACGGTTCCCATTACTGTTGT	65	this work	AB051865
		MGlym5-pgR	GCAATGCCAAATGAGACAGAAA			
		MGlym5-pgP	FAM-ACTTGTTTTCTGGCTTC-MGBNFQ			
	Glym30-MGB	Glym30-pgF	GAAGCGGTTGGGCGTTT	56	this work	DQ324851
		Glym30-pgR	TGCTATTGCATGTGCTGCTTCT			
		Glym30-pgP	FAM-CTGCCACGGGAGC-MGBNFQ			
	Glym30-TAMRA	Gly30-F	CACATGCAATAGCAACAGGA	94	Platteau et al., 2011	DQ324851
		Gly30-R	TGCCATCCATTGTAACAACC			
		Gly30-P	FAM-CTGAACAAGAAGCTCGTAGACTGTGTGAAGAAAGC-TAMRA			
	Lectin	Lectin-F	TCCACCCCATCCACATTT	81	UNI EN ISO 21570:2013	
		Lectin-R	GGCATAGAAGGTGAAGTTGAAGGA			
		Lectin-TMP	FAM-AACCGGTAGCGTTGCCAGCTTCG-TAMRA			

In bold: guanine according to AY007229, instead of cytosine reported in Hird et al., 2003; bp: base pairs; FAM: 6-carboxyfluorescein; TAMRA: tetramethylrhodamine quencher dye; MGBNFQ: minor groove binder-non fluorescent quencher.

ddPCR™ Supermix for Probes No dUTP (Bio-rad) and 5 µl of DNA template, extracted with the validated method of extraction, in a 20 µl reaction volume. Primer and probe concentrations were the same applied in real-time PCR. Droplets were generated and read using QX200™ Droplet Digital™ PCR System and QuantaSoft™ software version 1.7 (Bio-Rad) applying the ABS mode. Accepted numbers of droplets were 10,000 per reaction and mean copies per droplet did not exceed 0.4 molecules per droplet. Positive droplets that contained the amplification products were discriminated from negative droplets without the amplification products, by applying a threshold set manually.

The emulsion PCR was performed on a C1000 Touch™ Thermal Cycler (Bio-Rad) following thermal cycling conditions recommended: initial step of 10 min at 95 °C, then 40 cycles of 30 s at 94 °C and 60 s at 60 °C and final step of 10 min at 98 °C, then cooled to 4 °C.

For each selected target, thirteen serial dilutions to 1:2 were performed, each in duplicate, starting from 6 ng (merge value) of peanut and soybean DNAs extracted in validation test.

2.6. Enzyme-linked immunoassay (ELISA)

The immunoassay detection and quantification of peanut and soybean allergens were performed using respectively the ELISA kits Ridascreen® Fast peanut, Art. No. R6202 “Enzyme immunoassay for the quantitative determination of peanut” (R-Biopharm Italia Srl, Milano, Italy) and Euroclone Soy, Art. No. EAE010096 “Enzyme immunoassay for the quantitative determination of soy in food” (Euroclone SPA, Milano, Italy). The immunoassay procedures are directed to the detection and quantification of whole peanut and soybean seeds.

The determinations were achieved, reagents included,

according to the ELISA Kits instructions. The absorbance reading were performed using the Multiskan™ FC microplate photometer (ThermoFisher Scientific Inc., Waltham, MA USA).

3. Results and discussion

3.1. Extraction methods

The DNA extraction yields were evaluated, during the first and the second elution steps of purification process, when the expected elution volume was 50 µl according to instructions (Supplementary Table 2, 3 and 4). The results showed the presence of DNA in variable quantity, but not enough to gather together the extracted DNAs.

Often the ΔCq value of the first extract was not so good and was improved in the second extract, giving evidence of inhibitors presence; in other cases, the value was not improved, perhaps due to a poor purification efficiency. Sometimes the first elution ΔCq values were good and those of the second elution get worse, this is probably due to a release of inhibitory substances.

The quantity and the quality of the extracted DNAs, evaluated photometrically (ng/µl and absorbance) were not always in agreement with the data obtained with real-time PCR (Cq and ΔCq) and were then considered less reliable (Supplementary Table 2, 3 and 4).

Therefore, the methods of extraction were evaluated with the data obtained in the first extract and the values of Cq and ΔCq, assigning them a score on a merit point scale of evaluation (Table 2). As the number of the extraction methods, tested for each matrix on two test portions, was 16, a scale ranging from 1 to 16 was assessed, assigning 1 to the best result and 16 to the worst one. When the acceptance criteria reported in Table 2 were not met, the

Table 2

Results of eight extraction methods for soybean flour and roasted peanut matrices tested on two different test portions of 1 g and 2 g.

		peanut					soybean					
fast real-time PCR		actin system										
extraction buffer	clean-up	mean Cq	Cq score	mean ΔCq	ΔCq score	score	mean Cq	Cq score	mean ΔCq	ΔCq score	score	tot score
extraction methods		1 g										
CTAB	(I)	26.06	3	1.59	6	9	20.47	6	1.52	11	17	26
	(II)	27.28	16	1.70	4	20	19.91	5	1.94	2	7	27
	(III)	28.11	9	0.52	16	25	19.81	4	1.92	3	7	32
	(IV)	27.00	16	1.52	8	24	20.49	7	2.01	1	8	32
guanidine hydrochlor	(I)	27.13	6	1.75	3	9	23.48	13	1.86	5	18	27
	(II)	31.65	16	0.51	16	32	27.57	16	0.52	16	32	64
	(III)	27.92	16	0.23	16	32	22.19	16	1.44	16	32	64
	(IV)	27.81	7	1.60	5	12	22.26	9	2.34	10	19	31
		2 g										
CTAB	(I)	24.74	1	1.80	2	3	19.68	2	1.77	8	10	13
	(II)	26.21	4	1.40	16	20	19.78	3	2.13	4	7	27
	(III)	29.00	10	-0.40	16	26	19.57	1	2.21	7	8	34
	(IV)	25.65	2	1.37	16	2	21.10	8	2.19	6	14	16
guanidine hydrochlor	(I)	26.74	5	1.54	7	12	22.52	11	1.76	9	20	32
	(II)	nt	16	nt	16	32	nt	16	nt	16	32	64
	(III)	29.01	16	-1.09	16	32	23.02	12	1.11	16	28	60
	(IV)	27.83	8	2.01	1	9	22.37	10	2.49	12	22	31
acceptance criteria:		≤0.5 Cq*		2.00 ± 0.50			≤0.5 Cq*		2.00 ± 0.50			

(I): NucleoSpin® gDNA Clean-up.

(II): SureFood® Prep Advanced.

(III): Wizard® DNA Clean up System.

(IV): Foodproof® Sample Preparation.

ΔCq: difference between mean Cq of undiluted DNA and its dilution 1:4.

In bold: values out of acceptance criteria, associated at max score of 16.

Nt: not tested for not suitability due to column saturation.

Score: from 1 (best) to 16 (worse).

*: difference between Cq of each extract (first elution) of the same sample.

Table 3

Results of Allergen 40 and Allergen RM 800 DNAs obtained with eight extraction methods and with kits (II) and (IV).

		Allergen 40		RM800		elution vol.
extraction methods		200 mg				
extraction buffer	clean-up	actin system (fast PCR real-time)				
		Cq	ΔCq	Cq	ΔCq	μl
CTAB	(I)	20.00	2.29	24.28	1.81	50
	(II)	24.79	2.44	27.42	2.09	50
	(III)	22.95	1.62	23.67	1.52	50
	(IV)	21.14	2.21	26.00	1.53	100
guanidine hydrochlor	(I)	21.42	2.22	24.75	2.06	50
	(II)	21.26	1.75	26.11	1.76	50
	(III)	22.51	1.65	26.21	1.61	50
	(IV)	22.37	2.49	26.43	2.29	100
(II) SureFood®		21.37	2.21	26.12	1.61	50
(IV) Foodproof®		22.06	2.15	24.91	2.42	100
acceptance criteria:		2.00 ± 0.50		2.00 ± 0.50		

(I): NucleoSpin® gDNA Clean-up.

(II): SureFood® Prep Advanced.

(III): Wizard® DNA Clean up System.

(IV): Foodproof® Sample Preparation.

ΔCq: difference between mean Cq of undiluted DNA and its dilution 1:4.

highest value was assigned. In Table 2, the better extraction method observed for both soybean and peanut matrices was that achieved from a combination of the CTAB extraction buffer and the (I) NucleoSpin® gDNA Clean-up (Macherey Nagel) performed on 2 g of test portion. This method was efficient also as a micro-method (200 mg), using it for Allergen 40 and RM 800 extraction (Table 3, Supplementary Table 4). Since we have observed in this study that the presence of different matrices in a same sample lead to different results (Table 2), it was necessary to select an adequate and efficient method of extraction. When the size of the particles is wide and heterogeneous it is necessary to extract a test portion of 2 g, in order to obtain a better representativeness of the sample and a greater probability to detect hidden allergens, often present in low quantity and probably with a not uniform distribution in complex matrices.

Table 4 shows the results of some modifications (method b) done on the selected method of extraction (method a) used for samples collected from the market. In Table 4, the method b appears to be more repeatable (Δextract) than method a, probably due to the steps reduction, also critical for pellet loss and their replacement with purification kit, improves reproducibility, efficiency (mean Cq) and time consuming. This method was afterwards validated and the results, reported in Supplementary Table 5, show the suitability for the purpose.

3.2. Real-time PCR

Four systems for peanut and for soybean were tested in fast real-time PCR (Table 1). The systems have been chosen to targeting a species specific region (Lectin system) or major allergen genes

Table 4

Results of extraction method selected (method a) and the same with some modifications (method b). Each sample was extracted and verified in duplicated by fast real-time PCR with actin system.

sample	ID	mean Cq	Δreply	Δextract	ΔCq	sample	ID	mean Cq	Δreply	Δextract	ΔCq
polenta (corn meal)	A1 a	22.12	0.08	0.58	1.94	crackers	C2 a	26.68	0.14	5.08	1.99
	A1.a	22.70	0.41		1.81		C2. a	21.61	0.30		2.16
	A1 b	21.11	0.16	0.15	1.91		C2 b	21.46	0.06	0.07	1.96
	A1. b	20.97	0.01		1.64		C2. b	21.39	0.39		1.96
breadcrumbs	A2 a	23.76	0.12	0.23	2.36	waffles	C3 a	26.91	0.04	0.27	1.74
	A2. a	23.52	0.13		2.48		C3. a	27.19	0.16		1.95
	A2 b	23.34	0.02	0.30	2.02		C3 b	23.90	0.17	0.06	1.98
	A2. b	23.64	0.04		2.25		C3. b	23.84	0.06		1.94
baking powder	B1 a	22.72	0.09	1.00	2.09	peanut butter	D1 a	26.94	1.93	0.32	2.56
	B1. a	23.72	1.71		1.11		D1. a	26.62	0.36		1.88
	B1 b	23.14	0.30	0.47	1.77		D1 b	26.16	0.19	0.17	1.64
	B1. b	22.67	0.11		2.26		D1. b	25.99	0.50		1.56
nuts mix	B2 a	28.16	0.12	0.28	1.99	snack choco	D2 a	35.15	0.18	0.32	1.71
	B2. a	28.44	0.20		2.21		D2. a	34.83	0.01		1.47
	B2 b	26.03	0.02	1.62	1.99		D2 b	34.42	0.14	0.57	1.63
	B2. b	27.65	0.05		2.10		D2. b	33.86	0.34		2.14
soya drink	C1 a	24.45	0.08	2.22	1.95	roasted peanut	D3 a	26.27	0.70	0.12	2.43
	C1. a	22.23	0.21		2.07		D3. a	26.16	0.46		1.53
	C1 b	22.25	0.26	0.26	1.65		D3 b	25.47	0.32	0.45	2.33
	C1. b	22.50	0.10		1.56		D3. b	25.92	1.25		1.78
acceptance criteria:		≤0.5 Cq		≤0.5 Cq	2.00 ± 0.50			≤0.5 Cq		≤0.5 Cq	2.00 ± 0.50

Δreply: difference between Cq of two replicates of the same extracted DNA.

Δextract: difference between the mean Cq of two extracted DNAs from same sample.

ΔCq: difference between mean Cq of undiluted DNA and its dilution 1:4.

In bold: values out of parameters.

b: extraction method without isopropanol and ethanol 70% steps.

Table 5
Concentrations of primers and probes.

	peanut				soybean			
	system							
	Arah1		Arah2		Glym30		Glym5	Lectin ^a
	MGB	TAMRA	MGB	TAMRA	MGB	TAMRA	MGB	TAMRA
Primer -F (nM)	900	900	900	300	900	900	900	300
Primer-R (nM)	900	900	900	900	900	900	900	900
probe (nM)	250	250	250	250	250	150	200	250

^a Lectin system was previously validated, accredited and used in the GMO Laboratory.

(Arah1, Arah2 systems for peanut and Glym30 system for soybean) or allergen gene related to primary sensitization and severe allergic reaction (Glym5 system for soybean) (Verhoeckx et al., 2015). The Lectin system used in our laboratory was previously validated and accredited for GMO analysis. Some systems were published, as for allergen genes *Glym30* and *Arah2* using TaqMan probes labeled with reporter dye, FAM (6-carboxyfluorescein) and quencher dye, TAMRA (tetramethylrhodamine) (Table 1). The non-fluorescent quenchers as MGB (minor groove binder) at the 3' end increases the melting temperature (T_m) of probes, allowing the use of shorter probes, reduces cross-talk signals and allows the PCR instruments to measure the reporter dye contributions more precisely.

A shorter probe and then a shorter amplicon could be an advantage for complex matrices characterized by DNA fragmentation; moreover, a reduction of cross-talk is recommended in digital and multiplex PCR.

Therefore, for the major allergen genes, the new systems were developed with probe labeled with MGB at the 3' end and subsequently called also MGB systems to differentiate them from other systems, targeting the same gene but with probe labeled with quencher TAMRA, TAMRA system (Table 1).

All systems reported in Table 1 were tested in fast real-time PCR, with the aim to select the best PCR method between MGB and TAMRA systems of the same gene and among different targets of the same allergen (Arah1 and Arah2 for peanut, Glym30, Glym5 and Lectin for soybean). The first step of verification was the optimization of primer and probe concentrations and the results are shown in Table 5.

The following steps were verified for each PCR system: specificity, robustness, LOD, efficiency and linearity. Each system shows to be specific (Supplementary Table 1); the presence of not expected signals was not confirmed after repetition and experimentally evaluation was supported by in silico verifications. All systems show to be robust as highlighted by the total concordance of qualitative results reported in Supplementary Table 6. The amplification efficiency and linearity calculated for each system meet the acceptance criteria (Supplementary Table 7).

Some differences among systems were observed to determine the limit of detection (Supplementary Table 8, Table 6). The results obtained in LOD₆ test and verified in 60 replicates, were not confirmed in LOD₉₅ for Arah1-TAMRA system (Supplementary Table 8, Table 6). In Table 6, the best system for Arah1 allergen is TAMRA, for Arah2 and Glym30, MGB.

Then, systems with not-fluorescent quenchers are not always the best solution but the best efficiency of the system results from the selection of amplicon, as showed for example, by Arah1 systems in which, at the same LOD of TAMRA and MGB systems, corresponds a different mean Cq, probably due to a different efficiency of amplification of two different amplicons of the same target. The systems Arah2-MGB for peanut and Glym5-MGB for soybean show the lower mean Cq and the lower LOD. These systems were selected to be used with the Lectin system for applications in droplet digital PCR.

Table 6
Results of LOD₉₅ for each peanut and soybean system.

system	LOD ₉₅		allergen
	mean Cq ± rSD	LOD	
Arah1-TAMRA	35.53 ± 0.40	0.006%	peanut
Arah1-MGB	37.99 ± 0.63	0.006%	
Arah2-TAMRA	35.90 ± 0.60	0.006%	
Arah2-MGB	34.73 ± 1.00	0.003%	
Glym30-TAMRA	35.60 ± 0.82	0.002%	soybean
Glym30-MGB	36.35 ± 0.96	0.001%	
Glym5-MGB	35.28 ± 0.63	0.001%	

rSD: standard deviation of repeatability.
LOD: limit of detection.

In all real-time PCR runs, negative extraction and amplification controls resulted negative, while positive amplification controls were positive.

3.3. Droplet digital PCR

The 13 serial dilutions were tested to have a first evaluation of ddPCR performance for each target.

The results of systems Glym5-MGB, Lectin and Arah2-MGB showed a good linearity of 0.9995, 0.9987 and 0.9998 respectively (Supporting Figs. 1, 2, 3).

The Glym5-MGB showed non-specific signals, not detectable using real-time PCR with hydrolysis probes (Fig. 1). The discrimination between positive droplets (containing target) and negative droplets (not containing target) was based on a good separation between them, with different signals expressed as amplitude in fluorescence units. In Fig. 1, it is possible to distinguish three groups in droplets amplitude, more clearly decreasing the target concentration. In Fig. 1, are shown the clusters of the positive and the negative droplets and those between them. Another cluster was not

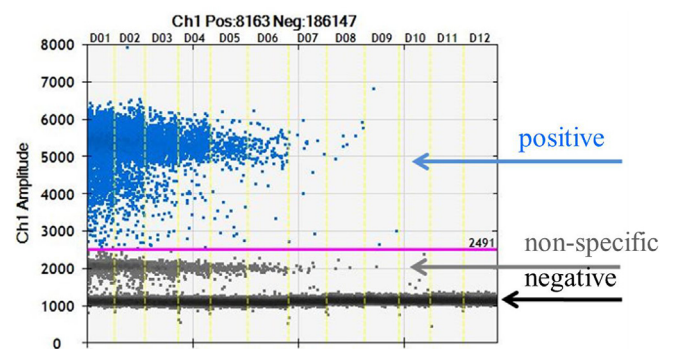


Fig. 1. Results of Glym5-MGB system. Three clusters are separated as amplitude in fluorescence units: positive, negative and non-specific droplets.

classifiable with positive or negative droplets and was defined as non-specific signal. This signal can be due to the differences in amplicon length or annealing temperature. The Glym5-MGB system was not further investigated and Lectin system was used to test the presence of soybean on market samples by ddPCR with Arah2-MGB system, having good separation of positive and negative droplets without further optimization.

The negative controls resulted negative.

3.4. Market samples

The Arah2-MGB and Lectin systems, selected in real-time PCR and in ddPCR, were tested in duplicate on market samples (Table 7).

Peanut was included among the ingredients in the label of three samples (D1–D3) and stated as PAL in another one (B2); the same was for soybean (C1–C3 and B1) and the remaining samples did not indicate the presence of these allergens in label.

The qualitative results of Arah2-MGB and Lectin systems by qPCR confirmed the presence of allergens when stated among the ingredients in label (Table 7). Peanut was not found in the samples which do not indicate its presence among the ingredients in label and do not state as PAL, while soybean was found in five of the six samples in which it was not stated in the label and in one sample with PAL.

To assess the results, all samples were tested with four commercial qPCR kits, two for peanut and two for soybean. The results shown in Table 7 confirmed the presence but not always the absence obtained with in-house qPCR systems. The qPCR kits were resulted more sensitive, indeed the mean Cq was lower of about 6–8 Cq for peanut and about 8–12 Cq for soybean, in samples with declared presence of these allergens.

Probably qPCR kits used multi-copy genes instead of single copy genes such as the in-house qPCR herein.

The advantages of single copy genes are the direct correlation with the target cells and the possibility to generate quantitative results (Tetzlaff & Mäde, 2016). Instead, the multi-copy genes allow to obtain a higher sensitivity and a better detection of the extracted DNA target in processed foods (López-Calleja et al., 2017).

Hence, the qPCR kits are greatly sensitive, but the target copy number per cell should not be too high in order to avoid cross-contamination phenomena between samples in routine analysis (Zagon et al., 2017). This is probably the case of S4 and C2 samples tested with R-Biopharm kit for soybean and Biotecon kit for peanut, respectively (Table 7). The results of peanut PCR kits for samples C1 and C2 and of soybean PCR kits for D1 and D2 could be close to the limit of detection, but the amplification reaction must be more thoroughly investigated, including the evaluation of specificity, not performed in this study.

Moreover, since the qPCR kits allow the possibility of quantification, the market samples were tested with qPCR kits and in-house qPCR, using a calibration curve from Allergen 40 and Allergen RM 800, in order to generate quantitative results (Supplementary Table 9).

The quantity of peanut and soybean allergens in market samples in which their presence as ingredients was stated in label showed a great variability; the other samples with a lower quantity of allergens showed less variability in a same qPCR system, but different results among the systems. Moreover, the amount in ppm obtained in qPCR does not correspond with the concentration obtained by ELISA kit, because the target (DNA in qPCR and protein in ELISA) is different (Supplementary Table 9). Thus, quantitative results in qPCR are not reliable and useful, considering that proteins are the cause of allergen reactions and

Table 7
Qualitative results of market samples tested with different systems and technologies.

market samples	ID	type	label	peanut										soybean									
				ddPCR		qPCR		ELISA		Biotecon		R-Biopharm		ddPCR		qPCR		Biotecon		R-Biopharm		ELISA	
				declared	not declared	declared	not declared	mean Cq	copy n.	mean Cq	copy n.	mean Cq	ppm	mean Cq	copy n.	mean Cq	copy n.	mean Cq	copy n.	mean Cq	copy n.	ppm	ppm
A1		polenta (corn meal)			soybean/peanut		soybean/peanut	undet		undet		undet		1.24		33.25		27.77		25.41		0.06 (<LOD)	
A2		breadcrumbs			peanut		peanut	undet		undet		undet		0.47		37.20		27.96		25.44		0.06 (<LOD)	
B1		baking powder			peanut		peanut	undet		undet		undet		0.24		37.27		29.80		26.48		0.09 (<LOD)	
B2		nuts mix			soybean		soybean	undet		undet		undet				undet		undet		undet			
C1		soya drink			peanut		peanut	undet		34.40		33.97				21.18		13.03		11.71			
C2		crackers			peanut		peanut	undet		42.31		undet				21.39		14.32		11.96			
C3		waffles			soybean		soybean	undet		14.26		14.76				24.15		16.95		13.94			
D1		peanut butter			peanut		peanut	19.85	2031.00**	19.85		14.76				undet		35.48		34.12		12.45	
D2		snack choco			soybean		soybean	27.75	166.00	27.75		21.72				undet		34.06		33.62			
D3		roasted peanut			soybean		soybean	21.74	5970**	21.74		16.87				undet		undet		undet			
S1		polenta (corn meal)			soybean/peanut		soybean/peanut	undet		undet		undet		2.60		32.46		25.60		24.81		15.30	
S2		polenta (corn meal)			soybean/peanut		soybean/peanut	undet		undet		undet		0.24		37.56		29.85		29.67		0.07 (<LOD)	
S3		crackers			soybean/peanut		soybean/peanut	undet		undet		undet		0.12*		37.52		29.49		27.67		0.06 (<LOD)	
S4		savoardo biscuits			soybean/peanut		soybean/peanut	undet		undet		undet		undet		undet		undet		34.68		0.05 (<LOD)	

PAL: precautionary allergen labelling.

*: doubt.

** : D1 diluted 1:10; D3 diluted 1:2.

Undet: undetermined.

n.: number.

LOD: limit of detection.

their concentrations are not required by Regulation (EU) No 1169/2011.

Instead, the results of qualitative PCR showed a better sensitivity when compared with those obtained with the used ELISA kits, for both processed and unprocessed products (Table 7).

Then, the market samples, except for C1 and C2, with a high level of soybean and surely positive, were tested in ddPCR.

The samples with declared presence of peanut and soybean, tested with the same systems in qPCR and ddPCR, were totally in agreement and the results presented a correspondence in the scalability of contents. Indeed, the Cq shows a good correlation with the target content, resulting low with a high target content. For example, in the sample D1, at the lower Cq of 19.85 of peanut target corresponds the higher content of 20310 copy numbers, followed by sample D3 and finally D2 with a lower content of 166 copy numbers of peanut target and a higher Cq of 27.75 (Table 7).

The samples with a not declared allergen presence in label (S4 for peanut and A1, A2, S1, S2, S3, S4 for soybean) or with a PAL (B2 for peanut and B1 for soybean) have confirmed when analysed in ddPCR, the qualitative results of qPCR. The sample S3 resulted doubtful in ddPCR but the cut-off of lectin system in qPCR was estimated to be 36.91 Cq (Accredia DT-07-DL/DS, 2013, p. 47), and then this sample would not be detected in qPCR. According to the lectin qPCR cut-off, the samples A2, B1 and S2 would not also be detected in qPCR, but would be in ddPCR. Therefore, the lectin system in ddPCR results more sensitive than the same in qPCR.

The market samples resulted positives for peanut had a high level of allergen, and the concordance among qualitative systems was total, with the lone difficulty observed in ELISA to quantify peanut in peanut butter.

Understanding the sensibility in ppm of PCR is hard because the same quantity of allergen (ppm of protein) in different matrices is not comparable with the correspondent quantity of DNA. Actually, the concentrations of samples A1, A2 and S3 resulted 0.06 ppm by ELISA, but the content of DNA, expressed in Cq, was quite different (Table 7).

4. Conclusions

This study proposes an efficient method of DNA extraction from various food matrices that represents the most important phase of sample preparation, besides homogenization and collection of the portion test, in improving sensitivity and reliability of PCR methods. The study showed the possibility of using one single extraction method for many matrices and several DNA targets. The evaluation of various systems of different genes and sequences of the same gene tested by real-time PCR were useful to select the most efficient system to work.

Furthermore, the verification of the proposed objectives leads to the conclusion that digital PCR is a new technology suitable and reliable for the detection of peanut and soybean allergens in foods. This study represents a preliminary assessment, but digital PCR assays for Arah2-MGB and Lectin targets exhibit the same or a better sensitivity of real-time PCR, without setup in digital PCR and only transferring the validated methods from real-time PCR. Therefore, this new technology shows, in addition to the advantages of a qualitative real-time PCR, the ability to identify non-specific targets, such as Glym5-MGB, offering a possibility to reduce susceptibility to inhibitors and to save time and costs using multiplex analysis, without losing sensitivity such as in multiplex real-time PCR.

In addition, the results obtained in this study have allowed to show the contamination of mill and bakery products with soybean allergen, representing a potential source of risk for allergic people, detected with qualitative PCR methods but not always with ELISA

kit. Indeed, peanut and soybean PCR methods, although not suitable for quantification, offer, in contrast to ELISA, the advantages of a single efficient extraction method, with better sensitivity, ability of development of in-house methods and possibility of multiplex analysis. Finally, the digital PCR for this application does not solve the quantification problems of real-time PCR, due to the lack of correspondence between DNA and protein content and the expression of allergen amount in copy numbers. On the other hand, further studies for food allergen detection by digital PCR are needed to allow the simultaneous detection of many allergens, with a further reduction of analytical costs and improvement of sensitivity respect to multiplex real-time PCR to assure a fast and efficient control to protect sensitive consumers.

Declaration of interest

The authors declare that have no competing interests and by conducting this study does not imply endorsement of the technologies described in the manuscript.

Acknowledgements

This work was supported by Italian Ministry of Health [grant numbers IZS UM 07/14]. We would like to thank Dr. Giampiero Scortichini for his helpful comments for this study.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.foodcont.2018.04.039>.

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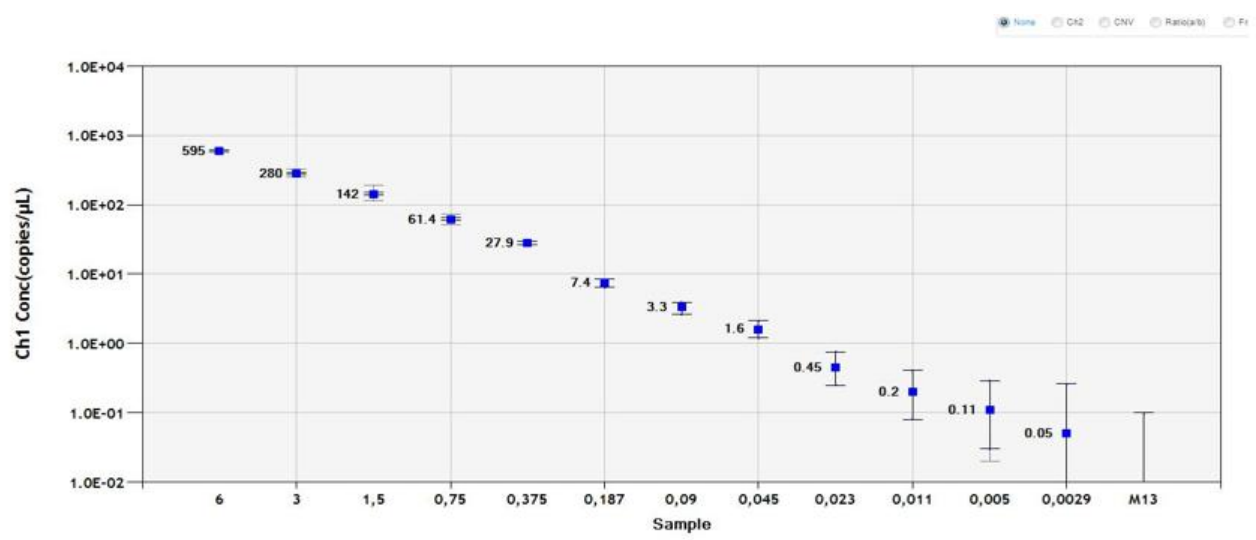
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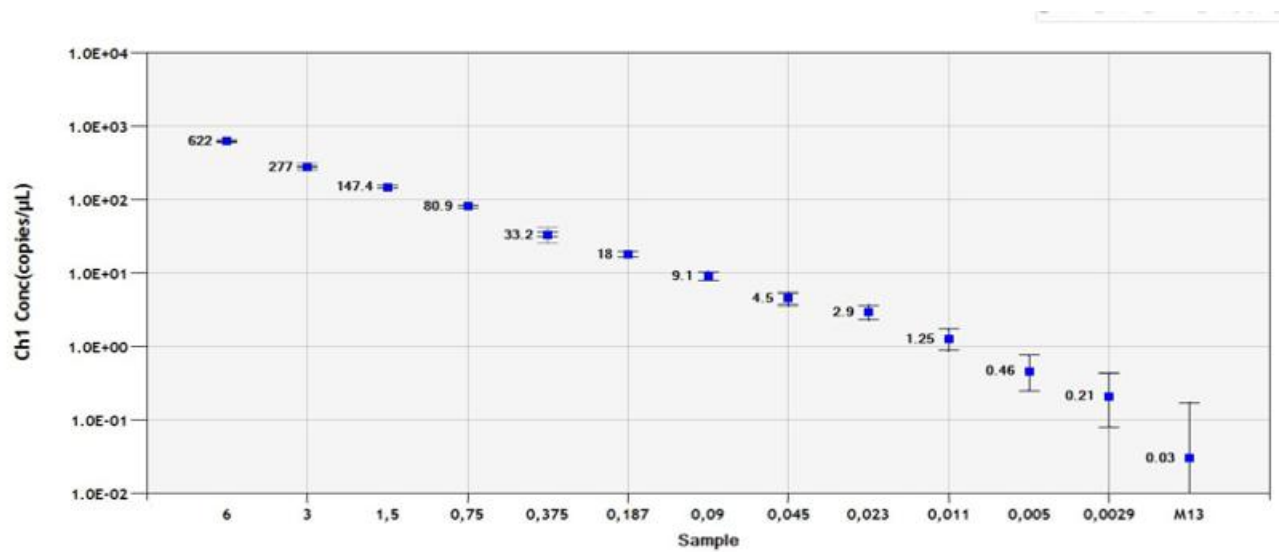
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2



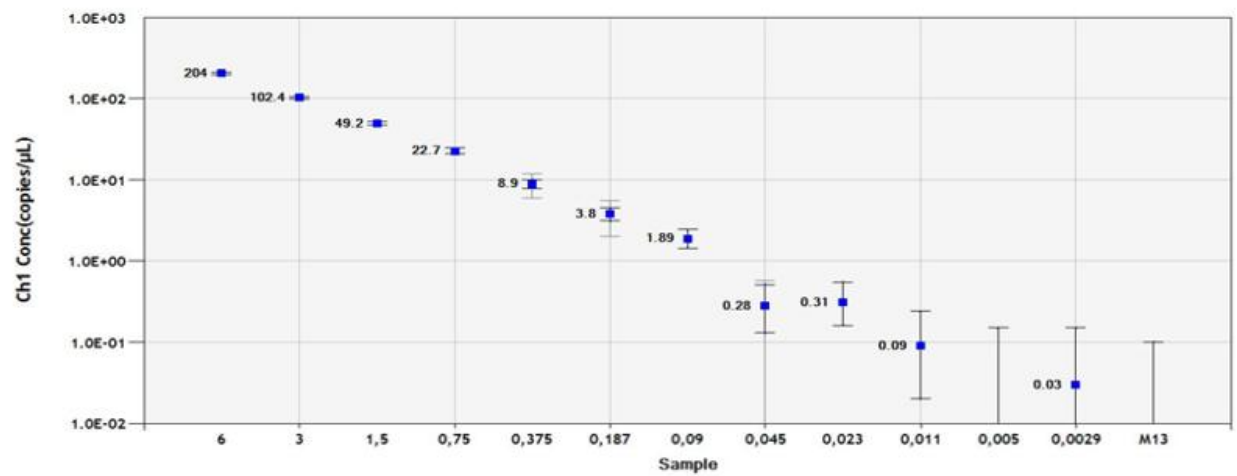
Supplementary Fig. 1 Results in copy number per microliter (merge data) against ng of scalar dilutions tested with Glym5-MGB system

3
4



Supplementary Fig. 2 Results in copy number per microliter (merge data) against ng of scalar dilutions tested with Lectin system

5
6



Supplementary Fig. 3 Results in copy number per microliter (merge data) against ng of scalar dilutions tested with Arah2-MGB system

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Supplementary Table 1. Specificity of each system tested on DNA samples extracted in duplicate and repeated twice for each DNA extracted when unexpected Cq was different from undetermined (-)

sample	peanut								raybean							
	Arah1				Arah2				Glym30				Glym5			
	TAMRA	MGB	TAMRA	MGB	TAMRA	MGB	TAMRA	MGB	TAMRA	MGB	TAMRA	MGB	TAMRA	MGB	TAMRA	MGB
	Cq	repeat	Cq	repeat	Cq	repeat	Cq	repeat	Cq	repeat	Cq	repeat	Cq	repeat	Cq	repeat
Caribean	39	--	--	--	39	--	--	--	--	--	--	--	--	--	--	--
Almond	37	--	--	--	38	--	--	37.0	36	--	--	--	--	--	--	--
Hazelnut	38	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Pistachio nut	39	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Walnut	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Brazil nut	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Macadamia nut	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Peanut	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Pine nut	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Lupin	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Chickpea	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Chickpea	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Barlatti bean	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Cannellini bean	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Lentil	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Barley	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Speltz	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Soybean	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Kamut	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Mile	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Rye	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Sunflower seed	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Sorghum	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Fava bean	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Nutmeg	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Paprika	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Cumin	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Apricot	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Strawberry	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Raspberry	--	--	--	--	--	--	--	--	38	--	--	--	--	--	--	--
Apple	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Pear	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Peach	--	--	--	--	--	37	--	--	--	--	--	--	--	--	--	--
Plum	--	--	--	--	--	--	--	--	38	--	--	--	--	--	--	--
Grape	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Vegetable soup	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Pepper	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Tomato	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Eggplant	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Tabacco	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Lettuce	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Herb	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Beef	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Chicken	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Goat	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Sheep	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Fish	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Deer	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Human	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Nutella spread	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Paalendagenaar	--	--	--	--	--	--	--	--	29.1	29.5	--	--	30.3	30.3	28.9	28.9
Soybean	--	--	--	--	--	--	--	--	22.0	22.1	--	--	22.3	22.6	20.9	20.9
Maize	nt	nt	nt	nt	nt	nt	nt	nt	--	--	--	--	--	--	--	--
Rice	nt	nt	nt	nt	nt	nt	nt	nt	--	--	--	--	--	--	--	--
Canola	nt	nt	nt	nt	nt	nt	nt	nt	--	--	--	--	--	--	--	--
Sugar beet	nt	nt	nt	nt	nt	nt	nt	nt	--	--	--	--	--	--	--	--
Cotton	nt	nt	nt	nt	nt	nt	nt	nt	--	--	--	--	--	--	--	--
Potato	nt	nt	nt	nt	nt	nt	nt	nt	--	--	--	--	--	--	--	--
Peanut	22.4	22.4	25.0	25.0	21.7	21.7	21.0	21.1	--	--	--	--	--	--	--	--
IRMM-481a	22.8	22.7	23.9	24.1	22.7	22.8	22.1	22.1	nt	nt	nt	nt	nt	nt	nt	nt
IRMM-481b	26.4	26.2	28.3	28.3	26.1	26.4	24.6	24.6	nt	nt	nt	nt	nt	nt	nt	nt
IRMM-481c	23.5	23.8	25.3	25.4	23.5	23.6	22.5	22.6	nt	nt	nt	nt	nt	nt	nt	nt
IRMM-481d	21.7	21.7	24.2	24.0	22.1	22.1	21.7	21.7	nt	nt	nt	nt	nt	nt	nt	nt
IRMM-481e	22.1	22.1	24.6	24.4	22.8	22.7	22.3	22.4	nt	nt	nt	nt	nt	nt	nt	nt
IRMM-481f	22.0	22.2	23.8	24.1	22.1	22.3	21.8	21.5	nt	nt	nt	nt	nt	nt	nt	nt

nt: not tested

paalendagenaar: maize, raybean, rice, canola, sugar beet, cotton, potato DNA

vegetable soup ingredients: potato, carrot, tomato, spinach, cauliflower, zucchini, green bean, barlatti bean, onion, parsley, basil, celery, garlic

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Supplementary Table 2. Results of different soybean flour test portion (1 g and 2 g), extracted in duplicate with two extraction buffers and four clean-up kits

Soybean	extraction buffer	1 g														2 g														elution vol.																															
		photometer							actin system							photometer							actin system																																						
		mean	rSD	mean	rSD	mean	rSD	mean	rSD	mean	rSD	mean	rSD	mean	rSD	mean	rSD	mean	rSD	mean	rSD	mean	rSD	mean	rSD	mean	rSD	mean	rSD																																
CTAB	clean-up	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ul																															
	(I)	124.00	109.50	20.31	1.94	1.95	0.01	1.65	0.34	20.37	20.47	0.14	1.12	1.52	0.00	151.00	157.50	9.19	1.96	1.96	0.00	1.40	0.40	19.76	19.68	0.11	1.57	1.77	0.28	50																															
	NucleoS	37.00	32.50	6.36	1.90	1.95	0.06	1.70	0.19	21.97	22.21	0.33	2.03	2.10	0.09	77.00	82.50	7.78	1.93	1.93	0.01	1.68	0.21	20.33	20.65	0.17	2.31	2.06	0.36	50																															
	pmB	95.00		1.95				1.53	0.23	20.57			1.32			164.00		1.96				1.59	0.45	19.60				1.97		50																															
	gDNA	28.00		1.99				1.58	0.08	22.44			2.16			88.00		1.92				1.64	0.24	20.77				1.80		50																															
	(II)	185.00	173.00	16.97	1.87	1.85	0.03	0.34	0.50	20.04	19.91	0.18	1.90	1.94	0.05	181.00	177.50	4.95	1.87	1.82	0.08	0.38	0.49	19.73	19.78	0.07	1.94	2.13	0.27	50																															
	SurfFos	56.00	66.00	14.14	1.61	1.44	0.25	0.38	0.15	21.55	21.96	0.57	2.48	2.40	0.11	82.00	89.50	10.61	1.40	1.41	0.01	0.35	0.22	23.51	23.21	0.43	2.58	2.40	0.26	50																															
	dB Prep	161.00		1.83				0.58	0.44	19.78			1.97			174.00		1.76				0.73	0.47	19.83				2.32		50																															
	Advance	76.00		1.26				0.54	0.21	22.56			2.32			87.00		1.41				0.59	0.26	22.96				2.21		50																															
	(III)	137.00	144.50	10.61	1.93	1.95	0.02	0.22	0.37	20.06	19.81	0.35	1.83	1.92	0.13	138.00	136.50	2.12	1.93	1.93	0.00	0.21	0.37	19.69	19.57	0.17	2.07	2.21	0.20	50																															
	Wizard	26.00	30.00	5.66	1.70	1.77	0.09	0.38	0.07	22.17	22.28	0.16	2.43	2.19	0.35	31.00	37.50	9.19	1.70	1.75	0.06	0.45	0.08	22.09	21.63	0.66	2.54	2.69	0.21	50																															
	gDNA	152.00		1.96				ev	0.41	19.56			2.01			135.00		1.93				0.33	0.36	19.45				2.35		50																															
	Clean up	34.00		1.83				0.22	0.09	22.39			1.94			44.00		1.79				0.40	0.12	21.16				2.83		50																															
	(IV)	80.00	80.00	0.00	1.97	1.96	0.01	1.49	0.22	20.58	20.49	0.13	2.00	2.01	0.01	61.00	54.50	9.19	1.88	1.86	0.04	1.49	0.17	20.87	21.10	0.33	2.03	2.19	0.22	100																															
	Foodpro	80.00		1.95				1.44	0.22	20.40			2.02			48.00		1.83				1.43	0.13	21.33				2.34		100																															
guanidine hydrochloride	clean-up	244.00	241.50	3.54	1.62	1.62	0.01	0.67	0.66	23.55	23.48	0.11	1.81	1.86	0.06	305.00	271.00	48.08	1.66	1.66	0.00	0.72	0.82	22.43	22.52	0.13	1.47	1.76	0.41	50																															
	(I)	179.00	186.50	10.61	1.59	1.61	0.02	0.56	0.48	23.58	23.35	0.33	3.17	2.98	0.27	167.00	161.00	8.49	1.57	1.58	0.01	0.57	0.45	24.66	24.28	0.54	4.19	3.59	0.85	50																															
	NucleoS	239.00		1.61				0.66	0.65	23.40			1.90			237.00		1.66				0.70	0.64	22.61				2.05		50																															
	pmB	194.00		1.62				0.59	0.52	23.11			2.79			155.00		1.59				0.62	0.42	23.89				2.99		50																															
	gDNA	ev	504.00	ev	1.56	ev	ev	ev	ev	27.88	27.57	0.44	0.24	0.52	0.59	ev		ev		ev	ev	ev	ev	ev	ev	ev	ev	ev	ev	50																															
	(II)	SurfFos	ev	ev	ev	ev	ev	ev	ev	undet.			ev			ev		ev		ev	ev	ev	ev	ev	ev	ev	ev	ev	ev	50																															
	dB Prep	504.00		1.56				ev	1.36	27.26			0.79			ev		ev		ev	ev	ev	ev	ev	ev	ev	ev	ev	ev	50																															
	Advance	ev	ev	ev	ev	ev	ev	ev	ev	undet.			ev			ev		ev		ev	ev	ev	ev	ev	ev	ev	ev	ev	ev	50																															
	(III)	610.00	577.50	45.96	1.72	1.71	0.01	ev	1.64	22.47	22.19	0.40	1.33	1.44	0.15	440.00	510.50	99.70	1.66	1.69	0.04	1.19	1.19	23.08	23.02	0.09	1.61	1.11	0.71	50																															
	Wizard	96.00	110.00	19.80	1.55	1.56	0.01	0.29	0.26	26.09	25.31	1.10	2.73	2.54	0.28	73.00	146.50	103.94	1.49	1.33	0.23	0.31	0.20	29.10	27.32	2.52	2.58	2.09	0.70	50																															
	gDNA	545.00		1.70				ev	1.47	21.90			1.54			581.00		1.71				1.56	1.56	22.95				0.60		50																															
	Clean up	124.00		1.57				0.52	0.35	24.53			2.34			220.00		1.16				0.55	0.61	25.54				1.99		50																															
	(IV)	40.00	41.00	1.41	1.78	1.79	0.01	0.91	0.10	22.30	22.26	0.06	2.30	2.34	0.05	41.00	43.50	1.54	1.77	1.73	0.06	0.93	0.11	22.22	22.37	0.21	2.67	2.49	0.26	100																															
	Foodpro	21.00	18.00	4.34	1.78	1.79	0.01	0.85	0.06	23.75	24.28	0.75	2.53	2.39	0.21	14.00	13.50	2.12	1.73	1.68	0.07	0.73	0.04	25.12	25.21	0.12	2.30	2.21	0.13	100																															
	Sample	42.00		1.79				0.84	0.11	22.21			2.37			46.00		1.68				0.88	0.13	22.51				2.30		100																															
	acceptance criteria:	RSD < 25%		1.8-2.0		~2.2		0.1-1.0		≤ 0.5 Cq*		2.00 ± 0.50		RSD < 25%		1.8-2.0		~2.2		0.1-1.0		≤ 0.5 Cq*		2.00 ± 0.50																																					
ΔCq: difference between mean Cq of undiluted DNA and its dilution 1:4																																																													
rSD: standard deviation of repeatability																																																													
ev: not tested																																																													
undet.: undetermined																																																													

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Supplementary Table 3. Results of different peanut flour test portion (1 g and 2 g), extracted in duplicate with two extraction buffers and four clean-up kits

Peanut	extraction buffer	1 g														2 g														elution vol.
		photometer							actin system							photometer							actin system							
		mean	rSD	mean	rSD	mean	rSD	mean	rSD	mean	rSD	mean	rSD	mean	rSD	mean	rSD	mean	rSD	mean	rSD	mean	rSD	mean	rSD	mean	rSD	mean	rSD	
CTAB	clean-up	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ul
	(I)	124.00	109.50	20.31	1.94	1.95	0.01	1.65	0.34	20.57	20.47	0.14	1.12	1.52	0.00	151.00	157.50	9.19	1.96	1.96	0.00	1.40	0.40	19.76	19.68	0.11	1.57	1.77	0.28	50
	NucleoS	37.00	32.50	6.36	1.90	1.95	0.06	1.70	0.19	21.97	22.21	0.33	2.03	2.10	0.09	77.00	82.50	7.78	1.93	1.93	0.01	1.68	0.21	20.33	20.65	0.17	2.31	2.66	0.36	50
	pmB	95.00		1.95				1.53	0.23	20.57			1.32			164.00		1.96				1.59	0.45	19.60				1.97		50
	gDNA	28.00		1.99				1.58	0.08	22.44			2.16			88.00		1.92				1.64	0.24	20.77				1.80		50
	(II)	185.00	173.00	16.97	1.87	1.85	0.03	0.34	0.50	20.04	19.91	0.18	1.90	1.94	0.05	181.00	177.50	4.95	1.87	1.82	0.08	0.38	0.49	19.73	19.78	0.07	1.94	2.13	0.27	50
	SurfFos	56.00	66.00	14.14	1.61	1.44	0.25	0.38	0.15	21.55	21.96	0.57	2.48	2.40	0.11	82.00	89.50	10.61	1.40	1.41	0.01	0.35	0.2							
sodium dodecyl sulfate	clean-up	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ul
	(I)	124.00	109.50	20.31	1.94	1.95	0.01	1.65	0.34	20.57	20.47	0.14	1.12	1.52	0.00	151.00	157.50	9.19	1.96	1.96	0.00	1.40	0.40	19.76	19.68	0.11	1.57	1.77	0.28	50
	NucleoS	37.00	32.50	6.36	1.90	1.95	0.06	1.70	0.19	21.97	22.21	0.33	2.03	2.10	0.09	77.00	82.50	7.78	1.93	1.93	0.01	1.68	0.21	20.33	20.65	0.17	2.31	2.66	0.36	50
	pmB	95.00		1.95				1.53	0.23	20.57			1.32			164.00		1.96				1.59	0.45	19.60				1.97		50
	gDNA	28.00		1.99				1.58	0.08	22.44			2.16			88.00		1.92				1.64	0.24	20.77				1.80		50
	(II)	185.00	173.00	16.97	1.87	1.85	0.03	0.34	0.50	20.04	19.91	0.18	1.90	1.94	0.05	181.00	177.50	4.95	1.87	1.82	0.08	0.38	0.49	19.73	19.78	0.07	1.94	2.13	0.27	50
	SurfFos	56.00	66.00	14.14	1.61	1.44	0.25	0.38	0.15	21.55	21.96	0.57	2.48	2.40	0.11	82.00	89.50	10.61	1.40	1.41	0.01	0.35	0.2							
SDS	clean-up	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ul
	(I)	124.00	109.50	20.31	1.94	1.95	0.01	1.65	0.34	20.57	20.47	0.14	1.12	1.52	0.00	151.00	157.50	9.19	1.96	1.96	0.00	1.40	0.40	19.76	19.68	0.11	1.57	1.77	0.28	50
	NucleoS	37.00	32.50	6.36	1.90	1.95	0.06	1.70	0.19	21.97	22.21	0.33	2.03	2.10	0.09	77.00	82.50	7.78	1.93	1.93	0.01	1.68	0.21	20.33	20.65	0.17	2.31	2.66	0.36	50
	pmB	95.00		1.95				1.53	0.23	20.57			1.32			164.00		1.96				1.59	0.45	19.60				1.97		50
	gDNA	28.00		1.99				1.58	0.08	22.44			2.16			88.00		1.92				1.64	0.24	20.77				1.80		50
	(II)	185.00	173.00	16.97	1.87	1.85	0.03	0.34	0.50	20.04	19.91	0.18	1.90	1.94	0.05	181.00	177.50	4.95	1.87	1.82	0.08	0.38	0.49	19.73	19.78	0.07	1.94	2.13	0.27	50
	SurfFos	56.00	66.00	14.14	1.61	1.44	0.25	0.38	0.15	21.55	21.96	0.57	2.48	2.40	0.11	82.00	89.50	10.61	1.40	1.41	0.01	0.35	0.2							
Triton X-100	clean-up	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ul
	(I)	124.00	109.50	20.31	1.94	1.95	0.01	1.65	0.34	20.57	20.47	0.14	1.12	1.52	0.00	151.00	157.50	9.19	1.96	1.96	0.00	1.40	0.40	19.76	19.68	0.11	1.57	1.77	0.28	50
	NucleoS	37.00	32.50	6.36	1.90	1.95	0.06	1.70	0.19	21.97	22.21	0.33	2.03	2.10	0.09	77.00	82.50	7.78	1.93	1.93	0.01	1.68	0.21	20.33	20.65	0.17	2.31	2.66	0.36	50
	pmB	95.00		1.95				1.53	0.23	20.57			1.32			164.00		1.96				1.59	0.45	19.60				1.97		50
	gDNA	28.00		1.99				1.58	0.08	22.44			2.16			88.00		1.92				1.64	0.24	20.77				1.80		50
	(II)	185.00	173.00	16.97	1.87	1.85	0.03	0.34	0.50	20.04	19.91	0.18	1.90	1.94	0.05	181.00	177.50	4.95	1.87	1.82	0.08	0.38	0.49	19.73	19.78	0.07	1.94	2.13	0.27	50
	SurfFos	56.00	66.00	14.14	1.61	1.44	0.25	0.38	0.15	21.55	21.96	0.57	2.48	2.40	0.11	82.00	89.50	10.61	1.40	1.41	0.01	0.35	0.2							
Tween 20	clean-up	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ul
	(I)	124.00	109.50	20.31	1.94	1.95	0.01	1.65	0.34	20.57	20.47	0.14	1.12	1.52	0.00	151.00	157.50	9.19	1.96	1.96	0.00	1.40	0.40	19.76	19.68	0.11	1.57	1.77	0.28	50
	NucleoS	37.00	32.50	6.36	1.90	1.95	0.06	1.70	0.19	21.97	22.21	0.33	2.03	2.10	0.09	77.00	82.50	7.78	1.93	1.93	0.01	1.68	0.21	20.33	20.65	0.17	2.31	2.66	0.36	50
	pmB	95.00		1.95				1.53	0.23	20.57			1.32			164.00		1.96				1.59	0.45	19.60				1.97		50
	gDNA	28.00		1.99				1.58	0.08	22.44			2.16			88.00		1.92				1.64	0.24	20.77				1.80		50
	(II)	185.00	173.00	16.97	1.87	1.85	0.03	0.34	0.50	20.04	19.91	0.18	1.90	1.94	0.05	181.00	177.50	4.95	1.87	1.82	0.08	0.38	0.49	19.73	19.78	0.07	1.94	2.13	0.27	50
	SurfFos	56.00	66.00	14.14	1.61	1.44	0.25	0.38	0.15	21.55	21.96	0.57	2.48	2.40	0.11	82.00	89.50	10.61	1.40	1.41	0.01	0.35	0.2							
Tween 80	clean-up	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ul
	(I)	124.00	109.50	20.31	1.94	1.95	0.01	1.65	0.34	20.57	20.47	0.14	1.12	1.52	0.00	151.00	157.50	9.19	1.96	1.96	0.00	1.40	0.40	19.76	19.68	0.11	1.57	1.77	0.28	50
	NucleoS	37.00	32.50	6.36	1.90	1.95	0.06	1.70	0.19	21.97	22.21	0.33	2.03	2.10	0.09	77.00	82.50	7.78	1.93	1.93	0.01	1.68	0.21	20.33	20.65	0.17	2.31	2.66	0.36	50
	pmB	95.00		1.95				1.53	0.23	20.57			1.32			164.00		1.96				1.59	0.45	19.60				1.97		50
	gDNA	28.00		1.99				1.58	0.08	22.44			2.16			88.00		1.92				1.64	0.24	20.77				1.80		50
	(II)	185.00	173.00	16.97	1.87	1.85	0.03	0.34	0.50	20.04	19.91	0.18	1.90	1.94	0.05	181.00	177.50	4.95	1.87	1.82	0.08	0.38	0.49	19.73	19.78	0.07	1.94	2.13	0.27	50
	SurfFos	56.00	66.00	14.14	1.61	1.44	0.25	0.38	0.15	21.55	21.96	0.57	2.48	2.40	0.11	82.00	89.50	10.61	1.40	1.41	0.01	0.35	0.2							
Tween 20 + Triton X-100	clean-up	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ul
	(I)	124.00	109.50	20.31	1.94	1.95	0.01	1.65	0.34	20.57	20.47	0.14	1.12	1.52	0.00	151.00	157.50	9.19	1.96	1.96	0.00	1.40	0.40	19.76	19.68	0.11	1.57	1.77	0.28	50
	NucleoS	37.00	32.50	6.36	1.90	1.95	0.06	1.70	0.19	21.97	22.21	0.33	2.03	2.10	0.09	77.00	82.50	7.78	1.93	1.93	0.01	1.68	0.21	20.33	20.65	0.17	2.31	2.66	0.36	50
	pmB	95.00		1.95				1.53	0.23	20.57			1.32			164.00		1.96				1.59	0.45	19.60				1.97		50
	gDNA	28.00		1.99				1.58	0.08	22.44			2.16			88.00		1.92				1.64	0.24	20.77				1.80		50
	(II)	185.00	173.00	16.97	1.87	1.85	0.03	0.34	0.50	20.04	19.91	0.18	1.90	1.94	0.05	181.00	177.50	4.95	1.87	1.82	0.08	0.38	0.49	19.73	19.78	0.07	1.94	2.13	0.27	50
	SurfFos	56.00	66.00	14.14	1.61	1.44	0.25	0.38	0.15	21.55	21.96	0.57	2.48	2.40	0.11	82.00	89.50	10.61	1.40	1.41	0.01	0.35	0.2							
Tween 80 + Triton X-100	clean-up	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul	ng/ul															

Supplementary Table 4. Results of Allergen 40 and Allergen RM 800 DNAs obtained with eight extraction methods and with kits (II) and (IV)														
extraction methods		Allergen 40						RM800						
extraction buffer	clean-up	200 mg												elution vol.
		photometer				actin system		photometer				actin system		
		ng/μl	A ₂₆₀ /A ₂₈₀	A ₂₆₀ /A ₂₃₀	A ₂₆₀	Cq	ΔCq	ng/μl	A ₂₆₀ /A ₂₈₀	A ₂₆₀ /A ₂₃₀	A ₂₆₀	Cq	ΔCq	
CTAB	(I)	205	1,81	1,63	0,554	20	2,29	40	1,85	2,05	0,105	24,28	1,81	50
		21	1,72	nv	0,02	24,26	1,85	5	5,201	nv	0,032	26,98	2,03	50
	(II)	20,9	1,52	0,18	0,06	24,79	2,44	41	1,36	0,28	0,112	27,42	2,09	50
		20,4	1,27	0,5	0,06	28,73	2,12	66	1,3	0,38	0,179	30,34	2,03	50
	(III)	17	1,43	0,72	0,045	22,95	1,62	47	1,75	0,35	0,125	23,67	1,42	50
		nt	nt	nt	nt	24,64	1,98	14	1,63	0,13	0,039	25,9	2,11	50
	(IV)	73	1,73	1,5	0,197	21,14	2,21	12	1,55	1,13	0,037	26,00	1,53	100
	guanidine hydrochlor	(I)	158	1,75	1,34	0,426	21,42	2,22	79	1,66	1,32	0,212	24,75	2,06
60			1,74	1,7	0,054	24,78	2,00	16	1,61	2,18	0,042	27,40	2,79	50
(II)		183	1,54	0,68	0,488	21,26	1,75	15	1,56	0,91	0,078	26,11	1,76	50
		61	1,25	0,52	0,165	25,34	1,86	nt	nt	nt	nt	nt	nt	50
(III)		143	1,74	0,16	0,386	22,51	1,65	73	2,22	nv	0,199	26,21	1,61	50
		33	1,63	0,2	0,087	26,23	1,44	22	1,87	0,62	0,055	28,42	2,73	50
(IV)		48	1,76	1,36	0,131	22,37	2,49	21	1,63	1,06	0,054	26,43	2,29	100
(II) SureFood®		198	nt	nt	nt	21,37	2,21	372	nt	nt	nt	26,12	1,61	50
Prep Advanced		49	nt	nt	nt	24,25	2,1	74	nt	nt	nt	28,53	2,03	50
(IV) Foodproof®		131	nt	nt	nt	22,06	2,15	539	nt	nt	nt	24,91	2,42	100
Sample Preparation	36	nt	nt	nt	25,78	1,63	91	nt	nt	nt	27,97	1,78	100	
acceptance criteria:			1,8-2,0	~ 2,2	0,1-1,0		2,00 ± 0,50		1,8-2,0	~ 2,2	0,1-1,0		2,00 ± 0,50	
(I): NucleoSpin® gDNA Clean-up														
(II): SureFood® Prep Advanced														
(III): Wizard® DNA Clean up System														
(IV): Foodproof® Sample Preparation														
nv: not value														
nt: not tested														
highlighted: second elution														
ΔCq: difference between mean Cq of undiluted DNA and its dilution 1:4														
vol: volume														

Supplementary Table 5. Validation of extraction method b ₁ by inhibition test									
days	test portions	roasted peanut				soybean flour			
		parameters				parameters			
		ΔC_q	R^2	slope	efficiency (%)	ΔC_q	R^2	slope	efficiency (%)
1	1	0.04	0.9980	-3.396	97.00	0.01	0.9998	-3.452	94.84
	2	0.07	0.9958	-3.351	98.80	0.02	0.9997	-3.374	97.87
	3	0.09	0.9987	-3.547	91.38	0.17	0.9984	-3.323	99.96
	4	0.20	0.9970	-3.096	110.38	0.06	0.9998	-3.318	100.16
	5	0.04	0.9990	-3.145	107.95	0.09	0.9995	-3.377	97.75
	6	0.31	0.9907	-3.186	106.00	0.03	0.9997	-3.481	93.76
2	7	0.17	0.9975	-3.225	104.21	0.14	0.9984	-3.459	94.58
	8	0.29	0.9935	-3.236	103.72	0.01	0.9996	-3.352	98.76
	9	0.01	0.9981	-3.519	92.39	0.04	0.9999	-3.602	89.51
	10	0.31	0.9962	-3.266	102.39	0.06	0.9996	-3.232	103.89
	11	0.27	0.9965	-3.367	98.15	0.03	0.9995	-3.365	98.23
	12	0.45	0.9922	-3.33	99.66	0.02	0.9993	-3.339	99.29
3	13	0.39	0.9920	-3.136	108.39	0.08	0.9994	-3.464	94.39
	14	0.30	0.9969	-3.336	99.42	0.15	0.9986	-3.399	96.88
	15	0.26	0.9972	-3.445	95.11	0.06	0.9999	-3.501	93.03
	16	0.22	0.9964	-3.279	101.82	0.00	0.9994	-3.404	96.69
	17	0.07	0.9965	-3.302	100.84	0.02	0.9997	-3.436	95.45
	18	0.09	0.9970	-3.591	89.88	0.09	0.9997	-3.358	98.52
acceptance		< 0.5	≥ 0.98	≥ -3.1, ≤ -3.6	90%-110%	< 0.5	≥ 0.98	≥ -3.1, ≤ -3.6	90%-110%
ΔC_q : difference between the measured C_q value and the extrapolated C_q value of the first diluted sample									

Supplementary Table 6. Robustness results of PCR systems for peanut and soybean detection																					
vol (μL) T _m T _a	peanut robustness									soybean robustness											
	Arsh1						Arsh2						Ghyn30						Ghyn5		
	TAMRA			MGB			TAMRA			MGB			TAMRA			MGB			MGB		
	1	2A	2B	1	2A	2B	1	2A	2B	1	2A	2B	1	2A	2B	1	2A	2B	1	2A	2B
	30.91	31.41	31.27	33.56	33.51	33.81	32.11	32.21	32.47	30.22	30.13	30.31	33.28	33.61	33.98	33.74	33.00	33.38	32.96	33.01	33.44
	30.91	31.49	31.51	33.66	33.33	33.52	32.06	32.20	32.23	30.10	30.50	30.16	31.45	31.52	31.70	33.50	31.11	33.97	32.83	33.13	32.55
30.98	31.66	31.51	33.56	33.55	33.51	32.05	32.12	32.61	30.30	30.35	30.15	32.70	31.00	33.28	31.65	31.10	31.70	33.07	33.29	32.75	
20	19	21	20	19	21	20	19	21	20	19	21	20	19	21	20	19	21	20	19	21	
	59/60°C			59/59°C			59/59°C			58/58°C			58/50°C			58/60°C			60/59°C		
	60°C		61°C	60°C		61°C	60°C		61°C	60°C		61°C	60°C		61°C	60°C		61°C	60°C		61°C
1: PCR run in 7900HT Fast Real-Time PCR																					
2A and 2B: PCR run in 7500 Fast Real-Time PCR																					
vol: reaction volume																					
T _m : melting temperature of primers																					
T _a : annealing temperature																					

Supplementary Table 7. Efficiency and Linearity (R^2) of peanut and soybean systems

system	parameters	A	B	C	mean
Arah1-TAMRA	slope	-3,47	-3,39	-3,48	-3,45
	efficiency (%)	94,28	97,05	93,90	95,08
	R^2	0,99	1,00	1,00	1,00
Arah1-MGB	slope	-3,57	-3,47	-3,50	-3,51
	efficiency (%)	90,68	94,13	93,11	92,64
	R^2	0,99	0,99	0,99	0,99
Arah2-TAMRA	slope	-3,31	-3,39	-3,33	-3,34
	efficiency	100,41	97,22	99,86	99,16
	R^2	0,99	1,00	1,00	1,00
Arah2-MGB	slope	-3,38	-3,39	-3,30	-3,36
	efficiency (%)	97,70	97,39	101,04	98,71
	R^2	1,00	1,00	1,00	1,00
Glym30-TAMRA	slope	-3,35	-3,38	-3,42	-3,38
	efficiency (%)	99,04	97,78	95,92	97,58
	R^2	1,00	1,00	1,00	1,00
Glym30-MGB	slope	-3,26	-3,32	-3,24	-3,27
	efficiency (%)	102,86	100,07	103,66	102,20
	R^2	1,00	1,00	1,00	1,00
Glym5-MGB	slope	-3,08	-2,98	-3,11	-3,06
	efficiency (%)	111,24	116,68	109,75	112,56
	R^2	1,00	1,00	1,00	1,00

Supplementary Table 8. Results of LOD₆ for each system of peanut and soybean

		LOD ₆											
Peanut	ng	1,5	0,75	0,375	0,1875	0,09375	0,04688	0,02344	0,1172	0,00586	0,00293	0,00146	0,00073
Arah1-TAMRA	mean Cq	27,36	28,46	29,51	30,55	31,62	32,68	33,58	34,91	35,73	37,00		
Arah1-MGB		30,41	31,67	32,58	33,78	34,78	35,87	36,86	38,32	39,08			
Arah2-TAMRA		27,57	28,63	29,66	30,70	31,68	32,77	33,71	34,63	35,72			
Arah2-MGB		26,14	27,18	28,19	29,18	30,19	31,25	32,24	33,43	34,19	35,47		
Soybean	ng	0,25	0,125	0,0625	0,03125	0,01563	0,00781	0,00391	0,00195	0,00098	0,00049	0,00024	0,00012
Glym30-TAMRA	mean Cq	28,66	29,69	30,73	31,77	32,71	33,76	34,76	36,18				
Glym30-MGB		28,99	30,08	31,05	32,02	32,95	34,04	34,97	36,04	36,97			
Glym5-MGB		27,34	28,40	29,26	30,20	31,03	32,03	33,02	33,97	35,36			

box empty: when one or more of the eighteen reactions does not present amplification

LOD: limit of detection

Supplementary Table 9. Quantitative results of market samples by qPCR and ELISA								
		qPCR Biotecon		qPCR R-Biopharm		qPCR Arah2-MGB		ELISA
		Allergen 40	RM 800	Allergen 40	RM 800	Allergen 40	RM 800	
peanut	slope	-3.39	-3.63	-3.52	-3.17	-3.68	-3.29	
	R ²	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	
	sample	ppm						
	D1	2350573.60	1174648.60	2022000000.00	13617796.00	148616.00	3099635.70	352953.00
	D2	121166.14	8552.44	143171.21	12361.63	1305.03	15633.59	170232.03
	D3	755052.93	406149.15	141257425.00	1935724.90	59197.48	1108475.15	120339.28
	C1	2.70	3.27	0.47	1.19	undet	undet	
	C2	0.01	0.02	undet	undet	undet	undet	
		qPCR Biotecon		qPCR R-Biopharm		qPCR Lectin		ELISA
		Allergen 40	RM 800	Allergen 40	RM 800	Allergen 40	RM 800	
soybean	slope	-3.62	-3.34	-3.40	-3.38	-1.45	-3.45	
	R ²	-1.00	-1.00	-1.00	-1.00	-0.91	-1.00	
	sample	ppm						
	A1	23.07	28.80	44.78	24.63	12.18	33.19	0.06 (< LOD)
	A2	20.41	25.22	43.91	24.15	1.13	3.98	0.06 (< LOD)
	B1	6.36	7.13	21.59	11.82	0.19	0.80	0.09 (< LOD)
	B2	undet	undet	undet	undet	undet	undet	
	C1	272860.61	747727.37	485003.44	281829.71	108041027.10	53860517.02	
	C2	120241.50	307625.28	408305.73	237019.68	74092770.25	38446225.05	
	C3	22462.79	49928.90	107305.07	61798.79	3298977.08	2382145.62	12.45
	D1	0.17	0.14	0.12	0.06	undet	undet	
	D2	0.42	0.38	0.17	0.09	undet	undet	
	S1	91.85	128.75	67.24	37.07	204.36	412.90	15.30
	S2	6.76	7.68	10.86	5.92	undet/0.38	undet	0.07 (< LOD)
	S3	7.74	8.82	9.69	5.28	0.62	2.33	0.06 (< LOD)
	S4	undet	undet	0.38	0.20	undet	undet	0.05 (< LOD)
in bold: out of acceptance parameters								
LOD: limit of detection								