



Quantification of the allergen soy (*Glycine max*) in food using digital droplet PCR (ddPCR)

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Received: 23 April 2018 / Revised: 22 October 2018 / Accepted: 27 October 2018
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Abstract

To meet the increasing need for quantification of allergens and to have an alternative to commercially available ELISA and PCR systems, the Austrian Agency for Health and Food Safety started establishing in-house PCR systems. To obtain low limits of detection (LOD) and quantification (LOQ), target sequences are preferably sought in multicopy genomes like mitochondrial- or chloroplast DNA. These molecules are of high but varying abundance even among tissues of the same organism. Beyond that, DNA might be degraded by processes of food manufacturing which additionally affects their quantification. Therefore, a reliable correlation of the allergen portion in a sample and its chloroplast-DNA concentration cannot be preassumed. This incoherence is not further considered (e.g., by a matrix-related reference material), and therefore, our quantitative results can only be understood as the mass of soy which maintained its biochemical activity, related to the soy content of the reference material used. To convert absolute results expressed in copies per microliter (Cp/μL) as obtained by digital droplet PCR (ddPCR) into a unit of mass fraction (e.g., milligram per kilogram), a conversion function is generated by the measurement of a reference material in the same run. For the specific detection and quantification of the allergenic ingredient soy (*Glycine max*) in food a primer/probe system has been developed which amplifies a 140 bp product of the *ndhH* gene of the chloroplast DNA. It is specific for soy and does not react with even closely related plant species. Digital droplet PCR (ddPCR) was selected for quantification for its particular advantages and the method has been validated in-house. It was found to be applicable to various matrices including meat products, flour, milk, and fatty creams, with recovery rates between 60 and 100%. The limit of detection and the limit of quantification (LOQ) are 0.16 mg/kg and 0.60 mg/kg, respectively. Repeated analysis of analyte-free food matrices spiked with reference material provided acceptable values for precision: The relative standard deviation (RSD_{overall}) of the whole method (including DNA extraction) is below 25%. The recovery of pure soy material (pulverized beans) was between 112.5 and 135.0%. The presented method is shown to be reliable and accurate, provided that samples and reference material are extracted and amplified in the same way.

Keywords Chloroplast DNA · Soybean · Quantification · Digital droplet PCR · Food allergens

Introduction

Epidemiology studies have shown that about 20% of the population of industrialized countries suffers from allergies, and its number is increasing [1].

Hypersensitivity reactions, which are also triggered by food components, are a serious food safety problem. The only way to prevent adverse effects is avoidance of their consumption [2]. To help consumers suffering from allergies

and intolerances, the European Union has provided a legislative framework for labelling of foodstuffs through regulation 1169 since 2011. Annex II of this regulation lists 14 groups of ingredients with known allergenic potential, including soy and products thereof [3]. There is a need to identify allergens specifically and to quantify them at levels as low as possible.

Apart from immunological methods, predominantly enzyme linked immunosorbent assays (ELISAs), DNA-based methods for detection and quantification prevail. Real-time PCR, also referred to as qPCR, is well established and widely used albeit some shortcomings should not be ignored. Digital PCR as a special form of endpoint PCR is a helpful alternative, because this method has three advantages over qPCR: (1) it is more tolerant to

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PCR inhibitors; (2) it shows higher sensitivity in low copy number ranges; and (3) it enables absolute quantification without the use of a standard curve [4, 5]. However, measurement results expressed in DNA copies per microliter (Cp/μL) are not very informative for customers or clients and need to be converted into units such as milligram per kilogram (mg/kg) or parts per million (ppm). This can be achieved by the measurement of a reference material, whose DNA is prepared by the same procedure as sample DNA and measured under identical conditions (i.e., in the same run).

Various qPCR methods for the detection of soy targeting nuclear DNA already exist [6–10] providing an LOD of 10 mg/kg with a probability of > 95% [10]. By taking advantage of the multicopy-gene adenosine triphosphate synthase (atpA) gene in the mitochondrial DNA of soy, the LOD and LOQ could be lowered to 1 mg/kg and 10 mg/kg, respectively [11].

To lower the limits of detection and quantification further, chloroplast DNA was chosen as target molecule, because its number of copies is even higher than that of mitochondrial DNA [11]. A plant cell encloses up to 40 chloroplast organelles, each of them with many copies of a typically circular genome approximately 120–200 kilobasepairs (kb) in length [12–14]. Meanwhile, complete chloroplast sequences of many plants are available [15] making their alignment for the identification of species-specific segments possible.

Materials and methods

All chemicals mentioned in this paper were of analytical or molecular-biological grade.

Primer and probe design

Using CLC Genomic Workbench 10.1.1, we created alignments of the whole chloroplast genome of soy (*Glycine max*, Accession no. NC_007942; 152,218 bp) with chloroplast genomes of common bean (Accession No. EU196765; 150,284 bp), peanut (Accession no. KX257487; 156,391 bp), carob bean (Accession No. KJ468096; 156,367 bp), macadamia nut (Accession no. KF862711; 159,714 bp) and chickpea (Accession no. NC011163; 125,319 bp). The alignments were screened for sections specific for soybean, and the sequences highlighted in Fig. 1 were identified as potentially specific.

PCR performed with the selected primers amplifies a 140 bp product in a region annotated as the *ndhH* gene, a subunit of the NAD(P)H dehydrogenase complex [16].

Both primers and the TaqMan[®] probe (with 6-carboxy-fluorescein as reporter dye and a minor groove binding quencher) were bought from Sigma-Aldrich and Eurogentec, respectively. The lyophilisates were dissolved in 1 × TE buffer (tris(hydroxymethyl)aminomethane $c=0,01$ mol/L, ethylenediaminetetraacetic acid-disodium salt $c=0,001$ mol/L; pH adjusted to 8.0 with hydrochloric acid) to obtain a stock concentration of 100 μmol/L. Information on nucleotide sequence and working concentration is given in detail in Table 1.

Samples and sample pretreatment

Material for specificity tests was bought at the local market or harvested from the author's garden. Great care was taken to ensure that the material originated from one plant species and was free from contamination in general and from soy contamination in particular. Whenever the purity of the material was dubious (e.g., in case of an unexpected



Fig. 1 Chloroplast-DNA alignment of *ndhH* gene of Fabaceae species and of macadamia with highlighted regions for forward primer, reverse primer and probe

Table 1 Information on nucleotide sequence and working concentration

Name	Sequence 5' → 3'	Final concentration in ddPCR
So115144a-F	GAATGGAATGAGTTTGAGTATCGATTT	0.90 µmol/L
So115144c-R	AAACCATTGATCCCTATTAGA	0.90 µmol/L
So115144e-S	<i>FAM</i> -ACCGTCTCCGACTTTTGA- <i>MGB</i>	0.25 µmol/L

cross reaction), it was washed carefully and in some cases (e.g., roots of curcuma and ginger) peeled, washed again and dried before further use.

The method of comminution was determined by the type of product. Leaves were frozen with liquid nitrogen and pulverized in a mortar. Dry kernels were ground in an ultracentrifugal mill, ZM 200, and moist products (fruits, tubers) were homogenized with a knife mill GM200 (both Retsch, Germany). When homogenization was impeded by high proportions of fat or oil (e.g., nuts, seed kernels), the material was chopped to coarse particles and defatted as described below.

Sample material (5 g) was mixed with 20 mL acetone using a Polytron PT3000 (Kinematica, Switzerland). The suspension was centrifuged at low speed (Eppendorf Centrifuge 5810 R), and the supernatant was discarded. This treatment was repeated twice with acetone and once with petroleum benzene (boiling range 40–60 °C), and the residue was air-dried at ambient temperature overnight. Using the recorded weights of the material before and after defatting, the proportion of fat free dry matter (FFDM) of the untreated material was calculated.

For the validation experiments, six soybean proficiency-test samples (for the quantification of GMO events in soy seed) from the International Seed Testing Association (ISTA) and one sample of maize kernels were used. Hazelnut cream was a real-life sample from our routine GMO testing programme.

Milk labelled as “Ja natürlich Bio Vollmilch länger frisch” containing 3.5% fat and 3.4% protein and lean pork meat were bought at the local market. Restriction enzymes Hae III and Hind III, along with the corresponding buffers, were bought from NEB (New England Biolabs).

Reference material

As reference material, we chose SureFood® QUANTARD Allergen 40 from R-Biopharm AG (Darmstadt, Germany), which is based on corn flour and contains all potentially allergenic food ingredients according to EU regulation no 1169/2011 (except for molluscs, lactose, and sulphur dioxide) at a concentration of 40 mg/kg.

DNA isolation and purification

1 g sample material (or the equivalent of FFDM) was mixed with 10 mL CTAB extraction buffer (hexadecyltrimethylammoniumbromide $c = 20$ g/L, sodium chloride $c = 1.4$ mol/L, tris(hydroxymethyl)aminomethane $c = 0.1$ mol/L, ethylenediaminetetraaceticacid-disodium salt $c = 0.02$ mol/L; adjusted to pH 8.0 with hydrochloric acid and sterilized), and 80 µL Proteinase K (Merck 1.07393). For the Quantard 40 standard, this preparation was scaled down to 200 mg. The suspension was incubated at 50 °C overnight under constant agitation and centrifuged for 10 min at 4000 rpm. Mixtures of the supernatant crude lysates were prepared for method validation (1) to simulate samples with low analyte concentration to evaluate precision and (2) to dilute lysates of pure soy material to evaluate the trueness of the method. Detailed information about the preparation of these mixtures is given in Table 2.

The (mixed) lysates (800 µL) were shaken vigorously with 800 µL chloroform and i-amylalcohol 24 + 1 (v/v). After centrifugation at 13,000 rpm for 10 min, 300 µL of the supernatant was collected and mixed with 5 µL RNase (Sigma-Aldrich, Ref: 11119915001) and 300 µL Lysis buffer, a component of the Maxwell® 16 FFS Nucleic Acid Extraction System (Custom-Ref. X9431, Promega, USA). After incubation at 65 °C for 15 min under constant agitation, the mixtures were cooled to ambient temperature and loaded into cartridges of the Maxwell® 16 Magnetic Particle Processor AS2000 for DNA isolation and purification. Finally, the DNA yield was determined by measuring the optical density at 260 nm on a QIAxpert photometer (Qiagen Hilden, Germany).

Each run of DNA isolation and purification was accompanied by two negative extraction controls.

These isolates were subjected to ddPCR without further processing.

ddPCR and data evaluation

ddPCR was carried out according to the manufacturer's recommendations [17, 18] and with the consumables provided (Bio Rad, USA): 50 µL ddPCR mixtures were made by combining 25 µL of ddPCR™ Supermix for probes (no dUTP),

Table 2 Mixtures of crude lysates and the resulting assigned analyte concentration

Lysate 1	Lysate 2	Mixing ratio (lysate 1 + 2)	Resulting mixture	Assigned analyte concentration [mg/ kg]
Quantard 40	None	–	Quantard 40	40
Quantard 40	Maize	1 + 1	Quantard 20	20
Quantard 40	Maize	1 + 3	Quantard 10	10
Quantard 40	Maize	1 + 9	Quantard 4	4.0
Quantard 20	Maize	1 + 9	Quantard 2	2.0
Quantard 10	Maize	1 + 9	Quantard 1	1.0
Quantard 2	Maize	1 + 9	Quantard 0.2	0.2
Maize	Quantard 40	3 + 1		10
Maize	Quantard 40	39 + 1		1.0
Maize	None	–		0
Meat (pork)	Quantard 40	3 + 1		10
Meat (pork)	Quantard 40	39 + 1		1.0
Meat (pork)	None	–		0
Hazelnut-cream def	Quantard 40 def	3 + 1		10
Hazelnut-cream def	Quantard 40 def	39 + 1		1.0
Hazelnut-cream def	None	–		0
Milk	Quantard 40	3 + 1		10
Milk	Quantard 40	39 + 1		1.0
Milk	None	–		0
6 “pure” soy samples	Maize	1:250,000		4.0
“Pure soy feed”	Maize	1:250,000		4.0

0.45 µL of each forward and reverse primer, 0.125 µL probe, 3.975 µL PCR-grade water and 20 µL sample DNA. A total of 20 µL of the ddPCR mixture was used for the PCR reaction in duplicate. Each PCR run was accompanied by one non-template-control (NTC). After formation of droplets and transfer of the emulsion to the microtiter plate (twin.tec PCR Plate 96, semi-skirted, colourless; Eppendorf, Germany), the plate was heat-sealed. PCR was carried out in a Mastercycler ep gradient S (Eppendorf, Germany) using the following time–temperature profile: 10 min at 95 °C, 40 cycles (30 s at 94 °C and 1 min at 63 °C), 10 min at 98 °C. Ramping rates between all holds were reduced to 50%. When the PCR had finished, the plate was conveyed to the QX200™ Droplet Reader running absolute quantification (ABS) with QuantaSoft™ Software version 1.7.4.0917 (Bio Rad, USA) for droplet counting.

For positive/negative droplet distinction, the threshold was set manually in the region between the two clusters, where the density of droplets was lowest. This threshold setting was applied uniformly to all reaction plots of a run (Fig. 2).

A two-point conversion function was created for each run obtained from measurement readings of the Quantard 40 standard and of the negative extraction control (Fig. 3).

Measurement results of single PCR wells with less than 10,000 total accepted droplets were excluded from

further data processing. The average droplet volume as stated by the manufacturer and used by the software is 0.85 nL. However, scientists measured deviating volumes depending on the way the droplets were created (automatically or manually) and the supermix used (supermix for probes or for EVA-green), but no significant intra- and inter-cartridge effects were reported [19–21]. Since the accuracy of absolute quantification is strongly dependent on droplet volume a bias would be introduced into the measurement. Our absolute results are related to a reference material which is prepared and measured under identical conditions; therefore, any deviation in droplet volume is adequately compensated.

Results and discussion

Several guidance documents on the validation of qPCR methods [22–25] and on the definition of minimum performance requirements [26] have also been accepted for digital PCR (dPCR). Additional criteria such as peak resolution, multiple amplification products, and intermediate fluorescence of droplets (“rain”) are to be considered when a dPCR method is validated [27].

Fig. 2 1D amplitude view of a ddPCR soy run. A01: non-template control; C01: negative extraction control; C03: Quantard 0.2; B03: Quantard 1; H02: Quantard 2; F02: Quantard 4; D02: Quantard 10; B02: Quantard 20; H01: Quantard 40; E03, H03 and A04 maize flour spiked with 0 ppm, 1 ppm, and 10 ppm soy. D04, F04, and H04: pork with spiking levels as above; B05, D05, and F05: milk with spiking levels as above; G05, A06, and C06: hazelnut cream with spiking levels as above. For better visibility, only one of the duplicate is displayed

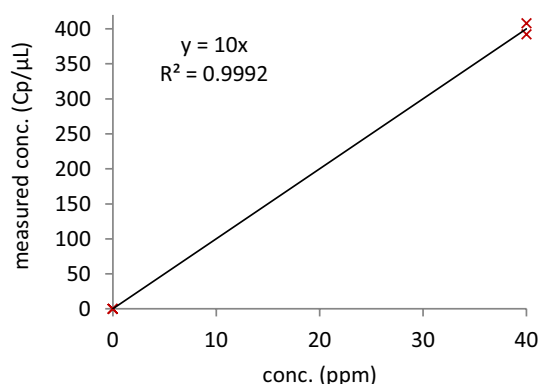
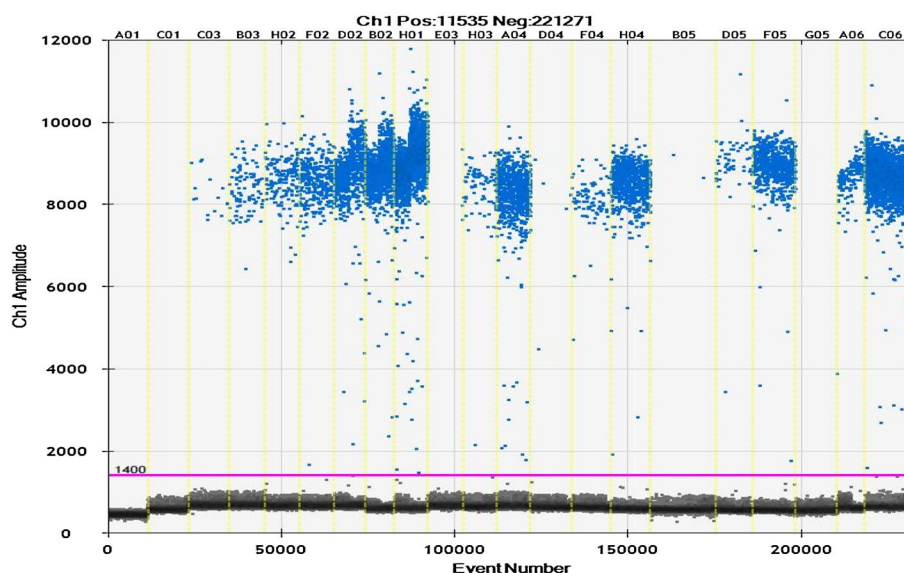


Fig. 3 Example of a two-point conversion function

Method optimization, cut-off value and specificity

The concentrations of forward primer, reverse primer and probe were used as recommended [17, 18] at 0.9 $\mu\text{mol/L}$, 0.9 $\mu\text{mol/L}$, and 0.25 $\mu\text{mol/L}$, respectively. Because these concentrations worked well, there was no need for further optimization.

To eliminate signals from cross reactivity with ginger, locust bean, curcuma and oregano, the annealing temperature was gradually raised to 63 $^{\circ}\text{C}$. This resulted in an improvement in specificity at the expense of droplet cluster resolution and increased the appearance of droplets with intermediate fluorescence.

To keep these adverse effects at a minimum, sample DNA was subjected to restriction enzyme digestion with Hae III and Hind III to linearize the circular molecules. However, no significant improvement was achieved, and therefore, this time-consuming procedure was omitted from further experiments.

Repeated measurements (104 in total) of analyte-free DNA preparations generated low positive droplet counts in some cases. The average of these measurements plus three times the standard deviation leads to a cut-off value of 0.6 $\text{Cp}/\mu\text{L}$. This corresponds to 0.06 mg/kg , a value less than 50% of our LOD. For specificity-test results below this value was classified as negative.

Test for specificity in silico

Primers and probe sequences were subjected to the Basic Local Alignment Search Tool (BLAST) provided by the National Center for Biotechnology Information (NCBI). For all three oligonucleotides, 100% identity was found with eight species of the plant genus *Glycine*, three species of *Pedimelum* and two species of *Psoraleidum*. None were found to be relevant for food production.

Test for specificity in vitro

DNA isolates from 72 plants were diluted to 0.1 $\mu\text{g/mL}$ and tested. None of the plant species listed in Table 3 yielded more than 0.4 $\text{Cp}/\mu\text{L}$.

Dynamic range, working range, LOQ, LOD average number of targets per droplet (λ), and resolution of droplet clusters

The dynamic range is the range of concentrations over which the method performs in a linear manner with an acceptable level of trueness and precision [28, 29].

Using a serial dilution of a soy-DNA solution with water, we found a linear relationship between measurement values ($\text{Cp}/\mu\text{L}$) and DNA concentration ranging

Table 3 Results of tests for specificity

English name	German name	Scientific name	English name	German name	Scientific name
Pigweed	Amarant	<i>Amaranthus</i> spp.	Garden leek	Lauch	<i>Allium ampeloprasum</i>
Anise	Anis	<i>Pimpinella anisum</i>	Lovage	Liebstöckel	<i>Levisticum officinale</i>
Garden bean	Bohne	<i>Phaseolus vulgaris</i>	Lentil	Linse	<i>Lens culinaris</i>
Summer savory	Bohnenkraut	<i>Satureja hortensis</i>	Laurel	Lorbeer	<i>Laurus nobilis</i>
Broccoli	Broccoli	<i>Brassica oleracea</i>	Lupine	Lupine gelb	<i>Lupinus arboreus</i>
Buckwheat	Buchweizen	<i>Fagopyrum esculentum</i>	Macadamia nut	Makadamianuss	<i>Macadamia</i> spp.
Cashew nut	Cashewnuss	<i>Anacardium occidentale</i>	Maize	Mais	<i>Zea mays</i>
Cayenne pepper	Cayennepfeffer	<i>Piperaceae</i> spp.	Sweet majoram	Majoran	<i>Origanum majorana</i>
Chinese cabbage	Chinakohl	<i>Brassica rapa</i>	Almond	Mandel	<i>Prunus dulcis</i>
Dill weed	Dille	<i>Anethum graveolens</i>	Oregano	Oregano	<i>Origanum vulgare</i>
Spelt	Dinkel	<i>Triticum aestivum</i>	Pak Choi	Pak Choi	<i>Brassica rapa chinensis</i>
Durum	Durum	<i>Triticum durum</i>	Paprika	Paprika	<i>Capsicum</i>
Sweet chestnut	Edelkastanie	<i>Castanea sativa</i>	Brazil nut	Paranuss	<i>Bertholletia excelsa</i>
Pea	Erbse	<i>Pisum sativum</i>	Parsnip	Pastinake	<i>Pastinaca sativa</i>
Peanut	Erdnuss	<i>Arachis hypogaea</i>	Pecan	Pecannuss	<i>Carya illinoensis</i>
Tarragon	Estragon	<i>Artemisia dracunculoides</i>	Parsley	Petersilie	<i>Petroselinum crispum</i>
Fennel	Fenchel	<i>Foeniculum vulgare</i>	Pepper	Pfeffer	<i>Piper nigrum</i>
Guar	Guar	<i>Cyamopsis tetragonolobus</i>	Pimento	Piment	<i>Pimenta dioica</i>
Cucumber	Gurke	<i>Cucumis sativus</i>	Canola	Raps	<i>Brassica napus</i>
Oat	Hafer	<i>Avena</i> spp.	Rice	Reis	<i>Oryza sativa</i>
Hazelnut	Haselnuss	<i>Corylus avellana</i>	Garden radish	Rettich	<i>Raphanus sativus</i>
Ginger	Ingwer	<i>Zingiber officinale</i>	Rye	Roggen	<i>Secale cereale</i>
Locust bean	Johannisbrot	<i>Ceratonia siliqua</i>	Rosemary	Rosmarin	<i>Rosmarinus officinalis</i>
Kamut	Kamut	<i>Triticum turgidum</i> × <i>polonicum</i>	Common sage	Salbei	<i>Salvia officinalis</i>
Cardamon	Kardamom	<i>Elettaria cardamomum</i>	Wild chive	Schnittlauch	<i>Allium schoenoprasum</i>
Cauliflower	Karfiol	<i>Brassica oleracea</i> var. <i>botrytis</i>	Celery	Sellerie	<i>Apium graveolens</i>
Carrot	Karotte	<i>Daucus carota</i> subsp. <i>sativus</i>	Black mustard	Senf schwarz	<i>Brassica nigra</i>
Potato	Kartoffel	<i>Solanum tuberosum</i>	White mustard	Senf weiss	<i>Sinapis alba</i>
Chickpea	Kichererbse	<i>Cicer arietinum</i>	Sesame	Sesam	<i>Sesamum indicum</i>
Garlic	Knoblauch	<i>Allium sativum</i>	Thyme	Thymian	<i>Thymus vulgaris</i>
Kohlrabi	Kohlrabi	<i>Brassica oleracea</i> var. <i>gongylodes</i>	Tomato	Tomate	<i>Solanum lycopersicum</i>
Cilantro	Koriander	<i>Coriandrum sativum</i>	Triticale	Triticale	<i>Triticale</i>
Horseradish	Kren	<i>Armoracia rusticana</i>	Walnut	Walnuss	<i>Juglans regia</i>
Cumin	Kreuzkümmel	<i>Cuminum cyminum</i>	White cabbage	Weißkraut	<i>Brassica oleracea</i> convar. <i>capitata</i> var. <i>alba</i>
Caraway	Kümmel	<i>Carum carvi</i>	Wheat	Weizen	<i>Triticum aestivum</i>
Curcuma	Kurkuma	<i>Curcuma longa</i>	Onion	Zwiebel	<i>Allium cepa</i>

from 0.109 ng/mL (1.0 Cp/μL; $\lambda = 0.0008$) to 444 ng/mL (5960 Cp/μL; $\lambda = 5.0662$) with a coefficient of regression (R^2) above 0.98.

The working range is located within the dynamic range and the RSD of measurement values within this range is below 25%.

Three different lots of the reference material were subjected to DNA isolation. DNA extracts were diluted in consecutive 1:2 ratios with a solution of maize DNA, yielding soy concentrations from 40 mg/kg (undiluted) to 0.0195 mg/

kg. Each dilution was measured repeatedly, giving 14 individual results in total.

The upper limit of the working range is determined by the allergen concentration of the SureFood® Quantard Allergen standard (i.e., 40 mg/kg). The lowest concentration for which RSD was below 25% was 0.625 mg/kg. This is the lower end of the working range and the LOQ of the method. We measured 7.1 Cp/μL with a standard deviation of ± 1.6 Cp/μL. Our absolute LOQ is 142 copies in 20 μL PCR volume containing 8 μL sample solution (Table 4).

Table 4 Working range with characteristics of measurement quality

Soy conc. [mg/kg]	Number of measurements	Copies per microliter PCR	λ	RSD $_{\lambda}$ (%)	Accepted droplets (mean)
40	14	468	0.3981	24.46	12,949
20	14	236	0.2000	24.50	13,772
10	14	115	0.0974	23.57	13,782
5	13	57	0.0483	22.15	13,178
2.5	14	30	0.0253	21.70	13,408
1.25	14	15	0.0127	22.06	13,654
0.625	13	7.1	0.0060	22.70	15,285
0.313	14	3.65	0.0031	31.12	14,946
0.156	14	1.64	0.0014	30.2	14,642
0.078	14	0.77	0.0007	35.95	14,533
0.039	13	0.40	0.0003	42.11	13,883
0.02	12	0.18	0.0001	76.75	14,728

The lowest concentration where 10 of 10 measurements were positive (i.e., above the cutoff) was 0.156 mg/kg. At this level 1.62 Cp/ μ L PCR with a standard deviation of ± 0.49 Cp/ μ L was measured. The absolute LOD is therefore 32.4 copies in a 20 μ L PCR reaction volume containing 8 μ L sample solution.

The average number of targets per droplet (λ) was calculated using the following equation [29]:

$$\lambda = -\ln \times \left(1 - \frac{k}{n}\right),$$

where k is the number of positive droplets and n is the number of accepted droplets.

Figure 4 shows a histogram of the ddPCR analysis containing 125 ng soy DNA per microliter (without any non-target DNA).

This histogram was used to manually estimate the peak resolution R_s according to the following equation [30]:

$$R_s = \frac{2 \times (t_p - t_n)}{w_p + w_n},$$

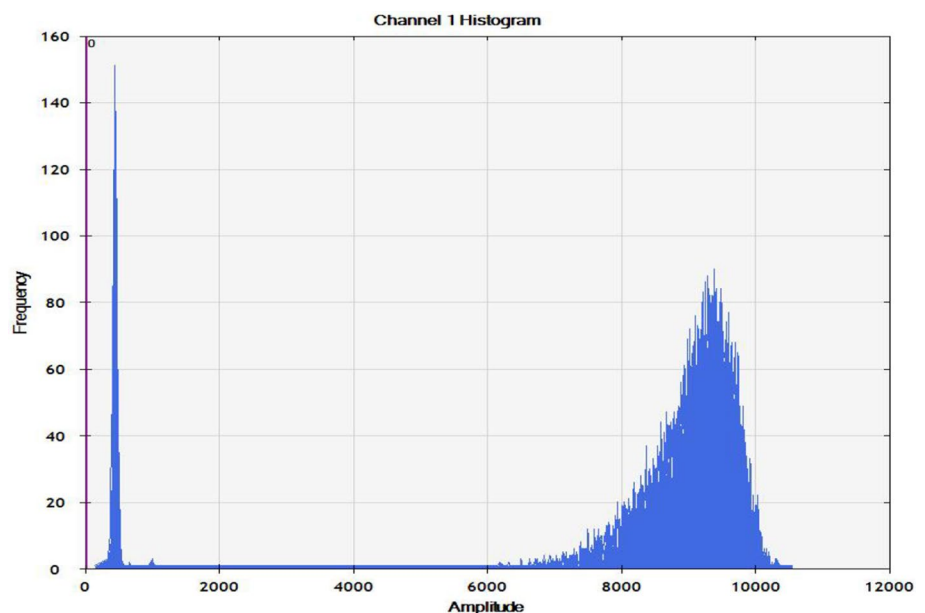
where t_p (9330) and t_n (440) are the amplitudes of positive and negative peaks and w_p (10240–6560) and w_n (588–290) represent the width of the peaks at their base. The calculated R_s is 4.47 which is well above the recommended minimum of 2.5 and allows tolerance necessary for partially inhibited samples (see Fig. 4) [30].

Selectivity

Selectivity is the degree to which a method can quantify the particular analyte accurately in the presence of interfering substances that could be present in a sample [31]. To investigate the effect of interfering substances on accurate quantification, we tested different types of matrices.

Due to the distribution of target DNA throughout many thousand partitions, dPCR is expected to be less impaired by inhibitory substances than qPCR. However, in our PCR protocol, the proportion of sample solution is high (8 μ L in 20 μ L total PCR volume), and the risk of introducing inhibitory substances into the PCR is therefore also elevated.

Selectivity was tested with four different types of matrices: (1) maize flour; (2) pork; (3) milk; and (4) hazelnut cream. We could not observe matrix-specific effects on the performance of the method. Only the LOQ in the milk

Fig. 4 Histogram for the estimation of peak resolution

matrix required adjustment to a level yet to be determined (presumably between 2 and 5 mg/kg).

However, when carrying out repeated extractions of maize samples, varying concentrations of total DNA (123–333 µg/mL) were measured in the isolates. This might be attributed to different lots of Maxwell cartridges used. When subjected to ddPCR, we found that increasing the amount of total DNA impaired the separation of droplet clusters. A repetition of the experiment using meat DNA as background confirmed this observation (Fig. 5). The DNA isolate from the Quantard 40 standard was mixed 1 + 3 (resulting in a Q10 sample; see Table 2) with diluents containing increasing concentrations of DNA from turkey, so that the mass of total DNA in the PCR ranged from 120 to 4680 ng.

According to these experiments, the amount of total DNA introduced into ddPCR should not exceed 1300 ng. This corresponds to approximately 160 µg/mL sample solution.

Recovery and repeatability

Lysates of the Quantard 40 standard were mixed with lysates of maize flour, pork, and milk, and the lysate of defatted hazelnut cream was mixed with a lysate of defatted Quantard 40 (see Table 2). DNA was isolated from these lysate mixtures and subjected to ddPCR measurement. This series of preparations was repeated *N* times, giving 2*N* results (odd numbers in Table 5 indicate that one measurement had to be excluded due to an insufficient number of accepted droplets). Each series of DNA preparations comprised two negative extraction controls and one extraction of Quantard 40 material, which were used to establish the conversion function.

Recovery data, even at 1 mg/kg (which is slightly above the LOQ), are within acceptable limits (80–110%). However, one milk sample (spiked with 1 mg/kg of soy) could only be

measured with lower recovery. The reason for this deficiency is subject to further investigation. As a consequence, the LOQ for this matrix is yet to be determined.

Data for repeatability expressed as RSD_{overall} are satisfying considering that two technicians worked over a 2-month period performing the testing procedure. An overview of these results is given in Table 5.

Trueness

To investigate the closeness of agreement between the average value obtained from a large series of test results and an accepted reference value [32], we tested seven soybean samples.

A reference material with a certified value of soy chloroplast-DNA copies does currently not exist but well-characterized material distributed for proficiency tests is an acceptable alternative [33].

Pure soybeans without any additives, admixtures, or impurities that had not undergone any denaturing treatment were provided by ISTA in the context of their GMO proficiency-test programme. We accepted them for this purpose because the molecular and biochemical intactness of the chosen material was demonstrated. The expected analyte concentration was 10^6 mg/kg.

Lysates of finely ground beans were mixed with lysates of maize flour to obtain a soy concentration of 4 mg/kg, the assigned concentration for trueness experiments and adequate for the working range of the method. From these lysate mixtures, DNA was isolated and subjected to ddPCR measurement. A series of preparations including two extraction controls and one extraction of Quantard 40 material were used to establish the conversion function. Each series

Fig. 5 Influence of non-target DNA (ntDNA) on the performance of ddPCR. Each sample (20 µL PCR volume) contained soy DNA from Q10 and an increasing concentration in mass of ntDNA from turkey. A01 and B01: NTC; C01 and D01: Q10 plus 120 ng ntDNA; E01 and F01: 800 ng ntDNA; G01 and H01: Q10 plus 720 ng ntDNA; A02 and B02: 1600 ng ntDNA; C02 and D02: Q10 plus 1320 ng ntDNA; E02 and F02: 3200 ng ntDNA; G02 and H02: Q10 plus 2520 ng ntDNA; A03 and B03: 4800 ng ntDNA; C03 and D03: Q10 plus 3720 ng ntDNA; E03 and F03: 6080 ng ntDNA; G03 and H03: Q10 plus 4680 ng ntDNA

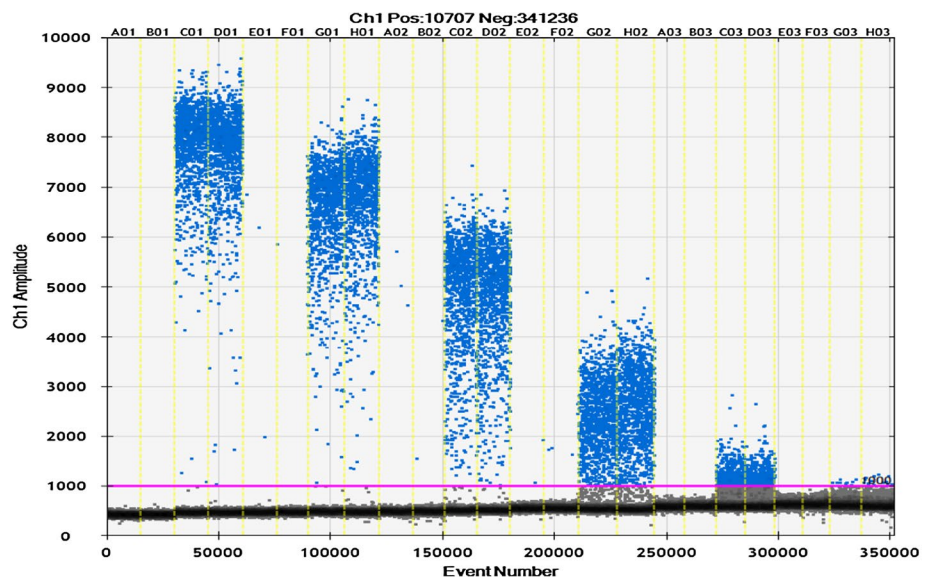


Table 5 Recovery and repeatability

Sample	Assigned analyte conc. [mg/kg]	2N	Cp/ μ L	Determined analyte conc. [mg/kg]	RSD _{overall} (%)	Recovery (%)	λ (mean)	Accepted droplets= (mean)
Q0.2 (outside of the working range)	0.2	15	3.38	0.34	72.4	170	0.00292	14,553
Q1	1.0	16	9.26	0.95	23.1	95	0.00787	14,493
Q2	2.0	16	17.1	1.76	11.4	88	0.01434	14,953
Q4	4.0	16	36.8	3.80	7.87	95	0.03119	14,863
Q10	10	16	92.8	9.61	5.16	96	0.07903	14,792
Q20	20	16	186	19.1	5.15	96	0.15826	14,479
Maize + 1 ppm	1.0	16	10.5	1.07	16.4	107	0.00886	14,946
Maize + 10 ppm	10	16	96.8	10.0	9.77	100	0.08228	14,685
Pork + 1 ppm	1.0	10	7.15	0.72	14.1	72	0.00839	15,421
Pork + 10 ppm	10	16	80.4	8.36	11.0	84	0.06831	15,821
Milk + 1 ppm	1.0	16	6.14	0.63	27.9	63	0.00541	15,190
Milk + 10 ppm	10	16	82.8	8.61	9.35	94	0.07038	15,265
Hazelnut-cream + 1 ppm	1.0	11	8.65	0.90	18.3	90	0.00740	15,240
Hazelnut-cream + 10 ppm	10	12	94.9	9.92	12.4	99	0.08057	15,181

was repeated N times giving $2N$ results. An overview of the results for the determination of trueness is listed in Table 6.

A possible explanation for the increased recovery might be the molecular and/or biochemical difference (e.g., copy number or material treatment) between soybeans under investigation and the soy material used in the Quantard 40 standard.

Comparison to another method

DNA isolates from 25 samples that had been tested negative for soy in 2017 using the method described in [10] (the LOD of this method is 10 mg/kg) were reanalysed with the method presented here. For 13 samples the first result was confirmed, 5 samples were found to be positive but below the LOQ and for 7 samples soy contents between 0.76 and 2.04 mg/kg were determined.

Conclusion

The method presented in this paper was tested extensively for specificity against a wide variety of plant species that are relevant for food production, and it was found to be specific.

Experts are of the opinion that detection limits for allergens in different food products need to be in the low ppm (mg/kg) range, depending on the respective allergen and food product [33].

Therefore, chloroplast DNA was chosen as the analyte, because it is known to have high copy numbers in plant cells.

By measuring a reference material that is certified in mass, a conversion function can be established to obtain results in units of mass fraction. As a consequence, all quantitative results can only be interpreted as the concentration of soy derived from the chosen reference material.

The LOD of the assay is 0.16 mg/kg and the LOQ is 0.63 mg/kg, provided that no additional dilution is necessary

Table 6 Overview of the results for the determination of trueness

Sample	Assigned analyte conc. [mg/kg]	2N	Cp/ μ L	Determined analyte conc. [mg/kg]	RSD _{overall} (%)	Recovery (%)	λ (mean)	Accepted droplets (mean)
1. Pulverized beans	4	12	43.2	4.5	20.0	112.5	0.05790	15,681
2. Pulverized beans	4	12	47.7	5.0	25.6	125.0	0.05115	15,620
3. Pulverized beans	4	12	48.5	5.1	12.2	127.5	0.04125	14,975
5. Pulverized beans	4	14	51.0	5.4	23.6	135.0	0.04338	14,867
6. Pulverized beans	4	14	46.7	4.9	9.18	122.5	0.03969	15,653
7. "Pure soy feed"	4	14	43.8	4.6	11.0	115.0	0.03725	15,097

to be in compliance with the maximum DNA mass of 1300 ng tolerated in 20 µL PCR volumes. The LOQ for milk, presumably between 2 and 5 mg/kg, has not been assessed yet.

The recovery from several spiked food matrices was 80–110% with an RSD_{overall} below 30% (except for low analyte levels in milk).

A recurrent challenge associated with biochemical analysis methods is the discrepancy of analyte condition between the reference material and unknown samples caused by degenerative effects of manufacturing processes. In addition, varying chloroplast-DNA copy numbers even in different parts of the same plant should be considered. The bias observed when running repeated analysis of pure native soy material was not above 30%; however, more deviation is expected with processed soy products, and such behaviour should be investigated.

The method presented in this paper is sufficiently specific, precise, and sensitive for the detection and quantification of the allergen soy at low levels in food. For comparison of quantitative results with other methods and for inter-laboratory ring trials, a stipulated reference material is needed.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects.

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