

Sensitive Detection of Soy (*Glycine max*) by Real-Time Polymerase Chain Reaction Targeting the Mitochondrial *atpA* Gene

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Detection of trace amounts of allergens is essential for correct labeling of food products by the food industry. PCR-based detection methods currently used for this purpose are targeting sequences of DNA present in the cell nucleus. In addition to nuclear DNA, a substantial amount of mitochondrial DNA (mtDNA) copies are present in the cytoplasm of eukaryotic cells. The nuclear DNA usually consists of a set of DNA molecules present in two copies per cell, whereas mitochondrial DNA is present in a few hundred copies per cell. Thus, an increase in sensitivity can be expected when mtDNA is used as the target. In this study, we present a reporter probe-based real-time PCR method amplifying the mitochondrial gene of the alpha chain of adenosine triphosphate synthetase from soy. Increase in sensitivity was examined by determining the minimal amount of soy DNA detectable by mtDNA and nuclear DNA (nDNA) amplification. Additionally, the LOD of soy in a food matrix was determined for mtDNA amplification and compared to the LOD determined by nDNA amplification. As food matrix, a model spice spiked with soy flour was used. Sensitivity of PCR-based soy detection can be increased by using mtDNA as the target.

Preventing health problems by food allergen avoidance can be very difficult for people suffering from food allergies, because hidden allergens can be present in the common daily diet. Trace amounts of allergenic impurities may enter food products during

the production process. This may occur by carryover of food components when different formulations of food products are produced on the same production line, if raw materials are already contaminated, or by allergens in the air. In the near future, specific threshold values will be established, making it possible to label products more precisely and to simplify law enforcement. Analytical methods will be needed to determine whether contents of allergens are above or below established threshold levels.

The Food and Agriculture Organization of the United Nations includes soy in its list of the eight most significant food allergens. Nevertheless, soy is widely used by the food industry in many different products for its functionality, such as texture improvement, viscosity building, protein filler, water and oil management, and gelling properties (1). The widespread use of soy by the food industry leads to a high risk of cross-contamination of food products not containing soy as an ingredient. Sensitive analytical methods are important to determine the presence of soy contamination in order to protect sensitized persons. Sensitive soy detection methods can also help to perform detailed allergen management during the production of foodstuffs, thereby helping to prevent occurrence of cross-contamination.

In recent years, several protein- and DNA-based methods for the detection of allergenic components in food have been developed (2). A comparison of protein and DNA-based detection of soy in different food products showed better results for DNA-based detection (3). This may be due to food processing. High temperatures during cooking, baking, or enzymatic treatments can denature proteins. As a result, the epitopes necessary for antigen recognition can be deformed, and binding of antibodies used for protein detection is less efficient. DNA is more stable than proteins; thus, PCR-based allergen detection is more reliable in processed foods.

Several PCR-based soy detection methods in food have been developed (4–7). These methods use the

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Table 1. Intensity of cross-reactivity for the *atpA* primers and probe with examined foodstuffs

Food ingredient	Cross-reactivity, ppm ^a	Cross-reactivity, % ^a	Food ingredient	Cross-reactivity, ppm ^a	Cross-reactivity, % ^a
Soy	(1000000)	(100)	Corn	1.2	0.00012
Potato	252	0.02520	Almond	1.2	0.00012
Carob	113	0.01125	Apple	1.2	0.00012
Eggplant	56	0.00560	Rice	1.1	0.00011
Pistachio	33	0.00327	Melon	1.0 ^b	0.00010 ^b
Tomato	31	0.00305	Oregano	1.0	0.00010
Blueberry	24	0.00238	Prawn	1.0	0.00010
Apricot	17	0.00165	Macadamia	0.9	0.00009
Bean	16	0.00160	Sesame	0.9	0.00009
Lupin	15	0.00152	Oat	0.7	0.00007
Cinnamon	14	0.00136	Coconut	0.6 ^c	0.00006 ^c
Juniper	8.8	0.00088	Cashew	0.5 ^b	0.00005 ^b
Nutmeg	7.5	0.00075	Blackberry	0.4	0.00004
Coffee bean	5.5	0.00055	Brazil nut	0.4 ^d	0.00004 ^d
Chickpea	4.6	0.00046	Caraway	0.3 ^b	0.00003 ^b
Beetroot	4.2	0.00042	Poppy seed	0.3 ^c	0.00003 ^c
Celery	4.1	0.00041	Wheat	0.3 ^d	0.00003 ^d
Linseed	3.8	0.00038	Anise	0.2 ^b	0.00002 ^b
Peanut	3.8	0.00038	Bovine milk	0.2 ^d	0.00002 ^d
Pepper	3.6	0.00036	Mustard	0.2	0.00002
Bay leaf	3.5	0.00035	Cucumber	0.2 ^d	0.00002 ^d
Pumpkin seed	3.3	0.00033	Grape	0.2 ^c	0.00002 ^c
Hazelnut	3.0	0.00030	Mushroom	0.2 ^c	0.00002 ^c
Lentil	1.9	0.00019	Paprika	0.1	0.00001
Ginger	1.5	0.00015	Mallow	NA ^e	
Pea	1.5	0.00015	Carnation	NA	
Sunflower	1.5	0.00015	Chive	NA	
Tarragon	1.4	0.00014	NTC ^f	NA	
Walnut	1.3	0.00013			

^a Values are the mean of four PCR reactions, performed in two PCR runs in duplicate, unless otherwise stated.

^b Values are the mean of three positive PCR reactions out of four, performed in two PCR runs in duplicate.

^c Values are the mean of two positive PCR reactions out of four, performed in two PCR runs in duplicate.

^d Value is of one positive PCR reaction out of four, performed in two PCR runs in duplicate.

^e NA = No amplification.

^f NTC = No template control.

amplification of nuclear DNA (nDNA) sequences. However, in eukaryotic cells, DNA can also be found within mitochondria (mtDNA) and chloroplasts (cpDNA). nDNA consists of the chromosomes, which, except for gonosomes, are present in a set of two homologous copies per cell. Accordingly, one cell has two similar copies of nDNA, which can be detected by a PCR reaction

with primers targeting a unique nDNA sequence. In mammalian cells, about several hundred copies of similar mtDNA molecules are present in the cytoplasm (8). Plant cells typically contain hundreds of copies of mtDNA and thousands of copies of cpDNA (9). Because there are more mtDNA and cpDNA copies/cell than nDNA copies, higher detection sensitivity can be expected by using

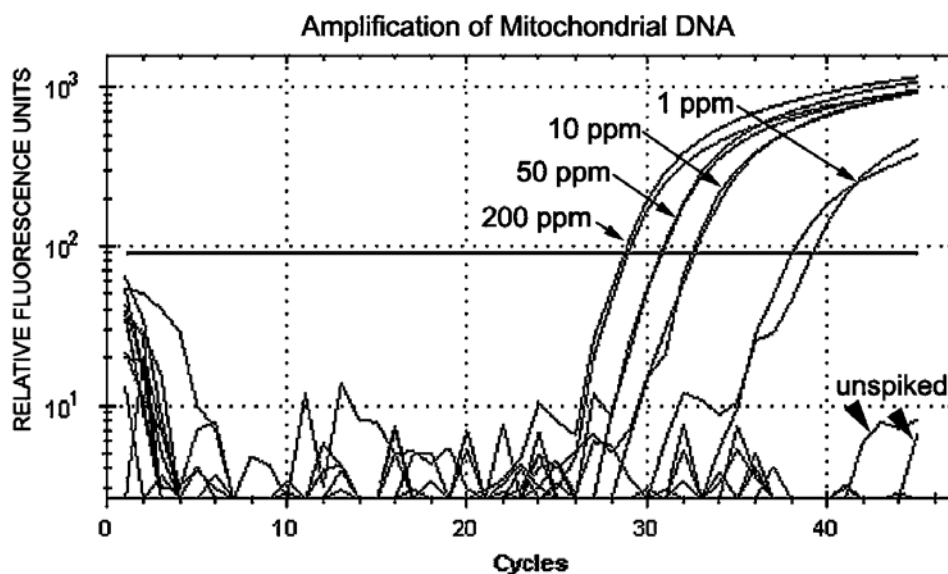


Figure 1. Amplification plots of real-time PCR obtained by mitochondrial soy DNA detection using primers and probe targeting the *atpA* gene. By applying DNA extract from model spice spiked with 200, 50, 10, and 1 ppm soy flour, amplification plots were obtained (arrows). Threshold was set at about 100 relative fluorescence units (horizontal line). DNA extract from blank spice did not result in the formation of amplification plots (arrowheads).

PCR-based test systems targeting mtDNA or cpDNA sequences compared to nDNA sequences. PCR-based allergen detection methods using mtDNA amplification (10, 11) and cpDNA amplification (12) have been reported, but not for soy.

The aim of the present study was to develop a PCR for the detection of soy in processed foods based on mtDNA amplification. The increase in soy detection sensitivity obtained by using mtDNA amplification compared to nDNA amplification was examined.

Experimental

Primer and Probe Design for the Detection of Mitochondrial Soy DNA by Real-Time PCR

At the time of primer and probe design (November 2008), six different mtDNA sequences of genes from *Glycine max* were available at GenBank. In order to reduce possible cross-reactivity with other plant species, available sequences were analyzed by Basic Local Alignment Search Tool (BLAST) using a query against the entire GenBank database. The sequence of the alpha chain of adenosine triphosphate synthetase (*atpA*; GenBank No. Z14031) offered some regions that did not show any similarity with sequences in GenBank, thus indicating suitable conditions for a sequence-specific detection of soy. Within the sequence area from nucleotide 2249 to nucleotide 2700 of the alpha chain of *atpA*, we were able to design a functional set of primers with corresponding reporter probe. Primers and reporter probe for the detection of mitochondrial soy DNA were created by the Primer3 open source software (v.0.4.0,

<http://primer3.sourceforge.net>). The following primers and probe producing an amplicon of 113 bp were used: forward primer “*atpA F*” 5'-tgattcccgaaacttgacctc-3'; reverse primer “*atpA R*” 5'-agtcgaggatcgctcgta-3'; and probe “*atpA P*” 5'-6-FAM-agcagagctcgctgttcgattgag-BHQ-1-3'. Primers and probes were purchased from biomers.net GmbH (Ulm, Germany).

Even though the genome sequence of soy was published in January 2010 (13), no additional annotated mitochondrial genome sequences of soy were added in GenBank since the time primer and probe design was performed. A BLAST search in October 2010 showed mtDNA sequences of other food-relevant plants added in May and June 2010 with similarity to the *atpA* sequence area used for primer and probe design. A 189 bp sequence area present in plants belonging to the family of solanaceans (*Solanum lycopersicum*, *S. commersonii*, and *S. somatic* hybrid sp.) showed a similarity of 69% to the *atpA* sequence area used for primer and probe design. To examine intensity of cross-reactivity of solanaceans, food-relevant plants belonging to the family of solanaceans (potato, eggplant, and tomato) were included in specificity tests of *atpA* primers and probe (Table 1).

Extraction of Total DNA from Food Samples and Soybeans

Food samples (Table 1), including soybeans which were used for LOD determination of pure total soy DNA, were purchased at local stores, except for lupin and soy flour, which were obtained from the State Institute of Chemical and Veterinarian Analysis, Sigmaringen, Germany. The

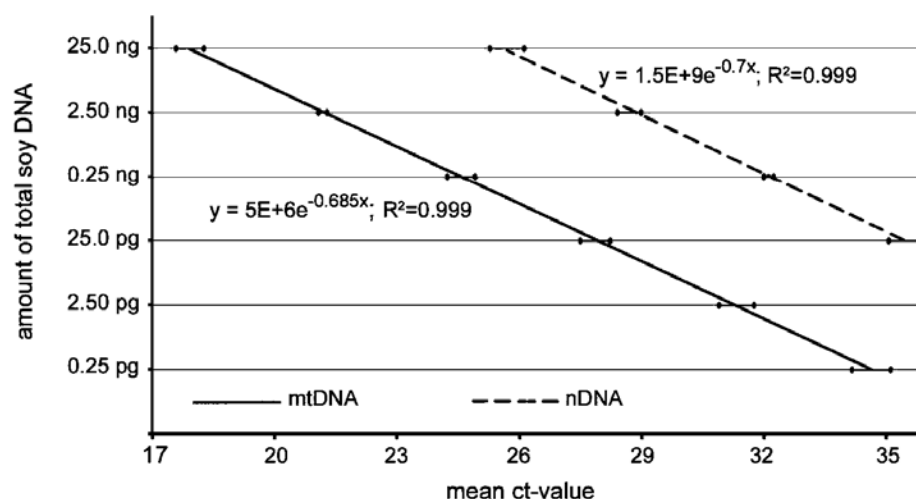


Figure 2. Calibration curves for mtDNA (continuous line) and nDNA (broken line) real-time PCR amplification of total soy DNA converting ct-values to the amount of target DNA in ng. Mean ct-value difference between mtDNA and nDNA amplification is 7.63 ± 0.12 ($n = 6$), reflecting an increased sensitivity of about two orders of magnitude. By mtDNA detection, all repeats and replicates showed amplification at a minimal amount of 0.25 pg total DNA. By nDNA detection, the minimal amount of total DNA resulting in amplification in all repeats and replicates was 100-fold higher at 25.0 pg. Sensitivity of soy detection was increased about two orders of magnitude by mtDNA amplification compared to nDNA amplification.

56 food samples were crushed, pulverized, or otherwise finely ground in a mortar prior to DNA extraction. Total DNA of all food samples, except for coffee bean, was extracted by the following cetyltrimethylammonium bromide (CTAB) method: Amounts of 0.2–0.5 g of food samples were mixed with 1500–1800 μL CTAB extraction buffer (2%, w/v) CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-OH/HCl, and 30 μL of 0.01 M proteinase K followed by incubation at 65°C for 90 min. After 10 min of centrifugation at $20\,000 \times g$, 500–700 μL of supernatant was transferred into a new tube, followed by addition of 5 μL 0.1 g/mL RNase. After 10 min of incubation at 37°C, 700 μL chloroform–isoamylalcohol (24 + 1, v/v) was added, and the samples were gently mixed and centrifuged at $20\,000 \times g$ for 10 min. The aqueous phase was then transferred into new tubes, and 700 μL of 100% isopropyl alcohol was added, followed by gently mixing and centrifugation at $20\,000 \times g$ for 10 min. The supernatant was discarded, and 700 μL 70% ethanol (v/v) was added. After 5 min of centrifugation at $20\,000 \times g$, the supernatant was discarded and the pellet was dried for 3 min in a vacuum centrifuge at room temperature. The pellet was then dissolved in 100 μL purified water, and DNA extracts were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. Concentration of DNA was determined by using a spectrophotometer (Implen, Munich, Germany). Coffee bean DNA could not successfully be obtained by the CTAB method, and was extracted using the QIAmp Stool Mini Kit (Qiagen) according to the manufacturer's instructions. DNA extracts were analyzed by agarose gel

electrophoresis in order to confirm the integrity of DNA (data not shown). All DNA samples were adjusted to a final concentration of 5 ng/ μL . DNA extracted from soy was diluted in 10-fold serial dilutions (5–0.5 fg/ μL) using purified water.

Real-Time PCR

The developed real-time PCR system for the detection of mtDNA of soy was arranged in amplification reactions (25 μL) containing 5 μL of sample extracts, 10 μL of $2 \times$ iQ Supermix (Bio-Rad Laboratories, Hercules, CA) and 10 pmol of each atpA primer and the atpA probe. Real-time PCR reactions were performed on CFX96 real-time PCR System (Bio-Rad Laboratories) according to the following thermal cycling program: initial denaturation at 95°C for 3 min, followed by 45 cycles of denaturation at 95°C for 10 s; annealing at 59°C for 30 s; and elongation at 72°C for 30 s. For all samples, the threshold was manually set into the exponential phase of the amplification curve at about 100 relative fluorescence units. Real-time PCR experiments were analyzed using the Bio-Rad CFX Manager Software version 1.0. Additionally, Rotor-Gene 6000 (Corbett Life Science, Mortlake, Australia) was used for analysis of robustness. The detection of soy nDNA was performed using the commercially available SureFood® Soy ALLERGEN kit (CONGEN, Berlin, Germany) according to the manufacturer's instructions. Threshold line was set at about 100 relative fluorescence units in all experiments. Threshold cycle (ct)-values were determined by the crossing point of the manually set threshold line and the amplification plots (Figure 1).

Table 2. Determination of LOD for mtDNA and nDNA amplification using a model spice spiked with defatted soy flour, and recovery rates for soy and copies of mtDNA molecules based on mtDNA detection

Content of soy flour in model spice	mtDNA detection				nDNA detection	
	Positive results/No. of reactions	Calculated content of soy flour in model spice ^a	Calculated copies of mtDNA molecules ^a	Mean ct-values $\pm$ SD	Positive results/No. of reactions	Mean ct-values $\pm$ SD
200 ppm	18/18	204.5 $\pm$ 91.1 ppm	2453 $\pm$ 1093	28.97 $\pm$ 0.67	14/14	36.58 $\pm$ 1.48
50 ppm	18/18	63.9 $\pm$ 23.3 ppm	639 $\pm$ 234	30.47 $\pm$ 0.31	12/14	37.87 $\pm$ 1.58
10 ppm	18/18	9.5 $\pm$ 2.9 ppm	86 $\pm$ 26	32.41 $\pm$ 0.37	5/14	38.77 $\pm$ 0.84
1 ppm	17/18			37.09 $\pm$ 1.31	3/14	38.61 $\pm$ 1.63
0.1 ppm	4/18			39.75 $\pm$ 0.45	NA ^b	
Unspiked	NA				NA	
NTC ^c	NA				NA	
LOD	1 ppm				65 ppm	

^a Only samples within the LOQ (≥ 10 ppm) were considered for calculations.

^b NA = No amplification.

^c NTC = No template control.

Determination of Cross-Reactivity for *atpA* Primers and Probe

To evaluate the applicability of the mitochondrial *atpA* real-time PCR test system for the specific detection of soy DNA in food, cross-reactivity with DNA of 56 different foodstuffs was examined. Foodstuffs were chosen on the basis of relevance as food allergen (14) and by the attempt to enclose a widespread range of different vegetable plant families to cover different variations of the *atpA* gene in a comprehensive way. Two PCR runs, with samples of 25 ng total DNA of soy and foodstuffs in duplicates, were performed. The degree of cross-reactivity was calculated by the following formula:

$$\text{Mean ct-value of analyzed foodstuff} - \text{mean ct-value of soy}$$

Percentage of cross-reactivity was calculated on the fact that a difference of 3.32 ct-values represents a 10-fold concentration difference in template DNA molecules (15). Intensity of cross-reactivity in ppm was done by calculating the intensity of cross-reactivity in percent of pure soy reflecting 10^6 ppm.

LOD Determination of Soy Spiked into a Model Spice

A model spice (blank spice) containing table salt (50%, w/w), yeast extract (10%, w/w), paprika (10%, w/w), Kyrosan[®] (10%, w/w), saccharose (10% w/w), black pepper (5%, w/w), and onion powder (5%, w/w) was prepared in a vacuum kneading machine (AV80, Fatosá, Sabadell, Spain) at a batch of 30 kg. To 49.6 g blank spice, 0.4 g defatted soy flour was added and manually mixed

for several min, resulting in a spiking concentration of 8000 ppm defatted soy flour. Model spice with 800 ppm was prepared by adding 45 g blank spice to 5 g 8000 ppm model spice. Model spice with 160 ppm was prepared by adding 75 g blank spice to 25 g 800 ppm model spice. Model spice spiked with 80 ppm was prepared by adding 1 g blank spice to 1 g 160 ppm model spice. Spiked model spice samples with 40, 8, 0.8, and 0.08 ppm were prepared by adding 3 g blank spice to 1 g 160 ppm model spice, 4 g blank spice to 1 g 40 ppm model spice, 9 g blank spice to 1 g 8 ppm model spice, and 9 g blank spice to 1 g 0.8 ppm model spice. Dilutions of spiked model spice were always prepared by adding blank spice in small portions and manually mixing after each addition for several min. Spices were obtained from S.A.M. (Stockstadt, Germany), and soy flour was obtained from Rubin Mühle GmbH (Lahr, Germany). Due to an average oil content of about 20% in soy (16), the spiked concentrations reflect 10 000, 1000, 200, 50, 10, 1, and 0.1 ppm full fat soy flour in the model spice. Amounts of 0.25 g of each dilution and blank spice were used to extract DNA by the SureFood[®] PREP kit (CONGEN) according to the manufacturer's instruction.

In PCR experiments, LOD can be defined as the minimal amount of target material still detectable with 95% confidence (17). Calculation of confidence was done by probit analysis (18). To obtain data for LOD calculation, DNA extracts of 200, 50, 10, 1, and 0.1 ppm soy flour in the model spice were prepared. For mtDNA amplification, 18 PCR reactions of DNA extracts of each concentration were performed. For nDNA amplification, 14 PCR reactions of DNA extracts of each concentration were performed. For each concentration, the numbers of positive PCR reactions per performed PCR reactions were

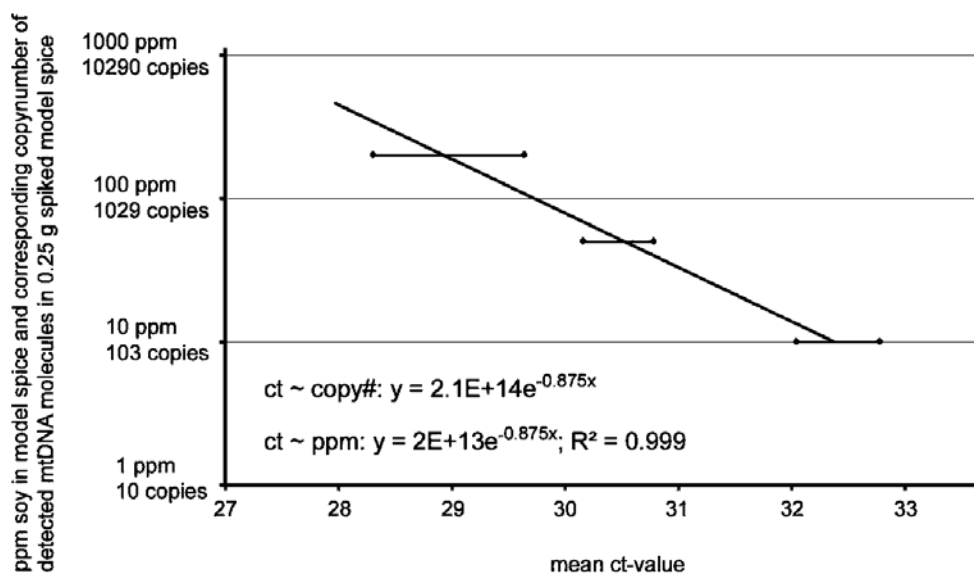


Figure 3. Calibration curve obtained by mtDNA detection of DNA extracts from model spice spiked with 200, 50, and 10 ppm soy flour. mtDNA copy numbers in DNA extracts of 0.25 g model spice spiked with 200, 50, and 10 ppm soy flour were calculated by a plasmid standard containing the *atpA* target sequence.

entered into the probit analysis. Probit calculation yielded the probability of a positive PCR outcome depending on the spiked amount of soy flour into the model spice. The soy concentration still detectable with 95% confidence reflects the LOD. Probit analyses were performed using the Minitab[®] Statistical Software, Version 15.1.30.0. (State College, PA).

mtDNA copy numbers present in DNA extracts of spiked model spice were determined by a standard curve obtained by dilutions of a plasmid (pUC19) with insertion of the *atpA* primer and probe target sequence. Length of the inserted *atpA* gene fragment was 307 bp, ranging from nucleotide 2171 to nucleotide 2478 of the *atpA* gene sequence (GenBank No. Z14031). Restriction sites were added to the gene fragment by PCR and insertion was performed using the BamHI restriction site of the pUC19 plasmid. Serial dilutions from 10^6 to 10^1 copies of the *atpA*-pUC19 plasmid were analyzed by *atpA* primers and probe, and were used as internal standard for determination of mtDNA copy numbers in spiked samples.

Variation in mtDNA Content

To examine variation in mtDNA content among different soy cultivars, DNA extracts of 15 different soy cultivars were prepared from soy flour obtained from the State Institute of Chemical and Veterinarian Analysis, Freiburg, Germany. DNA extracts were prepared from 0.25 g soy flour using the CTAB method described above. Four samples of each cultivar containing 2.5 pg total soy DNA were analyzed in a PCR run using the *atpA* primers and

probe to determine mtDNA content variation. Variation in nDNA content was examined by performing a PCR run using the commercial SureFood Soy ALLERGEN kit. Four samples of each cultivar containing 250 pg total soy DNA were used for examination of nDNA content variation. Obtained ct values for mtDNA and nDNA amplification were converted into mtDNA and nDNA copy numbers, respectively. mtDNA and nDNA copy numbers were calculated according to conversion factors obtained by PCR product standards. PCR product standard curves were generated by using serial dilutions of purified PCR products. PCR products were obtained by amplification of total soy DNA using the *atpA* primers and the SureFood Soy ALLERGEN kit. PCR reactions were run on agarose gels, and PCR products cut out followed by DNA extraction using Roti[®]-Spin MINI columns (Carl Roth GmbH, Karlsruhe, Germany) and subsequent purification of extracted DNA using the QIAquick PCR purification kit (Qiagen). Concentrations of purified PCR products were determined using a spectrophotometer (Implen), and PCR product copy numbers were calculated. Serial dilutions ranging from 10^6 to 10^1 copies of PCR products were amplified by the *atpA* primer and probe system and by the SureFood Soy ALLERGEN kit, respectively.

Results

Sensitivity of Soy DNA Detection by mtDNA Amplification

Three separate PCR runs with 10-fold serial diluted

Table 3. Ct-values and corresponding mtDNA and nDNA copy numbers including mtDNA to nDNA ration of 15 different soy cultivars from Canada, Brazil, Europe, and the United States

Soy cultivar	Country of origin	Mean ct-value of mtDNA detection in 2.5 pg total DNA (n = 4)	mtDNA copies in 2.5 pg total DNA	Mean ct-value of nDNA detection in 250 pg total DNA (n = 4)	nDNA copies in 250 pg total DNA	Ratio of mtDNA to nDNA copies in soy cultivar
Sigalia	Europe	29.10 ± 0.36	1837	30.66 ± 0.16	1911	96.1
Cadet	Canada	29.36 ± 0.11	1463	30.96 ± 0.17	1549	94.4
Vital	USA	29.98 ± 0.20	850	30.82 ± 0.29	1708	49.8
BRS258	Brazil	30.05 ± 0.17	800	30.50 ± 0.23	2137	37.4
Gallec	Europe	30.14 ± 0.25	739	31.00 ± 0.09	1506	49.1
Satyna	Europe	30.20 ± 0.16	701	31.15 ± 0.21	1356	51.7
Christine	Europe	30.45 ± 0.52	564	31.25 ± 0.37	1264	44.6
CD216	Brazil	30.66 ± 0.21	469	31.90 ± 0.14	802	58.5
Primus	Europe	30.80 ± 0.25	415	32.05 ± 0.38	722	57.5
Cordoba	Europe	31.04 ± 0.44	336	32.73 ± 0.17	449	75.0
Opaline	Europe	31.23 ± 0.16	285	32.21 ± 0.23	646	44.1
Lissabon	Europe	31.33 ± 0.34	261	32.99 ± 0.13	374	69.8
Merlin	Europe	31.55 ± 0.22	215	33.17 ± 0.21	330	65.3
Sultana	Europe	31.63 ± 0.41	201	31.63 ± 0.21	969	20.7
Petrina	Europe	32.70 ± 0.23	79	33.55 ± 0.56	253	31.1

total soy DNA samples were performed, and samples were run in duplicates. By using amplification of mtDNA, a minimal amount of 0.25 pg total DNA was detected in all repeats and replicates (Figure 2), indicating an LOD of total soy DNA of about 0.25 pg. Amplification of 25 fg total DNA was also observed, but was not reproducible in all duplicates and different PCR runs. By using amplification of nDNA, a minimal amount of 25 pg total DNA was detected in all repeats and replicates, indicating an LOD of about 25 pg. A 100-fold lower amount of total soy DNA could reliably be detected by mtDNA amplification compared to nDNA amplification.

Calibration curves converting the ct-values to the amount of target DNA were generated (Figure 2). The ct-values obtained by atpA primers and probe into total soy DNA amount could be transformed by the calculation:

$$\text{Total amount of soy DNA in ng} = 5 \times 10^6 \times e^{-0.685 \times (\text{ct-value})}$$

The ct-values obtained by the commercial SureFood Soy ALLERGEN kit could be transformed by the calculation:

$$\text{Total amount of soy DNA in ng} = 1.5 \times 10^9 \times e^{-0.7 \times (\text{ct-value})}$$

Mean difference between ct-values obtained for mtDNA and nDNA detection was 7.63 ± 0.12 ($n = 4$) ct-values. In real-time PCR experiments, a difference of 3.32 ct-values corresponds to a 10-fold difference in target DNA molecules (15). Thus, the difference of 7.63 ± 0.12 ($n = 4$) ct-values corresponded to a 200-fold

higher amount of mtDNA copies than nDNA. An increase in sensitivity by mtDNA detection compared to nDNA detection of two orders of magnitude could be obtained.

Obtained mean amplification efficiency E ($E = [(10^{-1/\text{slope}}) - 1] \times 100\%$) of mtDNA amplification was $100.50 \pm 7.24\%$ ($n = 6$). The nDNA amplification experiments showed a mean amplification efficiency of $108.36 \pm 12.03\%$ ($n = 6$). Coefficients of correlation (R^2) of the standard curves obtained for mtDNA and nDNA amplification showed a mean R^2 of 0.998 ± 0.001 ($n = 6$) and 0.992 ± 0.005 ($n = 6$), respectively. In order to obtain accurate and reproducible results, real-time PCR reactions should have efficiency close to 100% and a correlation coefficient close to 1. These conditions were given for both the mtDNA and nDNA amplification, respectively.

Precision

Precision of the atpA primer and probe system was analyzed for ct-value variation within one PCR experiment (intra-assay precision) and ct-value variation of separate PCR runs (interassay precision). Ct-values obtained from 15 samples containing 25 pg total soy DNA analyzed in the same PCR run showed a mean ct-value of 30.95 ± 0.25 ($n = 15$). No false-negative results were observed. Analysis of seven separate PCR runs with samples of 25 pg total soy DNA in duplicates showed a mean ct-value of 30.98 ± 0.55 ($n = 14$). No false-negative results were obtained.

Robustness

Robustness of the developed real-time PCR method amplifying the mitochondrial *atpA* sequence area was examined by transferring the established thermal cycling protocol from one instrument to another. The influence of different annealing temperatures on the *ct*-value was also examined. Possible differences in performance of the soy detection method on different instruments were estimated by comparing the 15 replicates of 25 pg total soy DNA analyzed on the Bio-Rad CFX96 real-time PCR system to 15 replicates of 25 pg total soy DNA, which were analyzed on a Rotor-Gene 6000 real-time PCR system from Corbett Life Science. All 15 replicates were amplified without false-negative results on both instruments. Mean *ct*-value and its variation were almost identical on both systems with a mean *ct*-value of 30.95 ± 0.25 on the Bio-Rad instrument and of 30.90 ± 0.26 on the Rotor-Gene instrument. The influence of different annealing temperatures was examined by analyzing triplicates of 25 ng soy DNA at six different annealing temperatures equally distributed in a gradient ranging between 56 and 61°C. A variation of ± 0.18 *ct*-values ($n = 18$) was found within the examined range of annealing temperatures.

Cross-Reactivity of *atpA* Primers and Probe

During the PCR run, the DNA sequence enclosed by the two primers was amplified. Detection of the amplified DNA was performed by the single-stranded fluorophore-tagged DNA probe, which was binding to the amplified DNA sequence and emitted fluorescence after being hydrolyzed by DNA polymerase during the amplification process. Sequence specific probe binding allowed specific detection of the amplified DNA and prevented detection of unspecific PCR products. Formation of PCR product was measured during each PCR cycle. Although primer and probe annealing to the target DNA is sequence-specific, small sequence variations within the target DNA still allow primers and probe to bind, resulting in the formation of PCR products. Among all tested allergens, potato showed the strongest cross-reactivity, with 0.0252%, followed by carob, with 0.01125% cross-reactivity (Table 1). Mallow, carnation, and chive did not show any cross-reactivity in the real-time PCR reactions using *atpA* primers and probe (Table 1).

Comparison of LOD for mtDNA and nDNA Amplification

Based on the numbers of positive PCR reactions for each soy concentration (Table 2), the LOD of soy flour spiked into the model spice was calculated by probit analysis at 1 ppm for mtDNA-based soy detection. Detection of 1 ppm soy flour in the model spice showed a variability for the developed *atpA* primer and probe

system of ± 1.31 *ct*-values ($n = 17$; Table 2). At 10 ppm, the variability of the *ct*-value is significantly smaller, showing a variability of ± 0.37 *ct*-values ($n = 18$; Table 2). Because intra-assay and interassay variability show a maximum variation of ± 0.54 *ct*-values, the variation of 0.37 *ct*-values is in the range of the variation of the method itself. Thus, reliable results can be obtained for the detection of 10 ppm soy flour in the spice matrix, and the LOQ can be set at 10 ppm.

The LOD for soy detection by nDNA amplification was calculated by probit analysis at 65 ppm spiked soy flour in the model spice. Thus, mtDNA detection showed a 65-fold increased sensitivity in comparison to nDNA detection. Detection of 200 ppm soy flour in the model spice by using the commercially available kit showed a variation of ± 1.48 *ct*-values ($n = 14$; Table 2). Thus, quantification with the commercially available kit is not possible within the examined concentration range of the soy flour spiked model spice and determination of LOQ is not possible.

Conversion Factors and Soy Recovery

In DNA extract of model spice spiked with 200 ppm soy flour, 2058 copies of mtDNA molecules could be detected by the *atpA* primers and probe according to the *atpA*-pUC19 plasmid standard. With the determined mtDNA copy number of the 200 ppm soy flour spiked model spice sample, for each spiked amount of soy flour in the model spice, corresponding copy numbers of mtDNA molecules could be calculated, and a factor for converting *ct*-values to amounts of mtDNA copies could be determined (Figure 3). A conversion factor for calculating amounts of spiked soy flour in relation to *ct*-values was determined as well (Figure 3). Obtained *ct*-values can be converted to the amount of mtDNA copies by the following calculation:

$$\text{mtDNA copy number} = 2.1 \times 10^{14} \times e^{-0.875 \times (\text{ct-value})}$$

Soy content in the model spice can be calculated by the formula:

$$\text{Soy content in ppm} = 2 \times 10^{13} \times e^{-0.875 (\text{ct-value})}$$

Copy numbers of mtDNA can be converted to concentrations of soy flour in the model spice as follows: copy number of mtDNA $\times 0.0972$ = soy content in ppm. A coefficient of $R^2 = 0.999$ for correlation of *ct*-values to amount of spiked soy flour (Figure 3) indicated homogenous distribution of soy flour in the model spice. To calculate conversion factors, *ct*-values of soy spiked model spice at concentrations of 200, 50, and 10 ppm were considered. *Ct*-values of the soy spiked model spice samples at concentrations of 1 and 0.1 ppm were not considered because they were not in the range of the LOQ.

Recovery rates were calculated for calibration samples of 200, 50, and 10 ppm soy spiked into model spice. Calculations were made for measuring points of the calibration curves (Table 2). Measuring points for calculated concentrations were excluded and separate standard curves were calculated for each concentration of spiked soy into the model spice. Obtained recovery ranged from 95% for 10 ppm samples to 127.8% for 50 ppm samples; for 200 ppm, recovery was 102.3%. Additionally recovery was determined in a test sample of 100 ppm soy spiked model spice. DNA extract from one sample of soy spiked model spice at a concentration of 100 ppm extracted by the SureFood PREP kit was analyzed in two PCR runs at a total number of 10 replicates. Obtained mean ct-value was 29.8 ± 0.43 ($n = 10$), resulting in a calculated soy content of 98.2 ± 35.1 ppm ($n = 10$), representing a recovery rate of 98.2%.

Calculation of mtDNA copies in DNA extract of 200 ppm soy spiked model spice resulted in 2058 copies per PCR reaction (according to formula in Figure 3 at $ct = 28.97$). Calculation of total soy DNA in DNA extract of 200 ppm soy spiked model spice resulted in 0.012 ng total soy DNA per PCR reaction (according to formula in Figure 2 at $ct = 28.97$). Accordingly, numbers of copies of mtDNA could be converted to an amount of total soy DNA in ng by a conversion factor of 5.85×10^{-6} .

In 0.25 g spice mixture, spiked with 200 ppm soy, which was used for DNA extraction, 0.05 mg soy is present. A volume of 1/10 (5 μ L) of the DNA extract was used per PCR reaction, which corresponds to a soy amount of 5 μ g. In the DNA extract of the 200 ppm soy-spiked model spice 0.012 ng total soy DNA per PCR reaction could be determined by calculation, as described above. Accordingly, the amount of total soy DNA in ng could be converted into total amount of soy in μ g by a factor of 417.

Variation in mtDNA Content

Numbers of mitochondria per cell are different depending on the physical state of the examined organism. Knowledge about mtDNA content variation is important if allergen quantification is based on mtDNA amplification. Thus, ct-level variation and corresponding mtDNA copy number variation between 15 different soybean cultivars were analyzed (Table 3). Examination of 2.5 pg total soy DNA showed a maximum difference of 3.6 ct-values, with the lowest ct-value for the cultivar Sigalia ($ct = 29.10 \pm 0.36$, $n = 4$), and highest ct-level for the cultivar Petrina ($ct = 32.70 \pm 0.23$, $n = 4$; Table 3). Variation in nDNA copy numbers of the examined 15 soy cultivars showed a maximum difference of 2.89 ct-values in samples of 250 pg total soy DNA extracts (Table 3). Lowest ct-value was found for the cultivar BRS258 ($ct = 30.50 \pm 0.23$, $n = 4$) and highest ct-level was found for the cultivar Petrina ($ct = 33.55$, $n = 4$;

Table 3). Within the examined 15 different soy cultivars, a 22-fold difference in the content of mtDNA could be found, as well as a 11-fold difference in the content of nDNA copies present in the examined amount of 2.5 and 250 pg total soy DNA extract, respectively. Variation in the ratio between mtDNA and nDNA content varied from 96.1 for soy cultivar Sigalia to 20.7 for soy cultivar Sultana.

Discussion

Sensitivity of detection of total soy DNA could be increased about two orders of magnitude by using mtDNA detection compared to nDNA detection. Content of mtDNA copies in the examined total soy DNA extracts was about two orders of magnitude higher than content of nDNA copies reported in the literature. Plant cells contain hundreds of copies of mtDNA (9). Comparison of mtDNA copies and nDNA copies in total soy DNA extracts showed a 200-fold higher amount of mtDNA copies than nDNA copies. Since soy plants (*Glycine max*) underwent genome duplication (13), four copies of the nDNA target sequence are present in one cell. Accordingly, a 200-fold higher amount of mtDNA copies, i.e., 800 copies of mtDNA molecules, were present in one cell of soybeans used in this study.

One sensitive soy DNA detection method using multicopy targets on nDNA recently reported a successful amplification of a minimal amount of 500 fg total DNA extracted from soy (6). Using our PCR method based on mtDNA amplification, a minimal amount of 25 fg total DNA extracted from soy could be successfully detected. By mtDNA detection of pure total DNA, a 20-fold increase in sensitivity could be obtained.

Although cross-reactivity of atpA primers and probe is completely absent only for mallow, carnation, and chive, specificity was sufficient to examine a wide range of foodstuffs for the presence of soy contamination. As expected from the BLAST search result, which showed some areas of the soy atpA target sequence with similarity to solanacean plants, potato DNA extract shows the strongest cross-reactivity of all examined foodstuffs. Cross-reactivity of 0.0252% of potato DNA extract would simulate the presence of 252 ppm soy in a pure potato product. Thus, specificity of the atpA primers and probe is not sufficient to examine pure potato food products and other pure products with high cross-reactivity. However, small traces of potato or other contamination with foodstuffs of high cross-reactivity do not affect soy detection. In pure wheat products with cross-reactivity of 0.00003%, an amount of 0.3 ppm soy would be simulated by the atpA primer and probe system. Since LOD of soy detection by the atpA primer and probe system was determined at 1 ppm, specificity is sufficient to detect soy contaminations in pure wheat products. In general, careful evaluation of cross-reactivity has to be

done prior to application of the *atpA* primer and probe soy test system considering contents of food ingredients and intensity of cross-reactivity of each ingredient. Absence of any amplification curve obtained for the analysis of the model spice not containing soy showed the applicability of the *atpA* primer and probe system for the detection of soy in real food samples.

So far there is only scarce information about plant mtDNA available within the databases, and finding species-specific sequences is difficult. However, with the constant increase of mitochondrial and chloroplast DNA sequences available in the databases, it will be possible to establish sensitive and more specific PCR test systems to detect food allergens based on extrachromosomal DNA amplification. Species-specific, PCR-based detection of mtDNA of animal origin is described for the identification of cows' milk in goats' milk (19) which shows applicability of species-specific analytics by PCR using extrachromosomal target sequences.

The reliability of mtDNA detection by the *atpA* primer and probe system was confirmed by the good precision of *ct*-values obtained for the amplification of pure soy DNA. Detection of soy in the model spice showed good results for precision as well. Calculated recovery rates of soy in the model spice were acceptable in terms of precision and amount of recovered soy for all samples examined. Within the examined range from spiked amounts of soy flour from 200 to 10 ppm, a maximum deviation of 27.8% compared to the spiked amount of soy flour was found, which allowed accurate quantification of spiked amount of soy based on mtDNA detection.

PCR-based allergen detection methods recently published show an LOD of 10 ppm soy flour in corn flour (7) and 10 ppm soy flour in wheat flour (5). Using our developed real-time PCR system, soy could be detected even more sensitively in foods. In a model spice spiked with soy, we obtained an LOD of 1 ppm. Model spice used for LOD determination contained 50% table salt, which could affect soy detection due to disturbance of DNA extraction and disturbance of PCR amplification. A previously reported comparison between spiked pure wheat flour and a spiked mixture of rice flour, salt (50%), and pepper with buckwheat showed comparable results in the detection of a defined amount of spiked buckwheat flour by PCR for both samples (20). These results show reliable detection of foodstuffs in matrixes spiked with 50% table salt. Thus, the obtained LOD of 1 ppm for the soy-spiked model spice can be compared to results in the literature where spiking experiments were done on foodstuffs not containing high salt concentrations. DNA extracts of foodstuffs with high fat content (e.g., nuts, full-fat soy) could be prepared using the CTAB method. Fat was efficiently removed by the chloroform purification step. No additional defatting procedures were necessary for mtDNA detection of foodstuffs with high fat content.

Soy detection by PCR in processed food products using amplification of nDNA was examined in a previous study (7). Food samples of products that underwent different treatments like cooking, baking, and toasting were positive when tested for soy content by PCR. Comparisons of degradation of nDNA and mtDNA in mouse tissue showed that factors, such as cellular location and molecular features, positively influence preservation of mtDNA (21). Thus, mtDNA can be considered as useful target molecule for the detection of food allergens in processed food. However, the main factor determining the assay's applicability to processed food is the extraction efficiency. The influence of DNA extraction methods on food allergen detection has to be tested in a future study.

Reliable detection of soy in processed food by ELISA has been recently reported in an interlaboratory evaluation (22). The ELISA test system showed an LOD of 10 ppm soy in food samples. By using a direct sandwich ELISA test system, an LOD of 0.05 ppm of whole soy flake in a food sample was described (3). The advantage of ELISA test systems compared to PCR-based methods is the possibility to detect the agent itself causing the food allergy. The amount of detected DNA by PCR may not correlate with the amount of the allergy-eliciting protein within the analyzed food samples. Variation in extrachromosomal DNA content per cell especially influences PCR-based test results, which do not reflect content of the protein causing the allergy. But for ELISA test systems, discrepancies in obtained test results and amount of the agent causing the allergy can be found as well. Processing of food can alter the structure of proteins resulting in reduced or increased allergenicity and may also result in reduced or sometimes even increased allergen detection. Therefore, PCR methods are useful additional tools for food allergen detection.

In quantitative PCR-based assays using mtDNA detection, variation in mtDNA content among different cultivars has to be considered. Therefore, an examination of mtDNA content in 15 different soy cultivars was done. Up to a 22-fold difference in mtDNA content was found among 15 different soy cultivars. Variation of the ratio of mtDNA copy numbers to nDNA copy numbers varied from 20.7 for cultivar Sultana to 96.1 for cultivar Sigalia. Thus, increase in sensitivity by mtDNA detection is different depending on the soy cultivar. Examination of nDNA contents also showed considerable variation. Variation of nDNA content was up to 11-fold different within the 15 examined soy cultivars. Variation of nDNA content can be explained by variation in mtDNA and cpDNA contents. Total plant DNA extract consists of a mixture of nDNA, mtDNA, and cpDNA. In a given total DNA amount, variation in percentage of one DNA component (nDNA, mtDNA, or cpDNA) affects percentages of other DNA components.

The widely used genetically modified glyphosate

tolerant soybean line created by the Monsanto Co. in 1995 (23) has a modification into nuclear DNA. Because the mitochondrial *atpA* gene sequence is unmodified, detection of genetically modified and nonmodified soy in food is also possible by the *atpA* primer and probe system.

In the current discussion about the establishment of regulatory threshold levels, limitations of analytical methods need to be considered (24). Methods that can fulfill these future requirements are necessary. A highly sensitive and reproducible detection of food allergens by amplification of extrachromosomal DNA might be helpful for the introduction of suitable threshold levels protecting highly sensitized allergic persons. Additionally, sensitive detection of food allergens below clinical relevant threshold levels is extremely helpful in optimizing allergen management during food production by the food industry to prevent the occurrence of cross-contamination.

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