SPECIAL GUEST EDITOR SECTION: FOOD ALLERGENS NEW METHODS

# Highly Sensitive Matrix-Independent Quantification of Major Food Allergens Peanut and Soy by Competitive Real-Time PCR Targeting Mitochondrial DNA

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**The development of two competitive real-time PCR assays for the quantitative detection of trace amounts of two major food allergens, peanut and soybean, is reported. In order to achieve very low detection levels for both allergens, we established PCR primers and probes targeting mitochondrial DNA sequences. We were able to demonstrate that this approach led to an increase in detection sensitivity in the range of at least 1 order of magnitude compared with published assays targeting nuclear DNA. Furthermore, we generated corresponding competitor molecules, which were used as internal standards to compete with matrix effects that are evident during DNA extraction and PCR amplification in heterogeneous analytical matrixes like food. According to the recently described competitive quantitative PCR method published by Holzhauser et al. (2014), we performed threshold calibration against milk powder spiked with 10 ppm peanut and soy. Matrix-independent quantitative determination of peanut and soy could be demonstrated for three different calibrated food matrix standards in a range between 1 and 100 ppm. The data presented indicate that both assay concepts are powerful analytical tools for the quantitative detection of trace amounts of peanut and soy in commercial food products.**

Food allergies are an important public health problem, and, in industrialized countries, around 3–4% of the population are affected (1–3). The reactions triggered by food allergens vary from mild urticaria to life-threatening anaphylactic shock (4). Irrespective of the widespread prevalence of such allergies, there is no therapeutic treatment available so far. Therefore, the only way for an allergic person to handle this situation is the strict avoidance of the allergen and symptomatic treatment after contact (5). This avoidance of allergens is problematic for those affected because the presence of allergenic ingredients in foodstuffs is, in some situations, not apparent to consumers (6). Accordingly, labeling rules of selected allergenic ingredients on food packaging have been issued by law in many countries, e.g., according to European Union Regulation No. 2011/1169/EC (7). The latter includes 14 major allergens or allergen groups that manufacturers must mandatorily declare. Despite this, trace amounts of allergenic impurities can be present unintentionally in food by carryover of food components during harvest, transport, storage, and the manufacturing process. Therefore, analytical methods are useful tools for purposes of compliance, allowing the reliable detection and quantification of allergenic food ingredients independently of the food matrix and in the relevant low concentrations.

Peanut and soy, which are known as major allergens, are widely used by the food industry, thereby leading to a high risk of cross-contamination of food products (8, 9). Detection of these allergens is generally performed using two analytical approaches. Immunological methods, such as ELISA, are the most commonly used (10–14), which allow for direct detection of allergenic proteins. However, detection and quantification of trace amounts of proteins in foodstuff with antibodies is often compromised by the modification of the epitopes, which becomes evident during food production and processing (15–17), and by potential cross-reactivity. Other approaches are based on PCR methods in which allergen-specific nucleic acids or DNA sequences specific for the allergenic organism are amplified (18). The high specificity of PCR-based techniques is determined by species-specific nucleotide diversification and the development of real-time PCR approaches; e.g., TaqMan probe chemistry has improved the quantitative detection of low template amounts. Regardless of the fact that DNA molecules are known to be very stable and robust against environmental factors, PCR methods, on the other hand, are inevitably affected by food processing, e.g., the acidic hydrolysis of DNA.

In recent years, several methods for the detection of peanut and soy based on conventional PCR (19–22) or on real-time PCR using fluorescent reporter probes were published (6, 9, 14, 23–28).

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Irrespective of the high specificity of these systems, quantitative determination of trace amounts of both allergens is limited due to matrix effects originating from the versatile nature of foodstuffs and their degree of processing and the purification and extraction methods of the corresponding DNA samples. Nevertheless, there are several publications that provide different possibilities to quantify trace amounts of soy and peanut under consideration of certain matrix effects. Quantitative determination of soy in the range of 800–1500 ppm could be achieved by establishing a real-time PCR-based standard-addition method (29). In another approach, soy could be quantified in samples prepared from cooked sausage in the range of 10–100 ppm by using incurred reference materials with comparable matrixes for calibration (30). In the case of peanut, Holzhauser et al. (31) published a method for quantitative detection based on competitive real-time PCR. The authors constructed an artificial internal competitor DNA molecule corresponding to the amplicon based on the Ara h2 detection system published by Stephan and Vieths (14). This internal competitor was used as a calibrator to normalize matrix effects occurring during DNA extraction and PCR amplification. The authors calibrated their competitor against a threshold concentration of 100 ppm peanut in food, which allowed the reliable quantification of peanut in various complex matrixes in the range of 10–1000 ppm. The utility of using an internal standard to compensate for matrix effects interfering with PCR amplification was also demonstrated by Zhang et al. (32). The authors established a detection system based on Ara h1 sequences and constructed an artificial internal amplification control (IAC) composed of a DNA fragment from *Listeria monocytogenes* flanked by Ara h1-specific primer binding sites. In order to preclude false-negative results, a defined amount of IAC molecules was coamplified during PCR, with a detection limit of 5 ppm. The authors showed that their approach largely compensates for matrix effects interfering with PCR amplification, thereby enhancing the reliability of their detection system. Taken together, the use of internal calibration standards provides an appropriate tool for the quantitative determination of allergenic ingredients independent of the food matrix analyzed.

In the present study, we report the development and validation of methods for the sensitive detection and matrix-independent quantification of peanut and soybean based on competitive real-time PCR primer pairs and probes targeting species-specific mitochondrial sequences: atp6 in peanut and bait8 in soybean.

## Materials and Methods

### *Food Samples and Spiked Reference Materials*

Spiked milk powder was prepared in collaboration with the Department of Pharmaceutical Technology at the University of Applied Sciences Albstadt-Sigmaringen (Sigmaringen, Germany). Peanut- and soy-free milk powder (verified by using peanut- and soy-specific real-time PCR) was obtained from a local dairy (OMIRA Oberland-Milchverwertung GmbH, Ravensburg, Germany) and peanuts and soy flour were provided by local distributors (Seeberger KG, Ulm, Germany; and Hensel, Magstadt, Germany). Peanut kernels were ground, subsequently defatted by a petroleum ether method, and ground again using a disposable disperser system (ULTRA-TURRAX; IKA-Werke GmbH & Co. KG, Staufen, Germany). The amount of extracted fat was determined gravimetrically and considered during the spiking procedure.

Relative amounts of 10 g of each defatted, finely ground peanut and partially defatted soy bean flour were mixed with 1 kg milk powder on a cube mixer (KB 15; ERWEKA GmbH, Heusenstamm, Germany) attached to a drive unit (ERWEKA AR 400 E; ERWEKA GmbH) for 10 min at 200 rpm. Serial dilutions were prepared by subsequent mixing with blank material to produce final allergen concentrations of 1, 5, 10, 20, 50, and 100 mg/kg. The homogeneity of the mixtures was verified by real-time PCR, whereas the DNA of three samples of each spiking level was prepared in duplicate and analyzed in three independent PCR reactions.

Rice cookies, Hollandaise sauce powder, and sausage spiked with 5, 10, and 100 mg/kg allergenic ingredients (including soybean flour and defatted peanut powder) were previously used as samples and for calibration in interlaboratory validation studies for the quantification of allergenic food (33). Levels of 1 and 20 mg/kg allergenic ingredients were prepared by appropriate dilution with blank material.

DNA samples of 15 different soy cultivars were previously prepared from soy kernels provided by German breeders (27). Kernels of 11 peanut varieties, as well as other food samples and food ingredients used for investigation of cross-reactivities, were obtained from local suppliers.

### *DNA Extraction*

Total DNA from food samples was extracted using a modified protocol of the cetyltrimethylammonium bromide (CTAB) method described by Kenk et al. (34). In brief, samples were ground to homogeneity in disposable grinding chambers using an analytical mill (IKA Tube Mill control; IKA-Werke GmbH & Co. KG). Sample aliquots of 1 g were incubated with 10 mL CTAB extraction buffer (2% CTAB, 1.4 M NaCl, 0.1 M Tris-HCl, and 20 mM EDTA; pH 8) in a thermomixer (MKR 13; HLC BioTech, Bovenden, Germany) for 90 min at 65°C at 400 rpm in the presence of 0.6 mg proteinase K (20 µg/mL, ready-to-use; Merck KGaA, Darmstadt, Germany). After incubation, samples were aliquoted in 2 mL reaction tubes and centrifuged for 10 min at 20 000 × *g*. Seven hundred microliters of the debris-free supernatant were transferred to 400 µL chloroform–isoamylalcohol (24 + 1, v/v) in a new 2 mL reaction tube. After mixing on a vortex mixer, the samples were centrifuged as described above, and 500 µL DNA containing aqueous phase were transferred into a 1.5 mL reaction tube and mixed with 500 µL isopropanol. After 30 min at room temperature, the DNA was precipitated by centrifugation for 15 min at 20 000 × *g*. The supernatant was discarded and the resulting DNA pellet washed with 500 µL of 70% ethanol, followed by an additional centrifugation step for 5 min at 20 000 × *g*. The supernatant was discarded again and the pellet air-dried at room temperature. The DNA pellet was resuspended in 100 µL TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8) supplemented with 2 µg RNase A (R6513; Sigma-Aldrich, Branchburg, NJ) for 60 min at 50°C. The DNA extracts were further processed with a column purification step using a QIAquick DNA purification system (Qiagen, Hilden, Germany). According to the manufacturers' instructions, samples were eluted with 50 µL elution buffer and the concentration of the purified DNA determined with a spectrophotometer

(Implen, Inc., Munich, Germany). Samples were subjected for PCR analysis or stored at –20°C.

Alternatively, DNA was extracted from 100 mg food samples using a SureFood PREP Advanced Allergen Kit (R-Biopharm/Congen) to prepare DNA from food products according to the manufacturer's instructions. Putative RNA contaminants were removed by RNase A digestion (1 µg; Sigma-Aldrich), followed by photometrical determination of DNA concentrations.

### Primers and Probes

Soy-specific primers and probes were identified by screening the mitochondrial genome of soy (35). The sequence information, comprising 402 558 bp, was split into 99 fragments consisting of 2250 bp (bait1), 4050 bp (bait2–46), 8100 bp (bait47–98), and 5733 bp (bait99). The mitochondrial DNA (mtDNA) fragments were analyzed with the Basic Local Alignment Search Tool (BLAST; 36, 37) using the National Center for Biotechnology and Information (NCBI) database (38). Nonconserved regions were identified with the Megablast program and further analyzed by BLASTN. Primers and probes were selected for the appropriate sequences by using primer3 open-source software (39). To avoid possible cross-reactivity, we performed a subsequent screen with primer-BLAST (40), and, finally, the amplicons were revised with an additional BLASTN screen. The identified amplicons were designated according to the DNA fragments used for the initial Megablast analyses.

Peanut-specific primers and probes were identified within the expressed sequence tag (EST) database, PeanutDB (41). Contigs of putative mitochondrial origin were obtained by using 12 conserved mitochondrial genes (cytochrome C biogenesis subunits C and B; cytochrome C oxidase subunits 1 and 3; NADH dehydrogenase subunits 3, 6, and 9; ATPase subunits 4, 6, and 9; ribosomal protein L16, rpl16; and maturase, matR) from soybean for the query. Nonconserved regions were identified by subsequent BLAST analyses, and primers and probes were selected as previously described for soy-specific oligonucleotides.

The probes for competitor detection were constructed by mutagenesis of the probe-binding sites of the target amplicons. To achieve identical amplification terms for both competitor and target DNA, we used NetPrimer open-source software (42), which allows the comparative monitoring of different primer characteristics, such as the melting temperature or the formation of secondary structures. Accordingly, the mutations of the probe regions were set to obtain identical binding conditions for target and competitor probes in regards to the melting temperatures and appropriate enthalpy values.

All primers and probes used in this study were purchased from biomers.net GmbH (Ulm, Germany). Sequences for the primers and probes, as well as fluorophores and quenchers used for probe labeling, are summarized in Table 1.

### Generation of Competitor DNA

Single-stranded oligonucleotides representing C-atp6 and C-bait8 DNA were commercially synthesized (biomers.net GmbH). Two femtomoles of the oligonucleotides were amplified by conventional PCR in the presence of 200 nM atp6- or bait8-specific primers, 100 µM of each dNTP, and 2.5 units Taq polymerase (New England Biolabs GmbH, Frankfurt, Germany) in the provided reaction buffer (New England Biolabs). Amplification occurred in a GeneAmp PCR System 2400 (PerkinElmer, Waltham, MA) with 30 three-step cycles at 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s. PCR products were analyzed by agarose gel electrophoresis and further processed for TOPO TA cloning (*see* below).

### Generation of DNA Standards

In order to obtain reliable DNA templates for the internal standards, all amplicons used in this study (arah2, arah3, atp6, C-atp6, bait8, C-bait8, and lectin) were amplified by conventional PCR (*see* above) and cloned in the pCR 2.1 TOPO vector (Invitrogen; Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's protocol. Positive clones were selected by blue–white screening, and plasmid DNA was extracted from bacteria according to standard protocols. All cloning was verified by sequencing (Eurofins MWG Operon, Martinsried, Germany), and plasmids containing the atp6, C-atp6, bait8, C-bait8, arah2, and lectin amplicon were linearized with *Hind*III; the arah3 plasmid was digested with *Eco*RI. After heat-inactivating the restriction enzymes, 10-fold serial dilutions from $10^7$ to $10^2$ copies were prepared and used as templates to generate the standard curves. Linearized C-bait8 and C-atp6 plasmids were further used for the calibration of threshold values for quantitative analyses.

### Determination of Cross-Reactivity

To test the specificity of the mitochondrial detection systems, atp6 and bait8, total DNA from 69 food ingredients (almond, aniseed, apple, apricot, azuki bean, bay leaf, beetroot, blueberry, brazil nut, caraway, carob, cashew, celery, chickpea, chive, cinnamon, cloves, cocoa, coconut, coffee bean, cucumber, egg, fava bean, ginger, grape, hazelnut, juniper, lentil, linseed, lupin, macadamia, maize/corn, margarine, melon, milk powder-A, milk powder-B, mung bean-prepA, mung bean-prepB, mushroom, mussels, mustard, nutmeg, oat, onion, oregano, paprika pepper, pea, peanut, pepper, pine nuts, pistachio-A, pistachio-B, poppy, potato, prawn, pumpkin seed, rice, sesame, soy, string bean-A, string bean-B, sunflower, tarragon, tomato, walnut-A, walnut-B, wheat-A, wheat-B, and wheat-C) was isolated and examined by real-time PCR. Twenty-five nanograms of DNA from each sample were amplified in duplicate in two independent PCR runs in the presence of atp6- or bait8-specific primers and probes. Starting quantities (SQs) were determined according to conversion factors obtained by atp6 or bait8 plasmid standards, which were used as calibration curves. The degree of cross-reactivity was calculated as the percentage of mean SQs obtained from amplified DNA from food material relative to the mean SQs obtained from equal amounts of amplified DNA from soy or peanut.

### Determination of Mitochondrial DNA Content

To examine possible variations in the mtDNA content of soybean, DNA extracts from 15 different soy cultivars were analyzed by real-time PCR. Aliquots of 2.5 ng total soy DNA from each cultivar were amplified in triplicate with the bait8 primers and probe to detect mtDNA and amplified in parallel with the lectin primers and probe (26) to detect nuclear DNA (nDNA). Cycle threshold (Ct) values for mtDNA and nDNA amplification were converted into copy numbers according to conversion factors obtained by bait8 or lectin plasmid standards, which were used in serial dilutions ranging from $10^7$ to

**Table 1. Primers and probes**

| Primers and probes[a] | Sequence (5′→3′) | Primer or probe size, bp | Amplicon size, bp | Ref. |
|---|---|---|---|---|
| | | Soy-specific | | |
| lectin-F | tccacccccatccacattt | 19 | 81 | Köppel et al. (26) |
| lectin-R | ggcatagaaggtgaagttgaagga | 24 | | |
| lectin-P | HEX-aaccggtagcgttgccagcttcg-BHQ1 | 23 | | |
| bait8-F | tctcatccctggattccttg | 20 | 114 | This work |
| bait8-R | tcgatacccccttacctgacg | 20 | | |
| bait8-P4 | FAM-cattcaccttcttttgaggagcg-BHQ1 | 23 | | |
| C-bait8-P4V4 | ROX-gtttctcgttgttttctggtcgc-BHQ2 | 23 | | |
| | | Peanut-specific | | |
| arah2-F | gctcgagagggcgaacct | 18 | 66 | Hird et al. (9) |
| arah2-R | tcctcgtcacgttggatcttc | 21 | | Köppel et al. (26) |
| arah2-P | HEX-aggccctgcgagcaacatctcatg-BHQ1 | 23 | | |
| arah3-F | gaagcttaccatatagcccataca | 24 | 105 | Scaravelli et al. (23) |
| arah3-R | cttgtcctgctcgttctct | 19 | | |
| arah3-P | FAM-tgctgtcctcgagggctaaattcacgctcttc-BHQ1 | 32 | | |
| atp6-F | cagggcatccttaactggag | 20 | 104 | This work |
| atp6-R | ggaaagacgggttggtgata | 20 | | |
| atp6-P | FAM-aaggcgaagaagggtcagat-BHQ1 | 20 | | |
| C-atp6-P | ROX-ttgccgaacaacgctcacta-BHQ2 | 20 | | |

[a] F and R represent Forward and Reverse for the primers and P represents Probe.

$10^2$ copies as calibration curves. In the case of peanut, DNA from 11 different peanut varieties were prepared, and 2.5 ng extracts were amplified in triplicate with the atp6 mitochondrial system and in parallel with the arah2 primers and probe (Accession Nos. FJ713110, L77197, and AY007229; 9) to detect nDNA. Copy numbers of mitochondrial and peanut nDNA were determined via standard curves prepared with arah2 and atp6 plasmid DNA. mtDNA and nDNA ratios were calculated by dividing the mean SQ values, and SDs were calculated using Bio-Rad CFX3.1 software (Bio-Rad Laboratories, Hercules, C). Variations were illustrated by calculating the deviation of mean SQ ratios as a percentage from the mean SQ ratios of all investigated soy cultivars (258.2) or peanut samples (194.3), respectively.

### *Competitive Real-Time PCR for Peanut and Soy DNA*

Amplification reactions (25 µL) were performed with 12.5 µL 2× SsoAdvanced Universal Probes Supermix, 5 µL template, and 400 nM of each primer pair (atp6, bait8, arah2, arah3, and lectin). Peanut mtDNA (atp6) was detected in the presence of 200 nM probe (atp6-P) and soy mtDNA (bait8) in the presence of 100 nM probe (bait8-P). Coamplification of competitor DNA occurred in the presence of equimolar concentrations of competitor probes (200 nM C-atp6-P or 100 nM C-bait8-P). The probes for the arah2, arah3, and lectin genomic amplicons were used at a concentration of 100 nM.

Real-time PCR reactions were run on a CFX96 real-time PCR System (Bio-Rad Laboratories) with an initial denaturation step at 95°C for 3 min, followed by 45 two-step cycles at 95°C for 15 s and 60°C for 30 s. Relative fluorescence signals were recorded after each cycle using the FAM channel for atp6, bait8, and arah3 detection; the ROX channel for the detection of C-atp6 and C-bait8 competitor DNA; and the HEX channel for detection of arah2 and lectin amplicons. Competitive real-time PCR was performed by using duplex PCR; other analyses were performed by conventional single-plex PCR.

Fluorescence signal thresholds were manually set during the exponential amplification phase, and experimental data were analyzed using Bio-Rad CFX 3.1 software. For competitive PCR, settings of Cts of FAM and ROX signals were performed by adjusting the signals of target and competitor standard curves in such a way that equal amounts of template resulted in identical Ct values.

### *Titration of Threshold Values of Competitor Molecules for Milk Powder Spiked with 10 mg/kg Soy and Peanut*

The numbers of copies of competitor DNA molecules that equal 0.1 g reference material spiked with 10 ppm peanut and soy were determined according to the procedure described by Holzhauser et al. (31). Two subsequent titration steps for C-atp6 and C-bait8 were performed by adding increasing amounts of competitor molecules during DNA extraction. Every titration level was prepared in three independent experiments, and each sample was analyzed by multiplex real-time PCR in triplicate. After setting FAM and ROX Cts, the logarithm of the ratios of Ct values of target signals (FAM channel) and corresponding competitor signals (ROX channel) were calculated and plotted against the logarithm of the initial competitor DNA molecule number. Threshold values were calculated by solving the equation of the linear regression analyses for $y = 0$. The initial titration was performed with $1 \times 10^3$, $2.5 \times 10^3$, $5 \times 10^3$, $1 \times 10^4$, $2 \times 10^4$, and $4 \times 10^4$ copies of competitor molecules C-atp6 and C-bait8. Subsequent titrations occurred in the presence of $8 \times 10^3$, $1 \times 10^4$, $1.2 \times 10^4$, $1.5 \times 10^4$, $1.75 \times 10^4$, and $2 \times 10^4$ copies of C-atp6 and $2 \times 10^4$, $2.5 \times 10^4$, $3 \times 10^4$, $3.5 \times 10^4$, and $4 \times 10^4$ molecules of C-bait8.

In order to proof the calculated threshold values, the determined amount of competitor molecules was added to the reference materials with varying allergen concentrations during the DNA extraction procedure.

### *Quantification of Peanut and Soy in Food Samples*

The quantities of peanut and soy in the food samples were calculated according to the equation published by Holzhauser et al. (31). The calculation implies that the target DNA and the competitor DNA are amplified in duplex reactions, with identical efficiencies of nearly 100%, which allows the application of the ΔCt approach. The threshold calibration procedure is carried out by multiplying the exponential ΔCt values by the concentration of incurred allergen in the reference material used for competitor titration.

$$\text{ppm allergen in sample} = 2^{\Delta Ct} \times (\text{ppm calibration})$$
$$= 2^{[Ct(\text{competitor}) - Ct(\text{target})]} \times (\text{ppm calibration});$$
$$\text{ppm allergen in sample} = 10 \times 2^{[Ct(ROX) - Ct(FAM)]}$$

## Results

### *Sequence-Specific Detection of Mitochondrial Soy and Peanut DNA*

In order to identify DNA regions restricted to soy mtDNA, we took advantage of sequence data that had been published in 2013, representing the complete mitochondrial genome of soybean (35). mtDNA was analyzed for homologies by using different BLASTN tools within the NCBI database, and primers and probes were selected by using primer3 open-source software (39). In sum, we could identify eight amplicons (bait2, bait7, bait8, bait37a, bait37b, bait38, bait57a, and bait57b), exclusively representing soy-specific sequences (*see* Supporting Information Figure S1), thereby providing consistent results with different online primer tools. Four of these (bait2, bait8, bait37b, and bait57a) were analyzed by conventional PCR and real-time PCR using SYBR Green (iQ SYBR Green Supermix; Bio-Rad Laboratories). Because no amplification was observed for amplicon bait57a, different probes were designed for the remaining three amplicons, which were examined for their robustness and amplification efficiency in real-time PCR approaches. Comparative analyses revealed that the bait8 amplicon (Figure 1) generated the most stable amplification profiles and the highest efficiency in combination with high sensitivity (Figure 2).

Despite the fact that genomic resources of peanut are still limited, EST data are more and more available. Since 2012, a comprehensive set of transcriptome sequences have been summarized in a public database, PeanutDB (41), with 58488799 EST single reads assembled to 32 619 contigs. These assembled contigs represent almost complete transcripts, including 5′ and 3′ untranslated regions (UTRs), which are putative sources of peanut-specific mtDNA. In order to identify transcripts originating from mitochondrial genes, we used the BLAST+ feature (43) in PeanutDB. Sequences of conserved mitochondrial genes were used as BLAST queries, and contigs harboring homologous

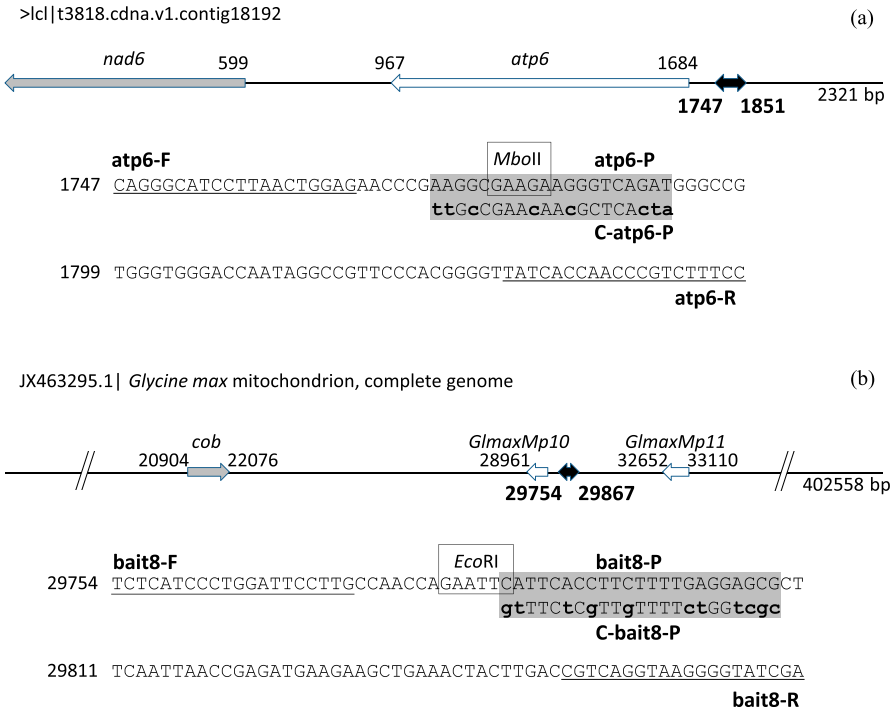


**Figure 1. Schematic view of the mitochondrial detection systems for the quantification of the food allergens, (a) peanut and (b) soybean. (a) The atp6 primers and probe were identified on contig 18192 in PeanutDB (41) and amplified a 104 bp fragment between positions 1747 and 1851 (black double-headed arrow). The amplicon is located upstream of the coding regions of the two conserved mitochondrial genes: ATPase subunit 6, *atp6*, and NADH dehydrogenase subunit 6, *nad6*. (b) The bait8 primers and probe are located between positions 29 754 and 29 867 of the mitochondrial genome of soy (35). The 114 bp intergenic region is located downstream of the gene encoding apocytochrome b, *cob*, between the two mitochondrial genes, *GlmaxMp10* and *GlmaxMp11*, with unknown function. The sequences of both amplicons are shown at the bottom of each map. Primer sequences are underlined and probe sequences are shaded in gray. Corresponding competitor sequences are shown below the probe regions, and the mutations that were introduced are in bold lowercase letters.**

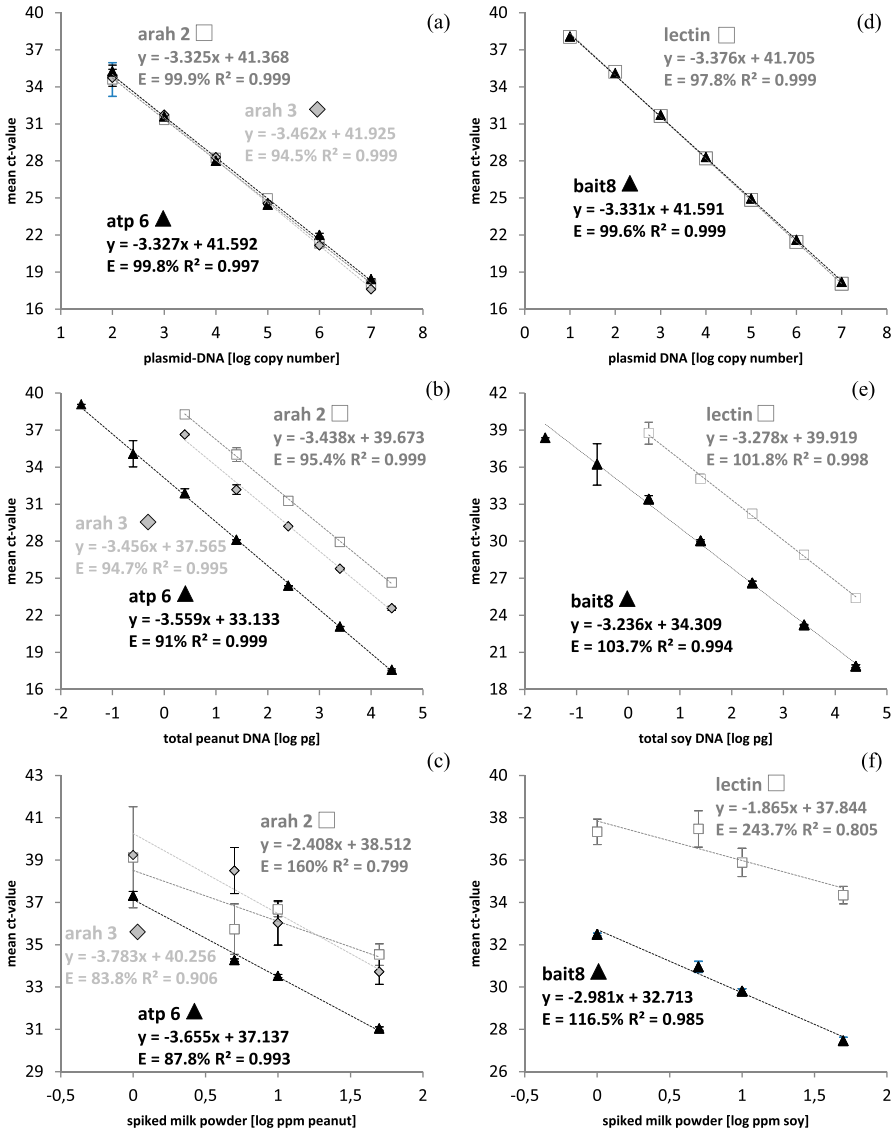


**Figure 2. Real-time PCR amplification of the mitochondrial detection systems for (a–c) peanut and (d–f) soy in comparison with published amplicons. Primers and probes of the arah2 (open diamonds; *see* ref. 9) and arah3 (gray diamonds; *see* ref. 23) peanut-specific systems were analyzed comparatively with the atp6 amplicon (black triangles). For the detection of soy, the lectin amplicon (open diamonds; *see* ref. 26) was compared with the bait8 primers and probe (black triangles). Amplification is shown as linear regression when 10-fold serial dilutions of $10^7$ to $10^2$ copies of (a and d) plasmid DNA or 25 ng to 25 fg total DNA prepared from (b) peanut or (e) soy were used as templates. Additionally, sensitivity was tested with DNA extracts prepared from milk powder spiked with 1, 5, 10, or 50 ppm (c) peanut and (f) soy. Slope values, coefficients of determination, and PCR efficiencies are indicated in the graphs.**

peanut sequences were further analyzed for nonconserved regions using the NCBI database. Peanut-specific sites and suitable primers and probes were identified as described for the soy-specific amplicons.

Two amplicons apparently representing peanut-specific sequences and mtDNA were identified. One is located in the 3′ UTR of the cytochrome C biogenesis C gene, *ccmC*, on contig2687 in PeanutDB between positions 1363 and 1480. The second amplicon was identified in the 5′ region of the coding region of the ATPase subunit 6 gene, *atp6*, located on contig18192 of PeanutDB between positions 1747 and 1851 (Figure 1). Real-time PCR analyses show identical amplification profiles when SYBR Green was used for detection. However, further investigations using probes revealed that the atp6 primers and probe were the most suitable.

### *Determination of Cross-Reactivity*

In addition to the BLAST homology searches, the specificity of the primer and probe sets was tested by using DNA extracted from 69 food ingredients (for details *see* Supporting Information Table 1). In the case of the atp6 amplicon, slight background signals were observed for 13 of the 69 samples examined, 7 of which showed cross-reactivities between 0.003 and 0.0001%, whereas the other 6 were <0.0001%. For the bait8 primers and probe, 13 DNA samples resulted in cross-reactivities between 0.01 and 0.001% and an additional 22 samples with values below 0.001%. Going into more detail, we found that DNA samples prepared from different varieties of identical species led to deviating results, such as those observed for DNA prepared from two different sources of pistachio in which only one of the

DNA samples led to amplification in the presence of the atp6 primers and probe, whereas in reactions with DNA from the corresponding preparation, no signals could be detected. Similar results were obtained with the bait8 primers and probe, when the DNA preparations of two different varieties of walnut and milk powder, respectively, or three different sources of wheat were analyzed. With DNA samples from mung bean or string bean, background signals were detected for both amplicons atp6 and bait8, which could not be observed in additional preparations. In summary, one can assume that false-positive signals are actually caused by impurities in the DNA preparations or the condition of the examined foodstuff, as by sequence similarities. This finding is supported by further studies in which bait8 primers were tested in combination with a modified version of bait8-P. The probe analyzed completely covers bait8-P sequences and has an additional 7 bp elongation at the 5′ end of bait8-P. Determination of cross-reactivities with this primer and probe set showed a completely different range of background signals for different food ingredients (data not shown), suggesting that these signals originate in a sequence-independent manner. Therefore, the strategy used for the identification of species-specific primers and probes was successful. It should be mentioned that, in competitive PCR approaches, background signals for bait8 or atp6 primers and probes were completely suppressed by the presence of C-atp6 or C-bait8 competitor DNA.

### *Optimization of Real-Time PCR Conditions*

The quality of DNA extracted from foodstuff is commonly suboptimal for PCR amplification because samples are frequently contaminated by inhibitors or other agents that influence PCR. In terms of quantitative analyses, there is also the problem that the DNA content in extracts from food varies considerably. Samples with high DNA content, as is the case for various cereals or meat-based dishes, can usually only be analyzed in diluted form. We took advantage of the recently available Sso7d fusion polymerase developed by Bio-Rad (U.S. patent Nos. 6,627,424; 7,541,170; and 7,560,260), which provides increased processivity and tolerance to PCR inhibitors and enables effective amplification of problematic templates. For comparative analyses, we amplified plasmid templates or DNA extracts prepared from a spice model spiked with 10, 20, 50, 100 and 1000 ppm allergens (34) in duplex reactions using the atp6/C-atp6 system (*see* below) with Sso polymerase (SsoAdvanced Universal Probes Supermix; Bio-Rad Laboratories) and with a standard polymerase mix (iQ Supermix; Bio-Rad Laboratories; *see* Supporting Information Figure S2). Both polymerases showed comparable PCR efficiencies when 10-fold serial dilutions of plasmid DNA were used as templates, whereas amplification of samples prepared from the spiked material clearly improved when using Sso polymerase. Similar results could be obtained when extracts from spiked wheat flour or spiked milk powder were analyzed under comparable conditions (data not shown). Therefore, all real-time experiments and quantitative analyses described in the present paper were carried out by using the SsoAdvanced Universal Probes Supermix.

### *Sensitivity of bait8 and atp6 Amplicons*

In order to determine whether bait8 and atp6 amplicons lead to a comparable increase in sensitivity, as already shown for other detection systems using amplification of mtDNA (27), we used published nuclear detection systems for the comparative analyses. In the case of peanut, we referred to the arah2 (9, 26) and arah3 (23) amplicons; for soy, we applied the lectin amplicon (26). Because the given systems are described to be used in single-plex or multiplex approaches and are established for different real-time PCR-systems, all amplicons were cloned into the pCR2.1 TOPO vector to obtain uniform DNA templates for the standard calibrations.

Comparison of standard curves of the peanut-specific detection systems based on arah2 (9) or on the arah3 multicopy gene (23) with the atp6 mitochondrial primers and probe show approximately identical amplification when plasmid DNA (10-fold serial dilutions of $10^7$–$10^2$ copies each) was used as the template (Figure 2A). However, when total peanut DNA (10-fold serial dilutions from 25 ng to 25 fg) was used as the template, a significant reduction in Ct values for the atp6 primers and probe could be observed and lower amounts of template (up to 25 fg) could be amplified compared with the nuclear detection systems (Figure 2B). Amplification of DNA extracts from milk powder spiked with different amounts of soy and peanut shows that all systems are able to detect peanut, even at a spiking level of 1 ppm (Figure 2C). However, only two out of three reactions were positive when the arah2 primers and probe were used, and, for the arah3 amplicon, only one out of three reactions could be detected. Additionally, high SDs in the Ct values could be observed when samples with spiking levels below 10 ppm were amplified with arah2 and arah3 primers and probes. Therefore, the atp6 mitochondrial detection system, which led to reproducible Ct values even when samples with a spiking level of 1 ppm were amplified, is most suitable for quantitative analyses of trace amounts of peanut. In sum, the atp6 system provides the expected improvement in sensitivity in the range of at least 1 order of magnitude compared with the published systems for the detection of peanut.

Similar results could be obtained when the bait8 primers and probe were compared with the lectin amplicon. Both amplicons amplify plasmid standards with almost equal efficiencies (Figure 2D), whereas amplification of total soy DNA results in lower Ct values when the bait8 primers and probe were used compared with the lectin primers and probe (Figure 2E). Analyses of spiked milk powder reveal that both amplicons detect soy at a spiking level of 1 ppm. However, only Ct values obtained with the bait8 primers and probe allow differentiation between spiking levels below 10 ppm (Figure 2F).

### *Generation and Characterization of C-atp6 and C-bait8 Competitor DNA*

An important prerequisite for quantitative analyses by competitive PCR is that the competitor be amplified with the same kinetics and efficiency as the endogenous target sequence. Therefore, length and sequence composition should be as close as possible to those of the molecules to be quantified (44). In the case of multiplex real-time PCR approaches in which the differential detection of molecules is achieved by individual probes labeled with distinguishable fluorophores, it is sufficient to generate a molecule that differs only in the probe-binding region of the target sequence. The competitor DNA used in this study (C-atp6 and C-bait8) was designed by virtual mutagenesis of the probe-binding sites of the target amplicons, with the aim to achieve identical amplification terms for target and competitor molecules.

In the case of bait8, 11 nucleotides of the binding region were exchanged, whereas 8 nucleotides of the probe region of atp6 were modified (Figure 1). Additionally, C-atp6 and C-bait8 competitor DNA have different restriction profiles compared with their corresponding target DNA, which, if needed, in addition to real-time PCR approaches, allows for easy discrimination between both molecules.

The competitor molecules, C-atp6 and C-bait8, were generated by PCR amplification of commercially synthesized single-stranded oligonucleotides (biomers.net GmbH) representing C-atp6 and C-bait8 sequences, followed by a subsequent cloning step. The plasmids, pCR-C-atp6 and pCR-C-bait8, were characterized by real-time PCR in comparison with their corresponding target DNA, atp6 and bait8, with single-plex (data not shown) or duplex PCR approaches. As shown in Figure 3, coamplification of internal standard mixtures of 10-fold serial dilutions ($10^7$ to $10^2$ copies of each plasmid) of pCR-atp6/pCR-C-atp6 or pCR-bait8/pCR-C-bait8 results in nearly identical PCR efficiencies close to 100%, with correlation coefficients of >0.99. After fluorescence threshold-setting, the calibration lines of both target and competitor sets showed almost complete superimposition, indicating that C-atp6 and C-bait8 competitor DNA are suitable for quantitative analyses.

All subsequent quantitative analyses were performed in duplex PCR approaches using internal standard mixtures of 10-fold serial dilutions ($10^7$ to $10^2$ copies of each plasmid) of pCR-atp6/pCR-C-atp6 or pCR-bait8/pCR-C-bait8. The resulting standard curves were used for the fluorescence threshold-setting of signals for the endogenous targets (FAM channel) and corresponding competitors (ROX channel).

### *Titration of Threshold Values of Competitor Molecules for Milk Powder Spiked with 10 mg/kg Soy and Peanut*

The calibration of C-atp6 and C-bait8 competitor DNA was performed according to the procedure described by Holzhauser et al. (31). The published quantification system is based on arah2 sequences (14), and the authors established an equivalent of competitor molecules of 4100 copies corresponding with target signals obtained with DNA extracts from 0.3 g milk chocolate spiked with 100 ppm peanut. In regards to the improved sensitivity of bait8 and atp6 mitochondrial detection systems compared with systems amplifying nuclear sequences (Figure 2), we used reference material with a spiking level of 10 ppm for threshold calibration. In a first titration step, we added $1 \times 10^3$, $2.5 \times 10^3$, $5 \times 10^3$, $1 \times 10^4$, $2 \times 10^4$, and $4 \times 10^4$ copies of C-atp6 and C-bait8 competitor molecules per 0.1 g milk powder spiked with 10 mg/kg soy and peanut during DNA extraction. Every titration level was prepared in three independent experiments, and each sample was analyzed by real-time PCR in triplicate. After setting fluorescence Cts, the logarithm of the ratios of the Ct values of FAM signals and their corresponding ROX signals were calculated and plotted against the logarithmic amount of competitor DNA. The equation of the linear regression plot was resolved for $y = 0$, resulting in 21 544 copies of C-bait8 DNA and 11 168 copies of C-atp6 DNA (Figure 4A and C). A subsequent

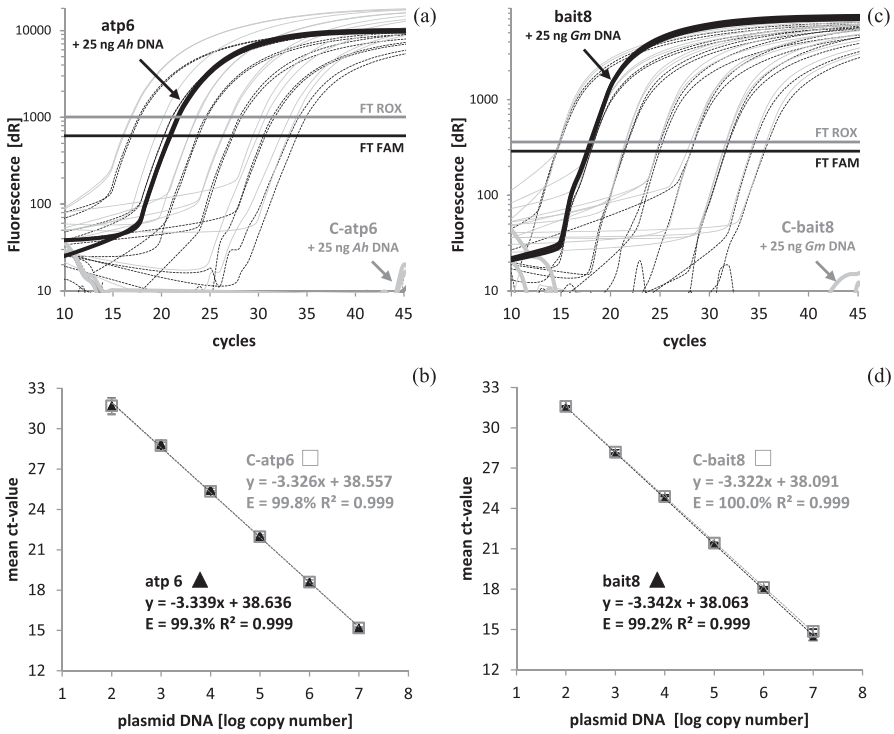


**Figure 3. Coamplification of competitor and wild-type DNA for the (a and b) atp6/C-atp6 and (c and d) bait8/C-bait8 quantitative detection systems by duplex real-time PCR. Amplification plots of 10-fold serial dilutions from $10^7$ to $10^2$ copies of (A, black dashed lines) pCR-atp6 and (a, solid gray lines) pCR-C-atp6 or (c, dashed black lines) pCR-bait8 and (c, solid gray lines) pCR-C-bait8 and (b and d) plasmids corresponding to calibration lines after fluorescence threshold-setting. Slope values and PCR efficiencies of nearly 100% indicate that wild-type DNA (black triangles) and competitor DNA (open squares) show almost identical amplification in duplex real-time PCR approaches ($R^2$, slope, and E-values are shown in the graphs). Signals are restricted to the FAM channel, when total DNA extracted from (a, +25 ng *Ah*DNA) peanut or (c, +25 ng *Gm*DNA) soy was amplified. Settings of fluorescence thresholds for signals recorded in the ROX channel (FT ROX) and the FAM channel (FT FAM) are indicated.**

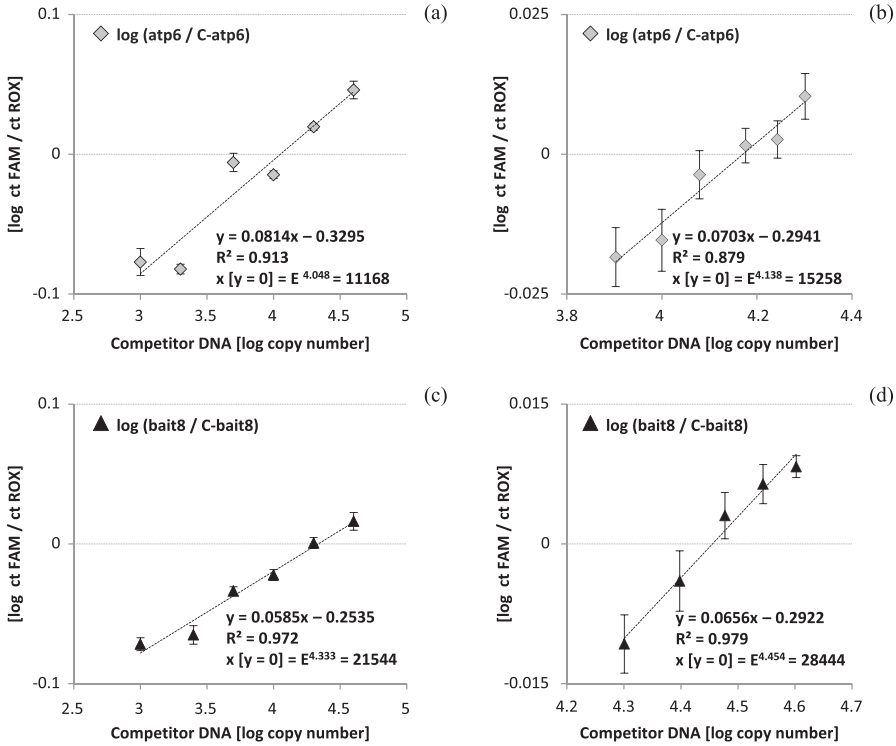


**Figure 4. Titration of competitor molecules equaling 0.1 g milk powder spiked with 10 ppm soy and peanut. The graphs show the linear regressions of two subsequent titrations for (a and b) C-atp6 and (c and d) C-bait8 molecules. The initial titration was performed within a range of $1 \times 10^3$ to $4 \times 10^4$ (A, gray diamonds) C-atp6 and (C, black triangles) C-bait8 competitor molecules. The final titration was performed with a finer screen and yielded (b) 15 258 copies C-atp6 or (d) 28 444 copies C-bait8, corresponding to wild-type signals originating from 0.1 g milk powder spiked with 10 mg/kg peanut and soy. Average values of three triplicate analyses ± SD are presented for each titration level.**

titration step with $8 \times 10^3$, $1 \times 10^4$, $1.2 \times 10^4$, $1.5 \times 10^4$, $1.75 \times 10^4$, and $2 \times 10^4$ copies of C-atp6 and $2 \times 10^4$, $2.5 \times 10^4$, $3 \times 10^4$, $3.5 \times 10^4$, and $4 \times 10^4$ molecules of C-bait8 resulted in 15 258 copy numbers of C-atp6 and 28 444 copy numbers of C-bait8 (Figure 4B and D).

### *Quantification of Peanut in Different Food Matrix Standards*

For the quantitative analyses, determined amounts of competitor molecules of C-atp6 (15 258 copies per 0.1 g food material) and C-bait8 (28 444 copies per 0.1 g food material) were added during the DNA extraction procedure to reference material spiked with different allergen concentrations ranging from 1 to 100 ppm. In addition to the spiked milk powder, we used three calibrated food matrix standards based on rice cookies, Hollandaise sauce powder, and sausage, which were previously characterized in interlaboratory validation studies (33). Each sample was extracted twice, and each extract was analyzed in triplicate by duplex real-time PCR. Threshold settings were performed according to serial dilutions of plasmid standards of target and competitor DNA, and the content of peanut (Figure 5) and soy (Figure 6) was calculated according to the equation published by Holzhauser et al. (31).

Comparison of Ct values of FAM and ROX signals obtained from the samples, which were amplified with atp6/C-atp6 primers and probes, reveals that the system is suitable for semiquantitative evaluation of trace amounts of peanut in the different food matrix standards analyzed. As shown in Figure 5A, spiking levels below the calibration threshold of 10 ppm lead to higher Ct values of FAM signals (peanut-specific amplification with the atp6 primers and probe) compared with ROX signals (competitor detection with the C-atp6 probe), whereas spiking levels above 10 ppm result in lower Ct values of FAM signals compared with ROX signals. In agreement with the titrated threshold values, nearly identical Ct values of FAM and ROX signals could be observed when samples prepared from reference materials containing 10 ppm peanut were amplified. The relations between the Ct values of FAM and ROX signals described are independent of the food matrixes analyzed, although peanut and competitor-specific Ct values obtained from samples prepared from different reference materials with identical spiking levels vary considerably. This indicates that C-atp6 competitor DNA is suitable to serve as an internal standard for PCR normalization.

Calculation of peanut concentrations according to the equation published by Holzhauser et al. (31; Figure 5B) results in 10.5 ppm peanut, with a CV of 14.4% when samples prepared from milk powder containing 10 ppm peanut were analyzed. This accordance was expected because milk powder was used for threshold calibration. Quantitative analyses of samples prepared from other food matrix standards with 10 ppm incurred peanut also showed good correlation between calculated and expected values, with 8.4 ppm for sausage (CV = 10.9%), 12.0 ppm for rice cookie (CV = 20.9%), and 9.8 ppm for Hollandaise sauce powder (CV = 46.9%). Quantitative analyses of materials with spiking levels of 1, 5, 20, and 100 ppm peanut showed that all spiking levels between 1 and 100 ppm could be quantified, indicating that the detectable amount of peanut lies within the range of 1 order of magnitude below or above the calibration threshold of 10 ppm. In case of larger deviations from the calibration threshold, either the peanut-specific signal

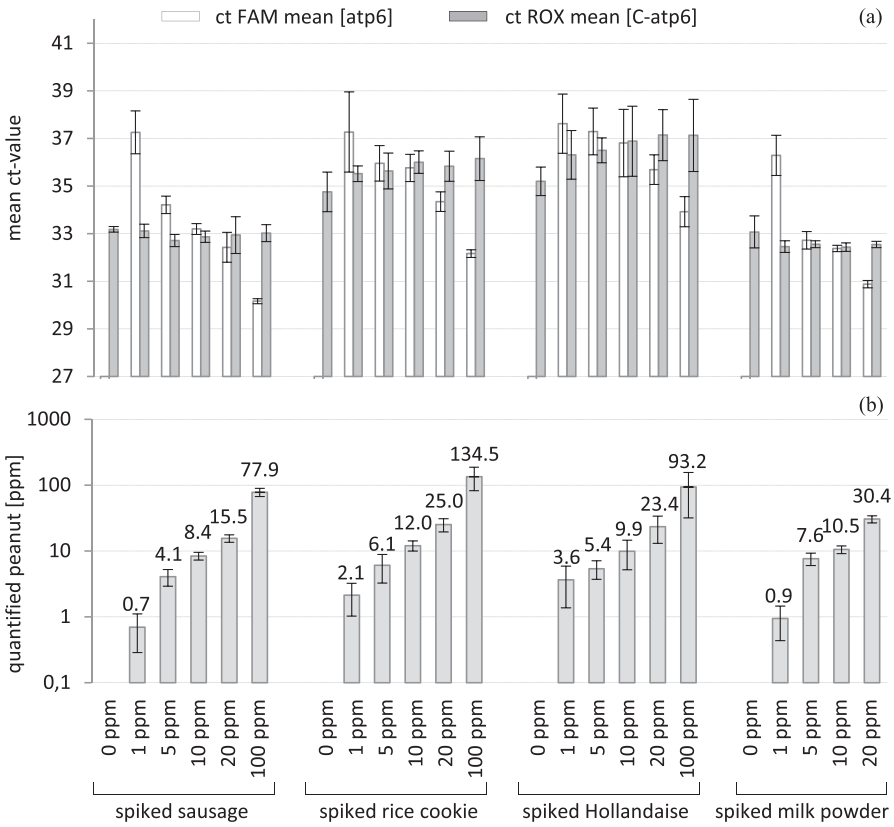


**Figure 5. Quantification of peanut in different food matrix standards with spiking levels between 1 and 100 mg/kg allergens by competitive real-time PCR. (a) Mean Ct values of triplicate analyses ± SD of ROX (competitor DNA C-atp6) and FAM (target DNA atp6) channels are shown for samples prepared from sausage, rice cookies, Hollandaise sauce powder, and milk powder spiked with 1, 5, 10, 20, and 100 ppm peanut. Amplification of samples prepared from blank material (0 ppm) result in signals restricted to the ROX channel (C-atp6). (b) Calculation of parts-per-million contents was performed according to the equation published by Holzhauser et al. (31). The calculated amounts of peanut (parts-per-million) are indicated above the bars.**

(with spiking levels >1 ppm) or the competitor-specific signal (with spiking levels >100 ppm) is suppressed, thereby precluding quantitative analyses. Comparing the recovered amounts within the quantitative range (1–100 ppm), the atp6/C-atp6 system provides distinctions even between slight differences of incurred peanut. It has to be mentioned that food matrix standards (rice cookies) have also been tested using a commercial ELISA kit in a previous work (2). For materials incurred with 5, 10, 20, and 40 ppm peanut, the recoveries ranged from 63 to 75% (data not shown). Thus, the deviation of the ELISA results from the real value is larger than the deviation obtained for all the matrixes tested with this method.

However, matrix effects cannot be entirely compensated for. For example, the quantitative analyses of samples prepared from spiked sausage reveals that all recovered values are slightly below the real peanut quantities, whereas, in preparations from spiked rice cookies, slightly elevated amounts of peanut were detected. Another point is that the SDs and, therefore, the CVs, are pronounced differently depending on the food matrix analyzed. For example, samples prepared from spiked Hollandaise sauce powder showed a significantly higher variance (e.g., $RSD_{10ppm}$ = 46.9%) compared with samples prepared from spiked sausage (e.g., $RSD_{10ppm}$ = 10.9%) or spiked rice cookie (e.g., $RSD_{10ppm}$ = 20.9%). In accordance with other PCR methods, the accuracy of competitive PCR still depends, to a certain degree, on the quality of the DNA to be analyzed. However, this problem can be largely avoided by multiple determinations, as is apparent from the mean parts-per-million values.

### *Quantification of Soy in Different Food Matrix Standards*

As already described for the atp6/C-atp6 system, semiquantitative evaluation of soy (Figure 6A) provides similar relations between the Ct values of FAM and ROX signals when samples prepared from different reference materials were amplified with bait8/C-bait8 primers and probes. In agreement with the data obtained with the peanut-specific system, FAM:ROX ratios are almost independent of the food matrix analyzed, as bait8-specific signals (FAM channel) and C-bait8-specific signals (ROX channel) show similar variations when different matrixes with identical spiking levels were compared. Evaluation of soy content reveals that all parts-per-million levels investigated could be clearly distinguished, even when samples prepared from food matrixes with minor differences in spiking levels of 5, 10, and 20 ppm were analyzed. However, quantification of soy concentrations showed that the calculated amounts are generally higher than the expected values (Figure 6B). This suggests that the amount of competitor molecules added during the sample preparation process was too low.

Although the threshold calibration for the bait8/C-bait8 system turned out to be slightly inaccurate (the titrated copy number of 21 544 molecules fit more for 5 ppm spiked materials rather than 10 ppm spiked materials), the data presented, in the

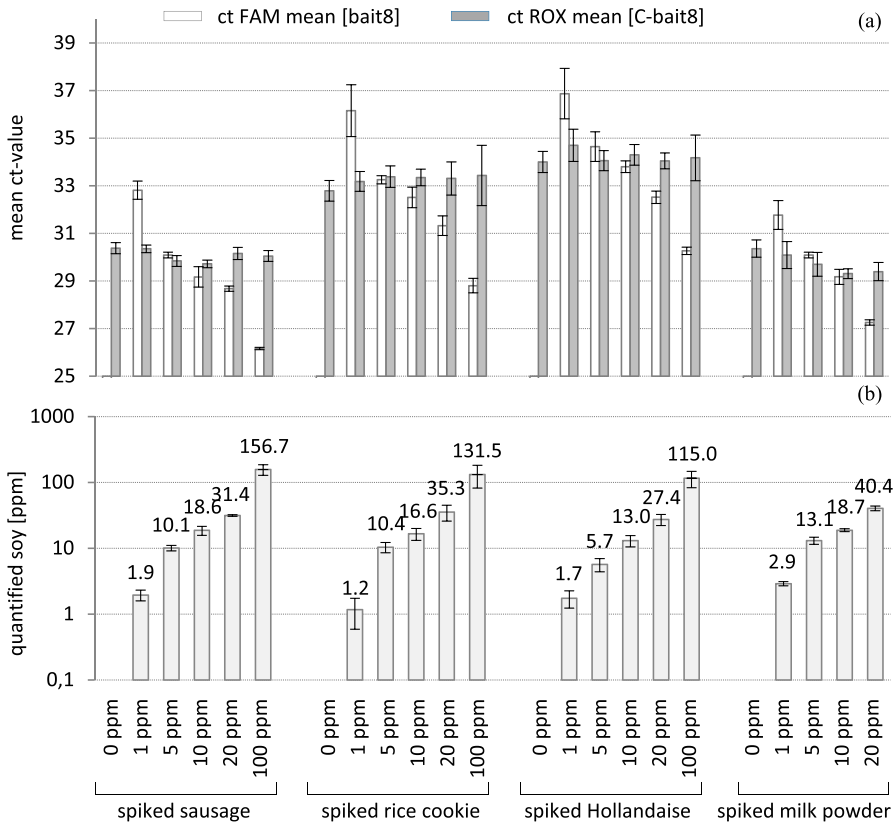


**Figure 6. Quantification of soy in different food matrix standards. (a) Comparative representation of mean Ct values detected in FAM (bait8) and ROX (C-bait8) channels for semiquantitative evaluation/analyses and (b) calculation of soy content according to Holzhauser et al. (31). Signals are restricted to the ROX channel when total DNA extracted from blank material (0 ppm) was amplified.**

context for proof of concept, clearly demonstrate that the described quantification systems for soy (bait8/C-bait8) and peanut (atp6/C-atp6) are feasibly methods to quantify trace amounts of both allergens. The threshold values of competitor molecules were determined with 0.1 g reference material spiked with 10 ppm allergens, which allows the quantification of soy and peanut in the range of 1–100 ppm. However, the calculated threshold values suggest that the two systems have the potential for the quantification of much lower amounts of allergens.

### *Determination of Mitochondrial DNA Content*

Because the bait8/C-bait8 and atp6/C-atp6 detection systems are based on mtDNA, we wanted to clarify whether there are significant variations in mtDNA content between different soy cultivars and peanut varieties.

To examine possible variations in the mtDNA content of soybean, DNA extracts from 15 different soy cultivars were analyzed by real-time PCR. Aliquots of 2.5 ng total soy DNA from each cultivar were amplified in triplicate with the bait8 primers and probe to detect mtDNA and amplified in parallel with the lectin primers and probe (Accession No. K00821; 26) to detect nDNA. Ct values for mtDNA and nDNA amplification were converted into copy numbers according to conversion factors obtained by bait8 or lectin plasmid standards, which were used, in serial dilutions ranging from $10^7$ to $10^2$ copies, as calibration curves. In the case of peanut, DNA from 11 different peanut varieties were prepared, and 2.5 ng extracts were amplified in triplicate with the atp6 mitochondrial system and amplified in parallel with the arah2 primers and probe (Accession Nos. FJ713110, L77197, and AY007229; 9) to detect nDNA. The copy numbers of peanut mtDNA and nDNA were determined via standard curves prepared with arah2 and atp6 plasmid DNA. Relative amounts of mtDNA were determined by calculating the ratios of mtDNA to nDNA. Ratios were calculated by dividing the mean SQ values, and SDs were determined using Bio-Rad CFX 3.1 software. Variations were illustrated by calculating the deviation of the mean SQ ratios as the percentage from mean SQ ratios of all investigated soy cultivars or peanut samples, respectively (258.2 for soy and 194.3 for peanut). As shown in Figure 7A, analyses of 11 different peanut varieties showed a maximum deviation of 25% from the mean mtDNA:nDNA ratio, and, in the case of soy cultivars (Figure 7B), we found maximal deviations of 17%, with 1 exception of 50% (Vital). In regards to quantitative analyses, these factors should be considered. However, in view of the trace amounts of allergens that can be quantified by both systems, the inaccuracies are sustainable.

## Discussion

### *Increased Sensitivity of Mitochondrial Targets Compared with Nuclear Detection Systems*

The atp6/C-atp6 and bait8/C-bait8 systems described in this paper show increased sensitivity compared with the nuclear detection systems analyzed in parallel. The advantage of targeting mitochondrial sequences for allergen detection is evident as higher

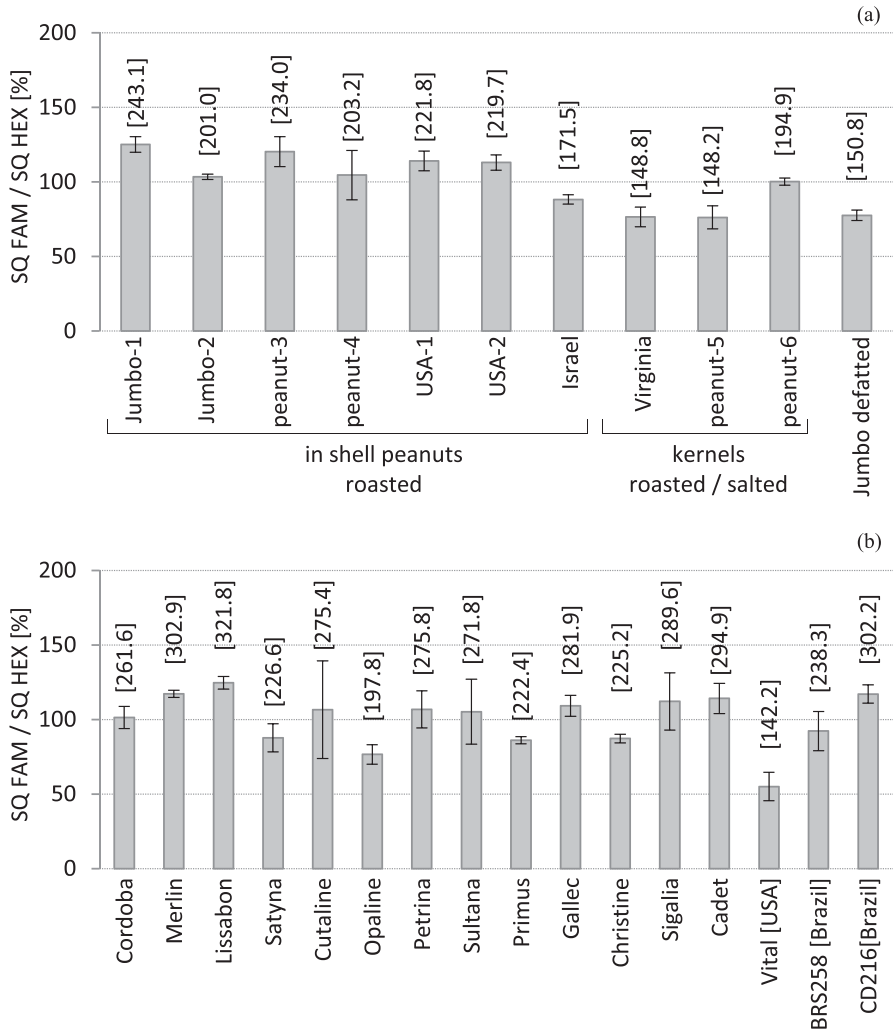


**Figure 7. Variation in mtDNA content in (a) peanut and (b) soybean. (a) Variation of mtDNA content is illustrated by bars representing the percentages of the mean ratios between starting quantities (SQs) obtained by atp6 amplification versus arah2 (9) amplification of samples prepared from 11 different peanut sources. (b) In the case of soy, extracts from 16 different cultivars were analyzed, and the percentages of the mean ratios of bait8 amplification versus lectin (9) amplification were calculated. The original ratios of the mean SQ values are indicated above the bars.**

detection sensitivity is achieved by the fact that there are more mtDNA copies than nDNA copies per cell. Methods targeting nDNA are mostly restricted to LOD values between 2 and 10 ppm. This is described for the detection of peanut based on arah2 (9, 14) or arah1 (32) sequences. In the case of soy, a method based on the lectin gene, Le1, sequences embedded in a multiplex PCR system for the simultaneous detection of peanut, hazelnut, celery, and soy result in an LOD value of 10 ppm (26).

Several approaches targeting multicopy genes or targets belonging to multigene families, which allow lower detection limits, are published. For peanut, two methods based on multicopy genes are described: a PNA system based on the agglutinin precursor gene, PNA (21); and the arah3 system (23), which was further refined by applying a nested PCR approach (28). Other PCR methods used targets representing repetitive elements or the polymorphic internal transcribed spacer regions, ITS-1 and ITS-2, of the 18S–5.8S–26S nuclear ribosomal cistron. In the case of soy, two methods based on conventional PCR are published: one targeting the interspersed repetitive element 1, SIRE-1 (22); and the other using sequences corresponding to the ITS-2 region (19). In addition to the soy ITS-2 system, Hirao et al. (19) established a method for peanut detection based on ITS-1 sequences (Accession No. HQ537458.1). The same genomic region was used to establish a real-time PCR system for the detection of *Arachis* species (24). The authors showed that the method allows the detection of 0.1 ppm peanut in different food matrixes. Comparable LOD values have only been described for the amplification of sequences specific to extrachromosomal DNA, such as mtDNA (27, 45) or chloroplast DNA (46, 47). With regards to the reference doses published by the Voluntary Incidental Trace Allergen Labelling (VITAL) Expert Panel (48), only these targets are suitable for compliance with the recommended analytical detection levels of selected allergens like peanut.

### *Requirements for Detection and Quantification of Soy and Peanut*

In 2014, the VITAL Expert Panel published reference doses of important food allergens derived from clinical challenge studies

of food-allergic subjects (48). Precautionary allergen labeling is recommended if, by consumption of a contaminated foodstuff, the respective reference dose is exceeded. Reference doses for peanut and soy (in milligrams of protein) were 0.2 and 1.0 mg. Therefore, assuming the consumption of 100 g contaminated foodstuff, the reference doses would be exceeded with peanut and soy concentrations of 2 ppm peanut protein and 10 ppm soy protein, respectively (about 8 ppm peanut and 25 ppm soybean flour). Therefore, labeling of commercial food is recommended at allergen levels from concentrations of 5 or 20 ppm for peanut or soy, respectively (49). This implies that analytical methods are required for the quantitative recovery of peanut with a quantitation limit below 10 ppm (50). The mitochondrial systems described in the present paper fulfill these requirements, as they provide quantitative determination of soy and peanut within a range between 1 and 100 ppm.

### *Are Mitochondrial Targets Suitable for Species-Specific Detection?*

In 2011, we published a mitochondrial detection system (the atpA system) for soy based on sequences corresponding to the ATPase subunit 1 gene, *atpA-1* (27). The paper demonstrated that the mitochondrial approach leads to an improvement of sensitivity in the range of 2 orders of magnitude compared with a commercial detection kit based on nDNA and allows the amplification of 25 fg total soy DNA with a detection limit of 0.1 ppm soy spiked in a spice model. However, a new database screen considering recently published sequence data of different leguminous plants (35, 51–54) revealed that the atpA target sequence shows redundancies compared with corresponding regions in *Vigna* and *Phaseolus* species. Because the complete mitochondrial genome of soy was published in 2013 (35), we took advantage of these data and performed a new database screen. Additionally, we were able to identify mitochondrial targets specific for peanut by using the EST data summarized in the PeanutDB transcriptome database (41). The number of completed plant mitochondrial genome sequences has rapidly increased in the last few years, and more than 100 are now available in GenBank database as of May 2017. Comparative analyses show that plant mitochondrial genomes are highly diverged in terms of genome size (ranging from 200 kb to over 2000 kb; 55), gene arrangement, and sequences in intergenic regions (56). However, within-species nucleotide polymorphisms are usually almost unobservable, as introns of plant mitochondrial genes are self-splicing, which implies that larger numbers of nucleotides are critical for proper splicing (57). Therefore, plant mitochondrial genomes provide a multiplicity of species-specific PCR targets that are available for the detection of plant allergens.

### *Are Mitochondrial Targets Suitable for Quantitative Approaches Like Competitive Quantitative PCR?*

One prerequisite for the quantitative determination of allergenic ingredients is that the amount of target molecules be comparable across various specimens. A particular feature of extrachromosomal DNA in plants is that the number of genome equivalents per diploid cell is highly variable during plant development (58), and the copy numbers of mitochondrial genes differ significantly in different plant tissues (59). Additionally, there is a linear increase in the number of mitochondria with increasing cell size (59). However, we could show that variations between the ratios of mtDNA and nDNA copy numbers are not very pronounced [up to 25% in different peanut species and up to 17% (one sample up to 50%) in different soy cultivars] when identical DNA samples of the same tissue of different species/cultivars were compared. Considering the low detection limits provided by mitochondrial targets and the fact that the occurrence of allergens is mostly restricted to one specific tissue, quantitative analyses via mtDNA are not significantly compromised by interfering variations of template amounts.

For quantitative determination, we designed and cloned IACs (C-atp6 and C-bait8) for competitive real-time PCR. According to the method published by Holzhauser et al. (31), we could show that both detection systems allow the quantitative determination of peanut and soy at a level of 1 ppm, which could be demonstrated independently for three different food matrix standards (33). In terms of a proof-of-concept study, the data presented in the present work were determined for a calibration threshold of 10 ppm peanut and soy. Considering the relatively high numbers of C-atp6 and C-bait8 competitor molecules equalizing this threshold, the sensitivity of both systems can be further extended to far lower allergen concentrations. In agreement with the data published by Holzhauser et al. (31), the competitive quantitative PCR approach is a timesaving possibility to quantify allergenic ingredients compared with other published methods (29, 30), as only a limited number of analytical replicates has to be prepared and analyzed to achieve reliable quantitative results. Another advantage is that the addition of competitor molecules during sample preparation and PCR amplification is an effective way to show false-negative results and, furthermore, false-positive signals are suppressed due the presence of the competitor. However, the number of replicates to be analyzed still depends on the quality of the DNA to be analyzed and, therefore, on the food matrix. As in other real-time PCR-approaches, high SDs occur in samples with insufficient DNA quality. We could show that this problem could be improved aside from optimizing DNA purification by using novel polymerases, like Sso polymerase, which was especially designed for complicated templates like food or food-related samples.

## Acknowledgments

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