



Analytical Methods

Matrix-normalised quantification of species by threshold-calibrated competitive real-time PCR: Allergenic peanut in food as one example

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ARTICLE INFO

Article history:

Received 16 May 2013

Received in revised form 4 December 2013

Accepted 21 April 2014

Available online 30 April 2014

Keywords:

PCR

ELISA

Quantitative

Species

Allergen

Threshold

Action level

ABSTRACT

A novel method to quantify species or DNA on the basis of a competitive quantitative real-time polymerase chain reaction (cqPCR) was developed. Potentially allergenic peanut in food served as one example. Based on an internal competitive DNA sequence for normalisation of DNA extraction and amplification, the cqPCR was threshold-calibrated against 100 mg/kg incurred peanut in milk chocolate. No external standards were necessary. The competitive molecule successfully served as calibrator for quantification, matrix normalisation, and inhibition control. Although designed for verification of a virtual threshold of 100 mg/kg, the method allowed quantification of 10–1000 mg/kg peanut incurred in various food matrices and without further matrix adaption: On the basis of four PCR replicates per sample, mean recovery of 10–1000 mg/kg peanut in chocolate, vanilla ice cream, cookie dough, cookie, and muesli was 87% (range: 39–147%) in comparison to 199% (range: 114–237%) by three commercial ELISA kits.

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1. Introduction

Allergies to food are considered a substantial health risk. Severe and potentially fatal reactions have been reported repeatedly (Hompes et al., 2011; Järvinen, 2011). Because therapeutic treatment of this immunological disorder is not available, avoidance of the allergenic food remains to be most effective for avoidance of an allergic reaction (Burks et al., 2011; Wang & Sampson, 2012). For better visibility of allergenic components, package labelling of selected allergenic ingredients is required in various countries (e.g. according to Directive 2007/68/EC; US FALCPA, 2004). Independently, allergens may unintentionally enter foods by so-called cross-contact (Röder et al., 2010a) and remain indiscernible or hidden.

At present, analytical verification of labelling requirements and the detection of allergenic proteins in foods is primarily protein-based ELISA (enzyme-linked immunosorbent assay). However, false positive results at low levels of detection (Nollet & Hengel, 2011) as well as large differences in quantitative results between available test kits have been reported (Poms et al.,

2005). Because of their potentially high specificity and sensitivity nucleic acid-based detection methods, such as polymerase chain reaction (PCR), can serve as an alternative or complementary method for the detection of allergenic species or crops used in food manufacture (Holzhauser & Röder, 2011). In Germany and Japan, both PCR and ELISA techniques are used as official methods for allergen detection in state control laboratories (BVL, 2003, 2013; Sakai, Adachi, Akiyama, & Teshima, 2013).

Quantitative determination of allergenic food ingredients using PCR has rarely been described. Hirao and colleagues demonstrated the successful quantification between 10 and 100 mg/kg buckwheat in unprocessed wheat or rice flour by normalisation against spiked statice (*Limonium sinatum*) in a duplex real-time PCR analysis. Recoveries between 57% and 80% were achieved (Hirao, Hiramoto, Imai, & Kato, 2006). The principle idea was adapted for lupine (*Lupinus angustifolius*) quantification by Demmel and co-workers: Levels below 10 mg/kg lupine in wheat flour were quantified at recovery rates between 146% and 186% (Demmel, Hupfer, Busch, & Engel, 2012). Moreover, 800–1500 mg/kg soybean was quantified at a 78–105% recovery rate in wheat or rice flour using a standard addition method and real-time PCR analysis (Eugster, 2010). Despite the same basic quantitative performance, all PCR methods described in literature required high numbers of replicates for samples or an external standard, have not been applied to processed foods or complex food matrices. Others quantified soy and mustard in cooked sausages by PCR. At or below

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100 mg/kg, recovery rates of 44–135% were achieved on the basis of external standards which were adjusted to the cooked sausage matrix (Siegel, Schnur, Boernsen, Pietsch, & Waiblinger, 2012). The applicability to matrices other than cooked sausage was not shown. However, the exponential nature of DNA amplification may result in significant variation between replicates and various sample types because of potential tube-to-tube variations in amplification efficiency and because nucleic acids extraction is likely variable in the case of complex multi-compound matrices such as food products. As a consequence, Siegel and co-workers developed standards which were adjusted to additional selected food matrices, such as rice cookie, wheat cookie, and sauce hollandaise (Siegel, Mutschler, Boernsen, Pietsch, & Waiblinger, 2013) but the authors did not demonstrate the general applicability of these food matrix standards to other food matrices which are rich in carbohydrates (e.g. cookie), protein (e.g. sausage), and fat (e.g. sausage, sauce hollandaise). Thus, the transferability of one matrix-adjusted calibration to another food product has not been shown. Moreover, effects on the quantitative determination arising from the degree of processing were shown for both ELISA and PCR analysis and need to be accounted for correct interpretation of the analytical results (Platteau, De Loose, De Meulenaer, & Taverniers, 2011). Considering the vast number of different food matrices and conditions of processing, a calibration that is independent from various external matrix-adjusted standards would be ideal for routine application in allergen quantification.

To overcome a reaction-to reaction variation because of differences in amplification efficiency, it has been proposed to normalise PCR by the addition of a known amount of an internal standard DNA or a reporter gene that is co-amplified with the target DNA (Zachar, Thomas, & Goustin, 1993). Having conserved primer binding sites, both target and internal standard DNA are amplified in a competitive way. This approach has also been successfully demonstrated for the quantification of genetically modified crops using both post-PCR gel analysis (Studer, Rhyner, Lüthy, & Hübner, 1998) and real-time PCR analysis (Shimizu et al., 2008). However, the addition of an internal competitive standard to the amplification reaction does not consider the effect of sample preparation. Based on the German official method for qualitative peanut detection with PCR according to Article 64 of the German food and feed law (BVL, 2013), this study explores the potential use of an artificial competitive nucleic acid sequence as an internal calibrator to normalise effects of DNA extraction and amplification in order to quantify peanut in various food matrices without the need of matrix adaption or the use of external standards. Furthermore, this study aims to quantify allergenic peanut in complex food matrices on the basis of a very limited number of replicates – an economic necessity for future routine application. The quantification of peanut in food products serves

as an example to present a novel method for the quantification of DNA or species in general.

2. Materials and methods

2.1. Samples

Milk chocolate with 2 and 100 mg/kg peanut, respectively, and dark chocolate with 20 mg/kg peanut, previously employed in a collaborative trial for performance testing as official German method according to Article 64 of the German food and feed law (BVL, 2003) were kindly provided by ifp (Institut für Produktqualität, Berlin, Germany) (Table 1).

Commercial food samples were purchased from a local retailer. Food products containing between 100,000 and 10 mg/kg incurred peanut were prepared in a ten-fold dilution series as described for chocolate: Peanut-free chocolate (verified using peanut-specific in-house ELISA and real-time PCR) was fortified with finely ground peanut cream that consisted of 100% peanut (unknown variety) from a commercial retailer (GranoVita, #1008, Heimertingen, Germany). 1 g of peanut cream was added to 9 g of milk chocolate, melted at 37 °C and mixed with a stirrer for sample homogeneity. Thereafter, 1 g of the incurred sample containing 100,000 mg/kg peanut was added to another 9 g of chocolate. The procedure was continued until a chocolate containing 10 mg/kg of peanut was obtained. Vanilla ice cream was prepared accordingly at 37 °C. Oat-based coconut muesli was homogenised in a blender and fortified with peanut cream accordingly. Self-prepared cookies having defined peanut content were baked from self-made cookie dough having defined peanut content. Cookie dough (50% wheat flour, 20% sugar, 14% water, 13% vegetable fat, 3% milk powder) and cookies baked thereafter at 200 °C for 15 min were prepared as described elsewhere (Röder, Filbert, & Holzhauser, 2010b).

The actual level of incurred peanut content in food matrix was experimentally determined using the in-house peanut specific ELISA. The quantitative read-out of peanut protein was normalised against peanut cream 'GranoVita'. Peanut protein from peanut cream was determined using the in-house ELISA. The peanut cream contained 29.15% extractable and quantifiable peanut protein, resulting in a conversion factor of 3.43 to calculate the peanut content from quantified peanut protein in the food samples.

2.2. Peanut specific ELISA tests

Three commercial peanut-specific ELISA test kits, all certified as 'AOAC Research Institute Performance Tested Method', were included in the study: BioKits Peanut Assay Kit (Tepnel BioSystems, via Coring Sytem Diagnostix GmbH, Gernsheim, Germany),

Table 1

Quantification of peanut using cqPCR and commercial peanut ELISA kits. The cqPCR was performed using external target and competitor DNA standards for adjustment of fluorescence signal thresholds.

Sample	mg/kg peanut quantified				
	cqPCR	ELISA A	ELISA B	ELISA C	ELISA, %CV
Milk chocolate (2 mg/kg)**	1.6*	2.0	4.2	<LOD	35.5
Dark chocolate (20 mg/kg)**	17.9	7.2	13.6	1.1	62.0
Milk chocolate (100 mg/kg)**	101.0	117.0	170.1	91.9	31.6
Milk chocolate***	11.4	13.4	11.5	0.9	78.3
Nougat chocolate***	47.7	47.4	15.2	38.5	49.3
Dark chocolate***	<LOD	<LOD	<LOD	<LOD	
Milk chocolate***	<LOD	<LOD	<LOD	<LOD	
Chocolate mousse***	<LOD	<LOD	<LOD	<LOD	

* 2/4 PCR reactions positive.

** Samples with known peanut level between 2 and 100 mg/kg originate from a previous collaborative trial.

*** Retail samples of unknown peanut level; %CV, coefficient of variation of quantitative results between three ELISA kits from vendor A, B, and C; LOD, limit of detection.

RIDASCREEN® FAST Peanut (R-Biopharm AG, Darmstadt, Germany), and NEOGEN Veratox® Peanut Allergen Test (Neogen Europe Ltd., Auchincruive, Ayr, Scotland). All three commercial ELISA tests featured peanut standards for direct assessment. Extraction buffers and protocols were included. Sample preparation and ELISA test procedures were performed according to the manufacturers' instructions. According to the manufacturers, the limit of detection (LOD) of the tests kits was 1, 1.5, and 0.9 mg/kg peanut, respectively, and the limit of quantification (LOQ) of the test kits was 1, 2.5, and 2.5 mg/kg peanut, respectively. The in-house peanut-specific sandwich-ELISA according to Stephan and Vieths (2004) had comparable LOD and LOQ of 0.5 and 2 mg/kg peanut, respectively. Applying the conversion factor experimentally determined, the LOD and LOQ would be 0.3 and 1.7 mg/kg peanut, respectively. The in-house ELISA was performed as previously described (Röder et al., 2010b; Stephan & Vieths, 2004).

2.3. Generation of DNA competitor

As competitor, a dsDNA of 107 bp length with conserved primer binding sites but divergent probe binding site was generated by PCR strategy. Details are given elsewhere (Supplementary material). The competitor dsDNA was serially diluted in TE buffer (10 mmol/l TRIS, 1 mmol/l EDTA, pH 8.5) containing 0.1% Tween 20, and stored at -20°C until use.

2.4. DNA extraction from food sample with/without internal competitor standard

The method was previously described elsewhere (Holzhauser, Wangorsch, & Vieths, 2000) and modified (Röder et al., 2010b). In this study, the method was further adapted for quantification against an internal competitor standard. Thus, the final protocol was as follows: The food sample was ground to homogeneity using an analytical mill (IKA M20, IKA Labortechnik, Staufen, Germany). 300 mg (± 15 mg) were transferred to a 2 ml micro reaction tube and suspended in 1.5 ml of CTAB buffer (55 mmol/l hexadecyltrimethyl-ammoniumbromide (CTAB), 1400 mmol/l NaCl, 20 mmol/l ethylenediaminetetraacetate (EDTA), 100 mmol/l TRIS (tris (hydroxymethyl)-aminomethane), pH 8.0, adjusted with 10% HCl, autoclaved). Initial extraction was carried out in a thermomixer (No. 5437, Eppendorf, Hamburg) at 65°C , 1000 rpm, for 5 min or until a homogeneous suspension was achieved. 20 μl of 20 mg/ml proteinase K (Merck, No. 1.24853, DNA grade, Darmstadt, Germany) were added. For threshold-based competitive PCR, the amount of competitor nucleic acid titrated (see Section 2.7.1 below), which was included in a total volume of 7.5 μl of 10 mmol/l Tris-HCl, pH 8.5, was pipetted to the extraction mixture.

The sample was digested at 65°C , 1000 rpm, for 30 min. For removal of undissolved material, the tube was centrifuged at 14,000 rpm for 2 min in a common desk top centrifuge for micro reaction tubes. Subsequently, digested proteins and Proteinase K were removed by chloroform extraction: 800 μl of the supernatant were transferred to a 2 ml micro reaction tube and 600 μl of chloroform were added. Peptides were precipitated by agitation using a vortexer. The tube was centrifuged as described above and 600 μl of the DNA-containing upper phase were transferred to another 1.5 ml micro reaction tube. After addition of 1 μl of 20 mg/ml glycogen (Roche, No. 10901393001, for molecular biology) and 500 μl of isopropanol (-20°C), the DNA was precipitated by carefully inverting the tube ten times or careful agitation using a vortexer. The precipitate was centrifuged as described above and the supernatant was discarded. The resulting DNA pellet was washed once with the addition of 500 μl of 70% ethanol, and the ethanol was removed. The pellet was dried at 50°C for 5 min in the thermomixer.

The dried pellet was dissolved at 4°C overnight in 100 μl of TE buffer or as much as needed to fully dissolve the pellet.

100 μl of the extracted DNA was further purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, No. 28106) according to the manufacturer's instructions. The purified DNA was eluted with 50 μl of buffer EB from the column. The column was once more eluted with 50 μl of the first elution. The purified DNA was subjected to PCR analysis or stored at -20°C until use.

2.5. Real-time PCR for peanut DNA and competitor DNA

The PCR chemistry described allows non-competitive analysis of peanut or competitor DNA in separate reaction tubes or simultaneous competitive amplification of both peanut and competitor DNA in a single reaction tube. PCR of peanut and/or competitor DNA was carried out in 0.2 ml thin-walled micro-tubes (Advanced Biotechnologies, Hamburg, Germany) in a final volume of 50 μl . The PCR mixture contained 1 $\times$ reaction buffer (20 mmol/l Tris HCl (pH 8.4), 50 mmol/l KCl; supplied with Platinum® Taq, Invitrogen, Karlsruhe, Germany), 5.0 mmol/l magnesium chloride (supplied with Platinum® Taq, Invitrogen), 200 $\mu\text{mol/l}$ each of the deoxyribonucleoside triphosphates (dNTP) dATP, dCTP and dGTP, and 400 $\mu\text{mol/l}$ of dUTP as substitute for dTTP, and derived from a dUTP-containing dNTP mix (Carl Roth, Karlsruhe), 0.25 $\mu\text{g/l}$ of bovine serum albumin (Sigma, No. A 8022, Deisenhofen, Germany), 300 nmol/l of each primer 'forward' (5'-GCAGCAGTGGGAACCTCAA GGAGACA-3') and 'reverse' (5'-GCATCACATGTTGCTCGCAG-3'), 200 nmol/l labelled peanut probe (5'-FAM™-CGAGAGGGCGAACC TGAGGCC-3'BHQ-1®), 200 nmol/l labelled competitor probe (5'-HEX-CCGGAGTCCAAGCGGGAGAGC-3'BHQ-1®), 0.5 units of uracil-N-glycosylase (AmpErase UNG, PE Biosystems, No. N 808-0096), 1.25 units of Platinum® Taq polymerase (Invitrogen), and either 5 μl of undiluted extracted DNA from samples, 5 μl of standards, or 5 μl of calibrator mixture. Primers and probes were commercially synthesised (biomers.net GmbH, Ulm, Germany). The PCR was carried out in an Mx3005P™ QPCR system (Stratagene, via Agilent Technologies Sales & Services GmbH & Co. KG, Waldbronn, Germany) using the following conditions: enzymatic decontamination reaction from dUTP-containing template at 50°C for 2 min, deactivation of AmpErase UNG and activation of Platinum® Taq polymerase at 95°C for 2 min, followed by 45 two-step cycles with a DNA denaturation at 95°C for 30 sec, and combined primer annealing and elongation at 62°C for 30 s. Fluorescence was recorded after each cycle using both the FAM and HEX channel.

2.6. Fluorescence threshold (F_t)-setting of thermo cyclers

For determination of cycle thresholds (C_t) of both peanut target DNA (FAM signal) and competitor DNA (HEX signal), a fluorescence signal threshold F_t needs to be defined. In competitive real-time PCR, specific F_t -values have to be set in a way that identical copy numbers of peanut and competitor DNA result in identical C_t values. Two different approaches of setting F_t were investigated.

2.6.1. External target and competitor standard curves

107 bp competitor DNA was prepared as described above. For the generation of an 86 bp peanut amplicon genomic DNA was purified from peanut cream as described above. As was described for the generation of competitor DNA (Section 2.3), an 86 bp peanut amplicon was generated in PCR with peanut genomic DNA. Two separate standard series between 100 and 1,000,000 copies were obtained by tenfold serial dilution made of peanut amplicon DNA and competitor DNA, respectively. Dilution was performed in TE buffer containing 0.1% Tween 20, and standard series were stored at -20°C until use. Both standard curves were run simultaneously in a non-competitive way in singlet analysis. F_t for peanut

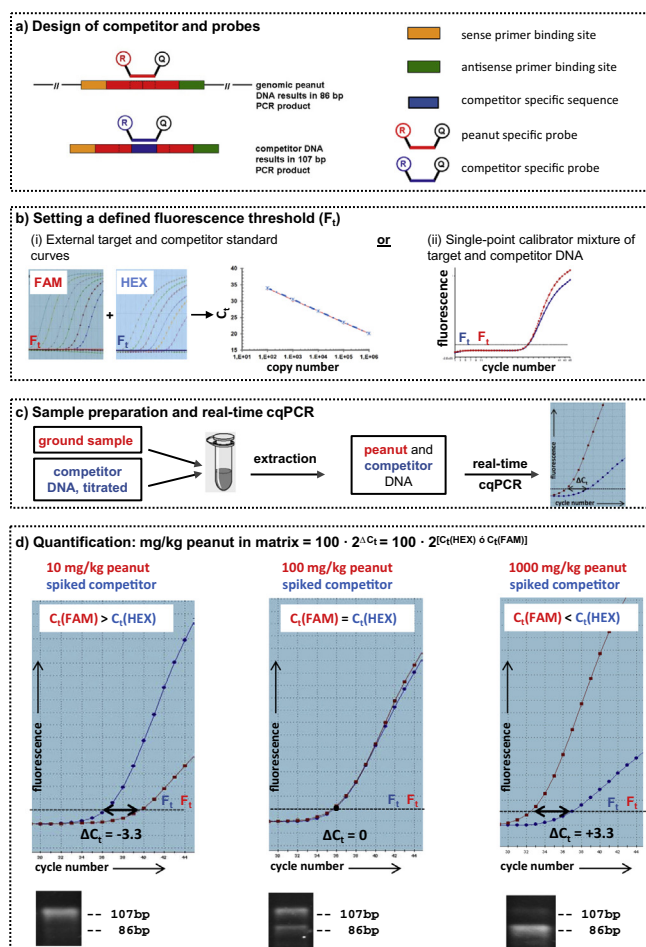


Fig. 1. Principle of real-time cPCR for threshold concentration-based quantification of peanut as an example.

DNA (FAM channel) and competitor DNA (HEX channel) was selected in a way that such F_t adjustment leads to a superimposition of both standard curves (Fig. 1b(i)). At this adjusted F_t -values, C_t -values of peanut DNA amplification (FAM channel) and of competitor DNA amplification (HEX channel) were recorded from investigated samples (see Section 2.7 below).

2.6.2. Single-point calibrator mixture of target and competitor DNA

200 copies/ μ l of each peanut and competitor amplicon were mixed in TE buffer. 20 μ l aliquots of this mixture, called single-point calibrator, were stored at -20°C in identical ready-to-use and single-use 0.2 ml thin-walled PCR micro-tubes for future experiments. This mixture of equivalent amounts of peanut DNA and competitor-DNA was run in triplicate to adjust the peanut and competitor specific F_t of the real-time PCR device in a way that such F_t adjustment leads to identical peanut C_t (FAM) and competitor C_t (HEX) for this single-point fluorescence calibrator (Fig. 1b(ii)).

2.7. Threshold-based cPCR for peanut

2.7.1. Titration of copy number of competitor equivalent to 100 mg/kg peanut

The principle procedure was described elsewhere for classic competitive PCR (Studer et al., 1998) and was adapted for real-time PCR in this study (Supplementary material).

2.7.2. Semi-quantitative evaluation or quantification of peanut with cPCR

For each study sample the determined copy number of competitor DNA (Section 2.7.1) was added to the sample for DNA extraction (see Section 2.4 above). Competitor spiking and sample extraction was done in duplicate. Each sample extract was amplified in two reactions. FAM (peanut DNA probe) and HEX (competitor DNA probe) signals from the samples spiked with competitor DNA were recorded in each real-time PCR allowing the determination of the C_t -values for the FAM and HEX signal at F_t that was set by making use of simultaneously run non-competitive external peanut DNA and competitor DNA standard curves (Section 2.6.1) or the single-point calibrator mixture of target and competitor DNA (Section 2.6.2).

The semi-quantitative analysis permitted a distinction between the three cases (A) peanut (FAM) and competitor (HEX) specific signal arise at identical C_t : the sample contains 100 mg/kg peanut; (B) C_t (FAM) > C_t (HEX): the sample contains less than 100 mg/kg peanut; and (C) C_t (FAM) < C_t (HEX): the sample contains more than 100 mg/kg peanut. At the level of equivalence of both peanut and competitor specific signal, the detection is considered quantitative. Divergent signals allow semi-quantitative evaluation of the result.

Moreover, the amount of peanut in the sample can be quantified based on the following equation, provided that the efficiency of both the amplification of peanut and competitor DNA is equivalent to or approximately 100%:

$$\begin{aligned} \text{mg/kg peanut in sample} &= 2^{\Delta C_t} (\text{mg/kg of calibration}) \\ &= 2^{[C_t(\text{competitor}) - C_t(\text{peanut})]} (\text{mg/kg of calibration}); \text{ hence: mg/kg} \\ \text{peanut in sample} &= 100 \cdot 2^{[C_t(\text{HEX}) - C_t(\text{FAM})]}. \end{aligned}$$

3. Results and discussion

3.1. Rational, principle and development of a threshold-based competitive quantitative real-time PCR

Zachar and co-workers suggest the addition of a known amount of an internal standard DNA or a reporter gene which is co-amplified with the target DNA (Zachar et al., 1993) to normalise tube-to-tube differences in PCR efficiency. However, this does not consider effects of sample preparation on the PCR efficiency. To overcome this limitation, in this study an internal standard DNA was added prior to DNA (peanut) extraction from a food sample.

Instead of gel analysis of PCR products (Zachar et al., 1993), which is cumbersome and highly prone to quantitative errors, we aimed at developing a competitive real-time PCR with objective read-out for both target and competitor DNA. For this approach identical or near-identical amplification efficiencies for competitor and target DNA are a prerequisite. By applying the concept of competitive PCR, a threshold concentration-based determination of the target DNA can be accomplished because at this calibrated threshold level the amount of amplified internal competitive DNA standard is equivalent to the amount of amplified target (peanut) DNA. This allows a single-point calibration of the cPCR to a defined threshold concentration of target commodity by the addition of the number of competitor DNA molecules that will provide equivalent amounts of amplified competitor and target DNA. The required amount of competitor DNA is determined by titration in preceding experiments. After the competitor is titrated, the amount of target commodity in a sample can be quantified in comparison to the competitor without the need of additional quantitative standards. The general principle of the real-time cPCR described for peanut quantification is depicted in Fig. 1. Conserved primer binding sites allow competitive co-amplification of natural peanut DNA and artificial

competitor molecule. Due to different size and probe binding sites, peanut and competitor amplicons are discriminated post-PCR in agarose gel electrophoresis by differences in migration or in real-time PCR by sequence specific probes that carry distinguishable fluorescent reporter dyes (FAM, HEX) (Fig. 1a). The fluorescent threshold (F_t) was set for both peanut and competitor amplicon using either the approach of external standard curve superimposition or superimposition of fluorescence curves at equimolar amounts of both molecules (single point calibrator mixture) (Fig. 1b). Such a fluorescent setting was run simultaneously in all experiments, i.e. titration of the amount of competitor necessary for calibration of a peanut threshold concentration, such as 100 mg/kg, and the down-stream investigation of any unknown sample. After titration, the amount of competitor molecules necessary for equimolar co-amplification with peanut target DNA at the defined concentration threshold (100 mg/kg) is added to the sample, and peanut and competitor DNA are co-extracted under the comparable conditions. Subsequently, the co-extracted peanut and competitor DNA is co-amplified and C_t of both amplicons is recorded applying the above described setting of F_t (Fig. 1c). The evaluation of results can be semi-quantitative or quantitative. If both peanut and competitor DNA are amplified at equimolar level, the amount of peanut in a sample corresponds to the calibrated threshold concentration, i.e. 100 mg/kg peanut in this study. In gel electrophoresis, both amplicons appear at comparable sensitivity. In real-time cqPCR, both amplification fluorescence curves are superimposed (Fig. 1d, middle). In case of more amplification of competitor molecules in real-time cqPCR, C_t of peanut is larger than C_t of competitor. Thus, the amount of peanut in a sample is below the threshold of 100 mg/kg peanut (Fig. 1d, left). If more peanut DNA is amplified, C_t of peanut is smaller than C_t of competitor. Thus, the amount of peanut in a sample is above the threshold of 100 mg/kg peanut (Fig. 1d, right). Providing efficiency of approximately 100% exists for both the amplification of peanut and competitor DNA, the amount of peanut in a sample can be calculated by the equation given in the materials and methods section (Section 2.7.2).

3.1.1. Design and generation of competitor and probes for cqPCR

The real-time PCR described by Stephan and Vieths (2004) that specifically amplifies an 86 bp sequence of the peanut Ara h 2 gene (NCBI GenBank acc No. L77197) required optimisation (Supplementary material). The optimised peanut specific PCR had an LOD of 2 mg/kg and an LOQ around 10 mg/kg peanut in chocolate. Thus, when compared to the studied peanut ELISA kits, this PCR was considered sufficiently sensitive for the detection of low milligram per kilogram levels of peanut.

A competitor molecule was designed on the basis of the 86 bp peanut-specific amplicon with conserved primer binding sites but divergent probe binding sequences (Fig. 1a and Supplementary material). Fig. S-1 displays complete overlay of the standard curves originating from peanut DNA and competitor DNA with identical PCR efficiency of nearly 100%.

3.1.2. Calibration against a virtual threshold concentration of 100 mg/kg peanut in food

4100 copies of competitor DNA were determined as the amount equivalent to 100 mg peanut per kg milk chocolate. Details are given elsewhere (Supplementary material). For further quantitative analysis of an unknown sample, 4100 copies of competitor DNA were added to each 300 mg sample for the DNA extraction as described above (Section 2.4).

3.2. Threshold-based cqPCR using F_t -setting by external standard curves

3.2.1. Determination of the dynamic range of cqPCR

The self-prepared milk chocolate material containing 100,000, 10,000, 1000, 100, and 10 mg/kg incurred peanut was quantified at 96,237, 8944, 988, 75.9, and 6.86 mg/kg peanut, respectively, using our in-house ELISA, which was additionally calibrated against the incurred peanut cream material. Thus, the desired levels of incurred peanut were principally verified.

The incurred milk chocolate samples were quantified in real-time cqPCR after addition of the titrated 4100 copies of competitor DNA (Section 3.1.2) and subsequent extraction. Each of the chocolates was extracted in duplicate, and each extract was analysed and quantified in two separate PCR reactions. The recorded C_t -values of peanut (FAM signal) and competitor (HEX signal) amplification as well as the calculation of peanut concentration of the individual reactions are provided as Supplementary data (Table S-1). The quantification was based on the assumption of 100% efficiency of the PCR for peanut and competitor DNA, as was demonstrated above (Section 3.1.1). The results were calculated as described in 2.7.2. and plotted against the amount of incurred peanut in Fig. 2: Taking the in-house ELISA results as a reference, 988, 75.9, and 6.86 mg/kg peanut were recovered at 122% (21.9% CV), 114% (15.8% CV), and 162% (40.2% CV), respectively. The CV (coefficient of variation) of four reactions is given in parenthesis. The chocolates which contained 8944 and 96,237 mg/kg were clearly overestimated, demonstrating that the quantitative dynamic range of the method spans approximately two orders of magnitude between 10 and 1000 mg/kg with each one order of magnitude above and below the calibrated level of a virtual 100 mg/kg threshold concentration.

3.2.2. Quantification of peanut using cqPCR in comparison to commercial peanut ELISA

All investigations were performed in 2007 with the available versions of the commercial peanut specific ELISA kits. The ELISA quantifications were each based on duplicate protein extractions and each singlet determination of extracts. In cqPCR, each sample was extracted in duplicate, and each DNA extract was quantified

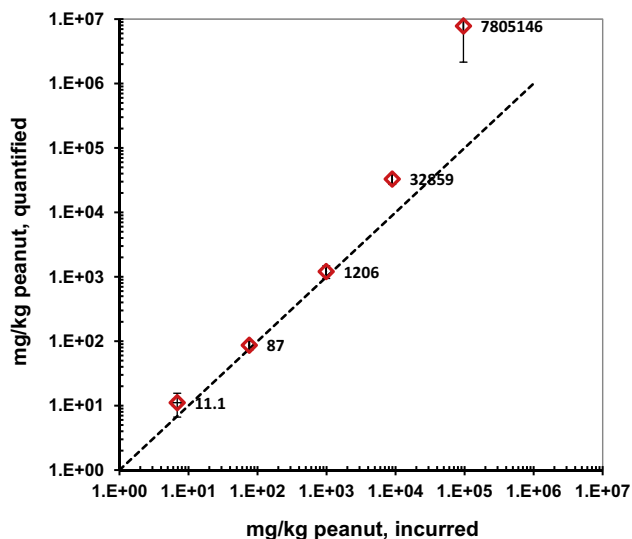


Fig. 2. Determination of the quantitative dynamic range of real-time cqPCR for peanut based on self-prepared incurred milk chocolate material and using external target and competitor DNA standards for adjustment of fluorescence signal thresholds. Error bars indicate standard deviation of displayed mean results that originate from four reactions. The dotted line indicates 100% recovery.

in duplicate PCR reactions. The results are summarised in Table 1. CqPCR was applied to three milk and dark chocolate samples from a previous German collaborative trial. Between 2 and 100 mg/kg incurred peanut was recovered between 80% and 101% by real-time cqPCR. However, it must be mentioned that 2 mg/kg peanut were detected and thus quantified in only 2/4 reactions, an expected result at the LOD (Section 3.1.1). In comparison, the three studied ELISA kits presented with between 5% and 68% clearly lower recovery rates than obtained by cqPCR. The milk chocolates having 2 and 100 mg/kg peanut, respectively, were well recovered by ELISA A (100–117%), and overestimated by ELISA B (170–210%). ELISA C recovered 100 mg/kg peanut well (92%) but was unable to detect 2 mg/kg peanut. Of five retail chocolate products with varying matrix (milk, dark, nougat and mousse chocolate) three did not have detectable peanut using cqPCR or the ELISA kits. Quantitative results of two products (milk and nougat chocolate) correlated well between cqPCR and ELISA A, which also gave the overall best mean recovery (84%) of the collaborative trial samples in comparison to cqPCR (90%). It was shown that cqPCR features good quantitative response also to unknown peanut that was not used for calibration. Moreover, cqPCR demonstrated applicability to food matrices other than the calibrated milk chocolate matrix, whereas some ELISA kits had either difficulty detecting peanut or quantifying it at an acceptable level of recovery.

3.3. Threshold-based cqPCR using F_t -setting by single-point calibrator mixture

The procedure of setting F_t was further simplified. Instead of two standard curves for peanut and competitor DNA, a single mixture of $1e^3$ copies of each peanut and competitor amplicon was run in triplicate. Thus, the number of reactions for F_t -settings was reduced from ten to three. Because of this change in protocol, the number of competitor molecules was again titrated against a newly prepared batch of chocolate containing 100 mg/kg incurred peanut (Fig. 3a).

On the basis of the single-point F_t -setting, peanut incurred at levels of 10, 100, and 1000 mg/kg was investigated in chocolate and four other food matrices, including vanilla ice cream, cookie dough, cookie made of cookie dough, and coconut muesli. In real-time cqPCR each sample was extracted twice and investigated in two reactions per single extract. Thus, quantitative cqPCR results were obtained from an average of four reactions, allowing also to calculate standard deviation as a means for imprecision. For comparison, all samples were also extensively quantified using in-house ELISA on the basis of three extracts that were each analysed in triplicate. Thus, each in-house ELISA result was obtained from an average of nine measurements.

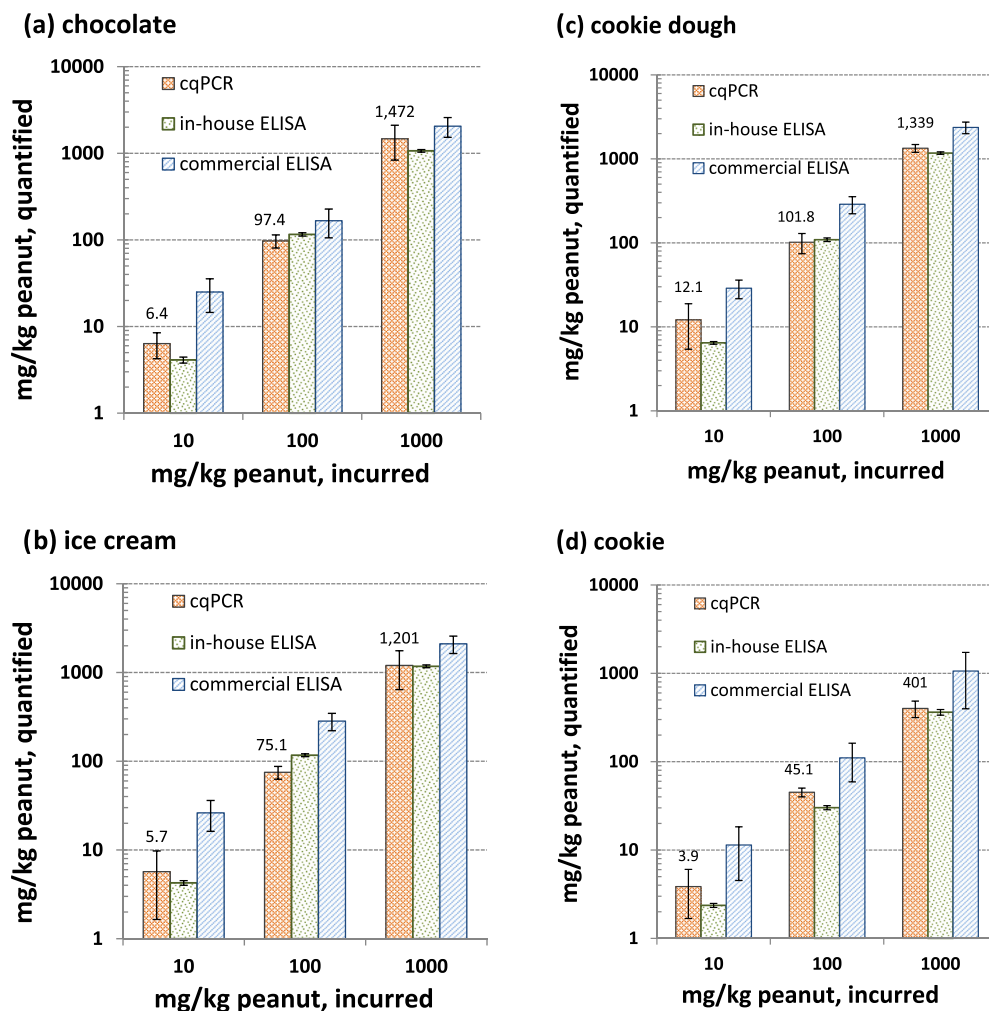


Fig. 3. Quantification of peanut between 10 and 1000 mg/kg using threshold concentration-based real-time cqPCR in comparison to in-house ELISA and commercial peanut ELISA kits in various matrices: (a) chocolate, (b) ice cream, (c) cookie dough, (d) cookie, and (e) muesli. (f) Summarises the recovery over all studied matrices. The dotted lines indicate 100% recovery of incurred material.

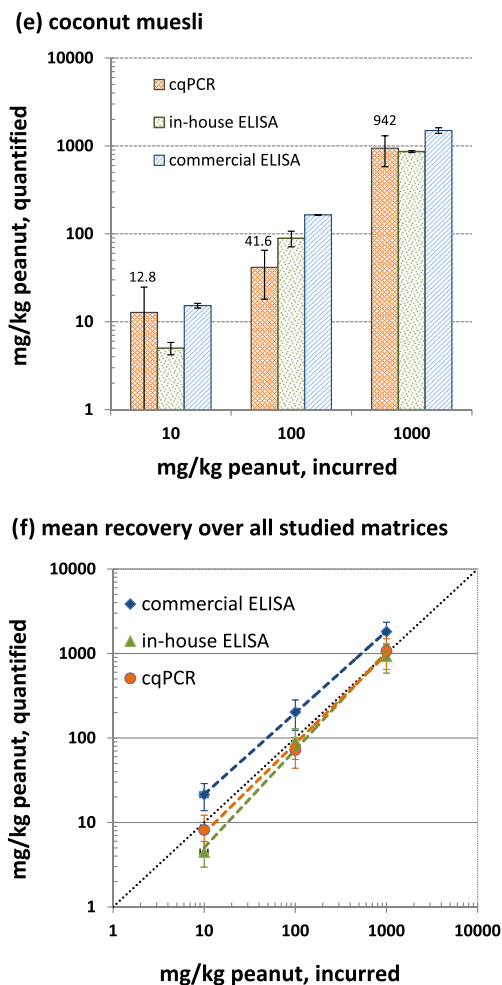


Fig. 3 (continued)

Finally, all samples were investigated with current commercially available peanut ELISA kits from recognised vendors. The rationale to include the commercial ELISA kits was to have comparable quantitative results from tests, certified as ‘AOAC Research Institute Performance Tested Method’, which are currently considered state-of-the-art methods in allergen detection. Because all samples were investigated in all three kits, single extraction and single well measurements were considered acceptable. Results were expressed as the mean of these three measurements per sample. The rationale of an ELISA average using three common kits was to obtain quantitative results by ELISA for comparison with PCR based on tendencies rather than identifying the best performing of the studied test kits. Thus, the study in principal focused on the performance characteristics of real-time cqPCR in comparison to ELISA ‘in general’. Moreover, instead of relying on the results of one single selected kit, an average of the results obtained by three common ELISA kits appeared to be more realistic, taking into account the experimental variations observed for commercial ELISA kits in this study (Table 1), and considering the reported variations of quantitative results between commercial ELISA kits for peanut allergens (Poms et al., 2005). All investigations were performed in 2009 and 2010 with the ELISA kits purchased in this period. Results of this multi-matrix and multi-method analysis are displayed in Fig. 3.

3.3.1. Comparability of single-point versus standard curve-based setting of F_t

PCR results of chocolate at the level of 10 and 1000 mg/kg were derived from four replicates each but, to serve additional purposes,

in a divergent way at the 100 mg/kg level. Of the chocolate containing 100 mg/kg chocolate, four extracts were analysed, three in duplicates and one in eight replicates. First, the CV of octa-replicate analysis of 100 mg/kg peanut from one extract was assessed with 17.3%. Second, four extracts from 100 mg/kg peanut were quantified at 97.4 mg/kg and 17.2% CV. Thus, with 97 mg/kg quantified peanut, the accuracy of the calibration procedure was verified. Moreover, a precision above 80% of replicate PCR analysis of one extract and between extracts was considered appropriate for an analysis at the level of calibrated threshold concentration.

In summary, peanut in chocolate was quantified using real-time cqPCR and single-point fluorescence setting at 6.4 mg/kg (33.2% CV), 97.4 mg/kg (17.2% CV), and 1472 mg/kg (43.3% CV) in the samples with incurred levels of 10, 100, and 1000 mg/kg (Fig. 3a). The coefficient of variation is given in parenthesis. Taking the in-house ELISA results as a reference for the purpose of comparability, 4.1, 116, and 1067 mg/kg peanut as quantified by in-house ELISA were recovered by real-time cqPCR at 156%, 84%, and 138%, respectively. On average, between 10 and 1000 mg incurred peanut per kg chocolate were recovered by 126% using the approach of single-point F_t -setting in comparison to 133% using standard curves for setting F_t (Section 3.2.1).

Independently, the quantitative cqPCR results obtained with the simplified procedure of setting F_t by simultaneous analysis of a single-point calibrator mixture were very much comparable to the results obtained by a standard curve-based setting of F_t .

3.3.2. Comparable quantification in non-calibrated matrices

Analysis of all five food matrices with in-house ELISA and commercial ELISA kits verified that incurred peanut was present at the three levels of magnitude, i.e. 10, 100, and 1000 mg/kg (Fig. 3a–e). In comparison to the detection of peanut in chocolate, the in-house ELISA obviously had a reduced recovery rate in baked cookie (Fig. 3d). Moreover, the in-house ELISA appeared to under quantify 10 mg/kg peanut in all matrices whereas the quantification by commercial ELISA kits appeared to overestimate all incurred levels of peanut in all matrices but baked cookie. Due to divergent ELISA results in general, the quantitative data obtained by ELISA were not taken as a reference. Hence, the theoretical levels of 10, 100, and 1000 mg/kg peanut were set as the 100% reference for the purpose of data comparison. The mean recovery of 10–1000 mg/kg over all five matrices was 87% (21% CV) for the real-time cqPCR, 77% (35% CV) for the in-house ELISA, and 199% (8% CV) for the commercial ELISA kits (Fig. 3f). In real-time cqPCR analysis all replicates were positive except for 10 mg/kg peanut in coconut muesli, where 3/4 replicates were positive. PCR results as displayed in Fig. 3 were derived from each two reactions of each two extracts for all matrices and investigated peanut concentration except for 100 mg/kg peanut in chocolate as described above (Section 3.3.1).

Notably, 10 to 1000 mg/kg peanut in baked cookie had a lower mean recovery of 41% (8% CV) in real-time cqPCR, but the three different levels of peanut concentration were appropriately quantified in a tenfold serial graduation (Fig. 3d). By contrast, the commercial ELISA kits over quantified peanut by approximately twofold in all matrices but cookie (Fig. 3d). In cookie the average recovery was 110% with some 40–60% variation between the kits. Taken together the results of ELISA and real-time cqPCR, both protein and DNA detection showed at comparable extent a reduced detectability in a strongly heated matrix such as cookie. This effect is fully clear by direct comparison of the peanut quantification in cookie dough (Fig. 3c) and cookie baked therefrom (Fig. 3d). Both in ELISA and real-time cqPCR, the level of recovery is reduced by approximately 60% between cookie dough and cookie. Consequently, if applied to strongly heated matrices the method calibration requires adequate adjustment against the effect of heat treatment. The presented real-time cqPCR was found suitable for

peanut quantification especially at the level of the calibrated threshold concentration of 100 mg/kg peanut. Also, it allowed a distinction between higher and lower levels of peanut in the range between 10 and 1000 mg/kg. At the level of 100 and 1000 mg/kg peanut in all investigated matrices excluding coconut muesli, the mean coefficient of variation of replicates was acceptable with 18% and 30%, respectively. At the 10 mg/kg level the average coefficient of variation increased to 54%, owing to the fact that only low copy numbers of amplifiable peanut DNA were present: C_t for peanut at this level ranged between 36 and 39. This indicates approximately ten or less copies of peanut DNA when comparing C_t -values to the standard curve displayed in Fig. S-1.

The error bars of coconut muesli analysis in real-time cqPCR apparently display the largest variation between replicates (Fig. 3e): In coconut muesli the mean CV of four replicates was the highest of all investigated matrices with an average of 63% over all three levels of investigated peanut concentration. An explanation for this is the presence of lower levels of amplifiable peanut DNA in comparison to all other studied matrices. On average, C_t -values for peanut (FAM signal) and competitor (HEX signal) DNA in coconut muesli matrix were increased by 3.3 cycles over C_t -values obtained in the other four matrices: Thus, C_t -values for 100 and 10 mg/kg peanut in muesli ranged between 36 and 39, which again translates into approximately ten or less copies of peanut DNA. Based on a slope of -3.4 for peanut amplification (Fig. S-1), an average increase of 3.3 cycles indicates that in coconut muesli only one tenth of amplifiable DNA in comparison to the other four matrices was available after extraction. Nonetheless, the approach of matrix normalisation with internal competitive standard DNA resulted in an average recovery of 88% (50% CV) of 10–1000 mg/kg peanut in coconut muesli which is very much comparable to the average recovery of 87% (21% CV) for all five matrices including coconut muesli. By contrast, common real-time PCR quantification with only external DNA standards would have resulted in a 90% reduced recovery in the studied muesli matrix due to 90% less amplifiable peanut DNA. This underlines the necessity to normalise both the DNA extraction and down-stream PCR analysis by the addition of an internal standard. Despite the fact that quantitative calibration of real-time cqPCR was only done for 100 mg/kg peanut in milk chocolate, and considering the fact that fluorescence settings were done with one simple equimolar mixture of peanut and competitor DNA, peanut was successfully quantified in five different matrices, i.e. milk chocolate, ice cream, cookie dough, cookie, and at three different levels, each by making use of a single internal competitive standard.

As a proof of concept, this study about the verification of a virtual peanut threshold concentration demonstrated the potential use of real-time cqPCR for any kind of DNA-based species quantification in complex matrices. Thus, potential regulatory thresholds or action levels may be verified using cqPCR. Likewise, the read-out can be adjusted as quantity of species or target copy numbers. If the calibration of an even lower level of concentration is required, real-time cqPCR can be adjusted accordingly by calibrating against a lower threshold concentration.

4. Concluding remarks

A novel DNA-based method for species quantification based on competitive real-time PCR with sequence specific probes was presented. As example, the detection and quantification of potentially allergenic peanut in various food matrices was demonstrated. In this study, the method allowed calibration against a distinct threshold concentration such 100 mg/kg peanut in food. Moreover, extended quantification between 10 and 1000 mg/kg was achieved. The accuracy of this real-time cqPCR was comparable

to that of in-house and commercial ELISA tests in this study. If higher sensitivity is required, an up to 100-fold more sensitive PCR can be developed, e.g. on the basis of high copy genes as demonstrated previously (Hirao, Watanabe, Temmei, & Hiramoto, 2009).

For quantitative analysis, a very limited number of analytical replicates ($n = 4$) was sufficient in comparison to other published methods in the field (Eugster, 2010; Hirao et al., 2006; Demmel et al., 2012; Siegel et al., 2012). Contrary to the published methods, no adjustment to any other matrix than the calibrated chocolate was necessary. This was achieved by calibration with an internal competitive standard. Thus, transferability of the calibration to other food matrices without further adjustment was demonstrated. Important characteristics of this PCR method include (i) the avoidance of external calibration standard curves, (ii) an internal calibration that normalises sample matrix effects and (iii) also serves as an inhibition control, and (iv) a threshold-based quantification that is efficient when verifying compliance with given analytical or regulatory threshold or action levels. The principle method can be adjusted for any type of species or DNA thus opening broad application in the fields of food and feed-related, as well as environmental, and clinical samples.

Conflict of interest disclosure

Thomas Holzhauser had consultant arrangements with Institut für Produktqualität and Monsanto Company; he is inventor of a method for quantitative detection of an organic substance in a sample (PCT/EP2009/061584).

Acknowledgements

Annabella Janise and Martin Röder were partly funded by the German Ministry of Economics and Technology (via AiF) and the FEI (Forschungskreis der Ernährungsindustrie e.V., Bonn) Project AiF 14362N.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2014.04.081>.

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1 **Supplementary material to:**

2 Matrix-normalised quantification of species by threshold-calibrated competitive real-time

3 PCR: Allergenic peanut in food as one example

4

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2. Material and methods

2.3 Generation of DNA competitor

Each 50 pmol of the synthesis oligonucleotides #1 and #2 with underlined overlapping revers complementary sequences were mixed in 20 µL of 1 × reaction buffer (20 mmol/l Tris HCl (pH 8.4), 50 mmol/l KCl; supplied with Platinum[®] Taq, Invitrogen, Karlsruhe, Germany), heated at 96 °C for 30 sec, and cooled to ambient temperature for hybridisation (sequence of oligo #1: 5'-GCAGCAGTGGGAACTCCAAGGAGACAGAAGATGCCAGAGCCAGCTGCAAGGGC GGCCGGAGTCCAAGCGGGAGAGC-3'; sequence of oligo #2: 5'-GCATGAGATGTTGCTCGCAGGCGCCTAGTGTGCTCTCCCGCTTGGACTCCGG-3'). 2 µl of the hybridisation mixture were added to a total volume of 20 µl of 1 × Klenow buffer (10 mmol/l Tris-HCL, 5 mmol/l MgCl₂, 3.75 mmol/l DDT, pH 7.5) which contains 4 units of Klenow Fragment (Roche Applied Science, # 11008404001, Mannheim, Germany) and 0.1 mmol/l of each dATP, dTTP, dGTP, and dCTP. After incubation at 37 °C for 30 min, the reaction mixture was purified with a Centri Spin 20 column (Princeton separation, # CS-200, via EMP Biotech GmbH, Berlin) according to the manufacturer's instructions. 5 µl of purified competitor nucleic acid were added to a total volume of 50 µl PCR reaction mix which contains 1 × PCR buffer, 800 µmol/l dNTP mix (each 200 µmol/l dATP, dTTP, dGTP, and dCTP), 2 mmol/l MgCl₂, and 1.25 units of Platinum[®] Taq (Invitrogen,). PCR was performed with 40 cycles of 30 sec at 95 °C, 30 sec at 60 °C and 30 sec at 72 °C. The 107 bp competitor PCR product was purified with a Centri Spin 20 column. The quality of the purified competitor DNA was investigated using agarose gel electrophoresis with subsequent ethidium bromide fluorescence staining: 5 µl of PCR product and 1 µl of 6 × gel loading concentrate (15% Ficoll[™], 0.25% bromophenol blue, 0.25% xylene cyanol in distilled water) were mixed, and PCR products separated on 3% agarose (Carl Roth, no. K 297.2, Karlsruhe, Germany) gels using rapid agarose gel electrophoresis (RAGE) (Cascade Biologics, via TEBU,

Frankfurt/M, Germany) according to the manufacturer's instructions. Gels soaked in 0.5 mg/l of ethidium bromide for 20 min were visualised using an UV-transilluminator (TFM20, UVP, via Merck). The size of the PCR-products was controlled in comparison to a 100 bp size marker (GenSura, SLL 101, via Carl Roth) and the results were documented using an instant camera (Polaroid GelCam, film type 667, 0.25 sec, 5.6f).

For determination of the concentration of genomic DNA, 5 µl of DNA was measured at 260 nm wavelength using a micro cuvette in a spectrophotometer. One OD of dsDNA at 260 nm was equivalent to 50 µg/ml of genomic DNA. From the amount of competitor DNA determined, the copy number was calculated considering the nucleic acid composition and length of the competitor dsDNA.

2.7.1 Titration of copy number of competitor equivalent to 100 mg/kg peanut

In this study, milk chocolate containing 100 mg/kg incurred peanut was used for the calibration of the competitive real-time PCR. The competitor DNA was serially diluted to allow addition to the incurred chocolate prior to DNA extraction. Identical replicate portions of each 300 mg chocolate that contained 100 mg/kg incurred peanut were fortified with increasing copy numbers of competitor DNA. After DNA extraction, the FAM (peanut DNA probe) and HEX (competitor DNA probe) signals were recorded in real-time PCR. The copy numbers of peanut DNA and competitor DNA in the chocolate samples that contained spiked competitor were quantified from C_t -values against the external standard curves of peanut DNA and competitor DNA, respectively, with fluorescence baselines adjusted according to the procedure described in section 2.6.1. The natural logarithm (ln) of the copy ratio of peanut DNA versus competitor DNA (y-axis) was plotted against the ln of competitor DNA copies (x-axis). After linear regression, the spiked copy number of competitor DNA that is equivalent to the copy number of peanut DNA in the sample was calculated from the equation of linear regression at the intercept with the x-axis ($y=0$).

3. Results and discussion

3.1.1. Design and generation of competitor and probes for cqPCR

One primer mismatch was corrected and the PCR performance was improved to obtain a PCR efficiency of almost 100%. This was achieved by additionally changing the chemistry of the PCR reaction mixture including the hot start polymerase and the parameters of thermal cycling. The optimised protocol is described in the 'Materials and methods' section. The optimised peanut specific PCR was further evaluated in an inter-laboratory collaborative trial and now serves as German official method for qualitative peanut detection with PCR according to Article 64 of the German food and feed law (BVL, 2013). With 62.5% true positives in 80 individual reactions, an LOD of 2 mg incurred peanut per kg chocolate was determined in the collaborative trial. 10 mg/kg peanut in chocolate were detected at a rate of 97.5% (78/80) true positives. Comparable to the studied peanut ELISA kits, the peanut-specific PCR thus provides appropriate sensitivity for the detection of low milligram per kilogram levels.

The competitor molecule with conserved primer binding sites but divergent probe binding sequences was generated by inserting an artificial 21 bp sequence between nucleotide position 54 and 55 of the peanut amplicon which corresponds to the middle of the hybridisation stretch of the peanut specific probe. A HEX-labelled fluorescent probe based on Taqman[®] chemistry was designed to specifically bind to the competitor but the target amplicon sequence. Vice versa, the binding site of the FAM-labelled Taqman[®] probe which is relevant for binding to the target DNA sequence was discontinued within the competitor sequence by the 21 bp insert. The quality of the purified competitor amplicon was evaluated by agarose gel electrophoresis and fluorescence staining with ethidium bromide: By visual inspection, the competitor amplicon comprised only one single DNA band of appropriate length (data not shown). Further, in a cross-wise amplification of peanut and competitor

DNA, no cross-binding of competitor probe (HEX signal) to target DNA and vice versa (FAM signal) was observed (data not shown). For the evaluation of PCR efficiency, serial dilutions of peanut DNA and competitor DNA were amplified in a non-competitive set-up, and the standard curves were generated by plotting the recorded C_t of each standard versus the logarithm of the copy number of amplifiable DNA. At 100% efficiency the slope would be - 3.322.

3.1.2. Calibration against a virtual threshold concentration of 100 mg/kg peanut in food

Using the approach of external target and competitor DNA standards for adjustment of F_t , a first rough titration with competitor DNA between 150 and 150,000 copies was carried out to identify the order of magnitude of spiked competitor copies which are necessary for equal amplification in the presence of DNA originating from 100 mg/kg peanut in milk chocolate (2.7.1.). The resolution of the equation of the linear regression at the intercept with the x-axis ($y=0$) resulted in 2940 copies of competitor DNA. Knowing the order of magnitude, a more narrow range of competitor DNA between 2000 and 4000 copies was chosen. The titration plot is given in Figure S-2. The resolution of the equation of the linear regression resulted in 3112 copies of competitor. Because the amount of peanut in the chocolate sample was quantified by in-house ELISA at 75.9 mg/kg, a total of 4100 copies were proportionally calculated to compete equally with exactly 100 mg/kg of peanut.

112 **Table S-1** Determination of the peanut concentration (mg/kg) in self-made incurred milk chocolate material using external target and competitor
 113 DNA standards for adjustment of fluorescence signal thresholds. (C_t cycle threshold, $\Delta C_t = C_t$ (HEX) – C_t (FAM), CV coefficient of variation).
 114

mg/kg peanut, incurred	extraction no.	PCR no.	C_t (HEX)	C_t (FAM)	ΔC_t	mg/kg peanut, calculated	mg/kg peanut, mean	% CV
10	1	1	31.44	34.19	-2.75	14.9	11.1	40.2
10	1	2	31.62	34.37	-2.75	14.9		
10	2	1	31.38	35.41	-4.03	6.1		
10	2	2	31.08	34.63	-3.55	8.5		
100	1	1	31.81	31.84	-0.03	97.9	86.6	15.8
100	1	2	31.32	31.59	-0.27	82.9		
100	2	1	30.97	31.51	-0.54	68.8		
100	2	2	31.20	31.25	-0.05	96.6		
1000	1	1	32.01	28.03	3.98	1578	1206	21.9
1000	1	2	31.71	28.14	3.57	1188		
1000	2	1	31.62	28.34	3.28	971		
1000	2	2	31.59	28.15	3.44	1085		
10000	1	1	32.72	24.51	8.21	29611	32859	17.3
10000	1	2	33.37	24.69	8.68	41019		
10000	2	1	32.37	24.22	8.15	28405		
10000	2	2	32.61	24.27	8.34	32403		
100000	1	1	36.82	22.16	14.66	2588807	7805146	72.0
100000	1	2	37.76	22.08	15.68	5249892		
100000	2	1	39.01	22.78	16.23	7686291		
100000	2	2	39.58	22.32	17.26	15695593		

115

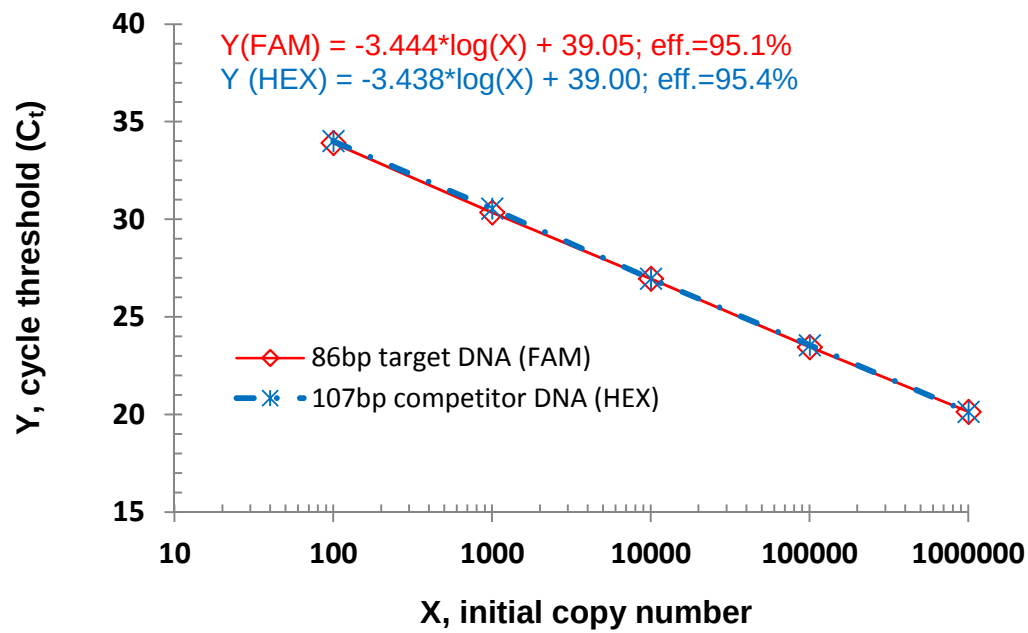
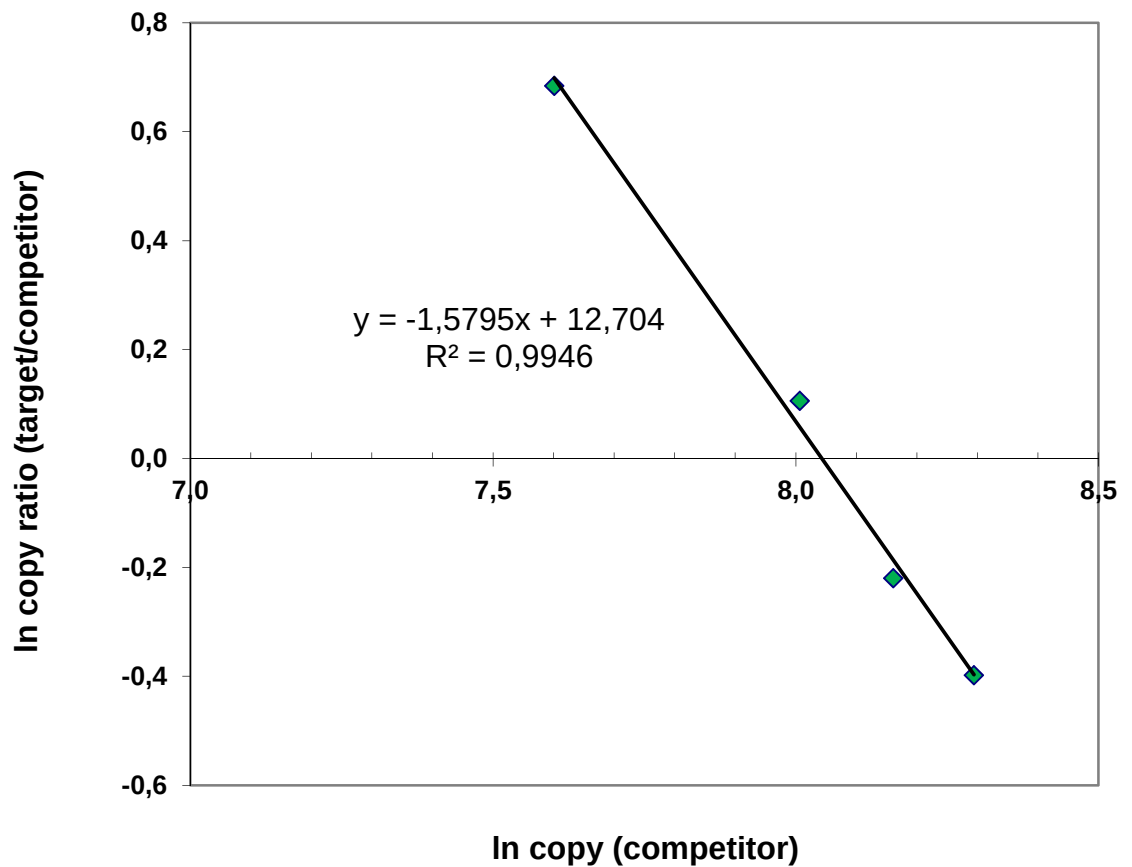


Fig. S-1. Standard curves of both competitor (HEX signal) and peanut (FAM signal) DNA with comparable PCR efficiency of nearly 100%.



120

121 **Fig. S-2.** Titration of copy number of spiked competitor DNA equivalent to 100 mg/kg peanut
 122 incurred in milk chocolate and using external target and competitor DNA standards for
 123 adjustment of fluorescence signal thresholds.