

The effect of heat treatment on the detection of peanut allergens as determined by ELISA and real-time PCR

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Abstract Peanut allergic reactions can result from the ingestion of even very small quantities of peanut and represent a severe threat to the health of sensitised individuals. The detection of peanut traces in food products is therefore of prime importance. Peanut traces which can be (unintentionally) present in food products have usually undergone one or more processing steps like roasting and baking. Therefore, methods designed to detect such traces have to be capable of detecting heat-treated peanuts. Commonly used methodologies designed to detect peanut traces in food products are enzyme-linked immunosorbent assays (ELISAs) that detect peanut-specific proteins, and polymerase-chain-reaction (PCR)-based methods targeting peanut-specific DNA. A comparative analysis of such methods was performed and the impact of heat treatment on peanut kernels as well as the impact on a peanut-containing food matrix are investigated. Our results show that heat treatments have a detrimental effect on the

detection of peanut with either type of method and that both types of methods are affected in a similar manner.

Keywords Real-time PCR · ELISA · Food allergy · Peanut · Heat treatment

Introduction

Food allergy is a serious public health problem. Around 1–2% of the population suffer from some type of food allergy and even higher prevalence levels (up to 8%) are estimated for children [1–4]. The severity of the reactions triggered by food allergens vary from mild urticaria to potentially lethal anaphylactic shocks, even after the ingestion of minimal doses of the allergenic food. It is estimated that in the United States about 30,000 anaphylactic reactions to food are treated in emergency departments and that food allergy results in 150–200 deaths each year [5]. Peanuts and tree nuts are responsible for the majority of deaths attributed to food allergies [6, 7].

Unfortunately, avoidance is the only means allergic consumers have to protect their health. Therefore, EU legislation has been issued aimed at informing the consumer about the content of food products by means of a mandatory labelling of allergenic food ingredients. The new EU Directive 2007/68/EC, which is an amendment to Directive 2000/13/EC [8, 9], provides a list of 14 groups of allergenic foods that manufacturers are required to declare on the labels if any of them are used as ingredients in pre-packaged foods, regardless of their quantity. Reliable analytical methods for the detection of hidden allergens in foodstuff are therefore required by the food industry and food control authorities to guarantee food safety for allergic consumers.

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Food allergens, the components capable of triggering allergic reactions, are usually proteins. The structure and chemical properties of proteins are known to be influenced by food-processing techniques such as boiling, baking, roasting, curing, and other types of food processing.

It has been reported that the allergenic activity of the peanut allergens Ara h 1, Ara h 2, and Ara h 3 increases with roasting while it decreases with frying or boiling [10, 11]. This decreased allergenicity was suggested to be a contributing factor in the lower prevalence of peanut allergy in China where peanuts are mainly boiled or fried compared with the United States where they are consumed roasted [11].

Thermal processing of foods is known to result in a degradation of food constituents, or in their modification. Such modifications can result from Maillard reactions, which are important in the development of flavour and colour in peanuts [10, 12]. In addition, interactions with other constituents of the food matrix may occur as a result of thermal processing.

All these modifications often result in a decreased protein solubility which affects protein extraction from food matrices. In peanut, it has been shown that protein solubility decreases due to industrially applied processing techniques like roasting [13–15]. Furthermore, the integrity of the target proteins could be changed during food processing leading to a further reduction in their detectability, since any analytical method based on the detection of markers for allergenic ingredients depends on an efficient extraction of such markers and their structural integrity.

Currently, enzyme-linked immunosorbent assay (ELISA) is probably the method that is most commonly used by the food industry and official food control agencies to assess the presence of allergens in food products. ELISAs are immunochemical based tests, which can be either designed to specifically recognise one allergenic protein, or to detect a pool of proteins contained in the protein extract of the allergenic food [16]. They usually employ IgG antibodies raised in animals, which bind allergen specific epitopes. But, in case of modifications of the protein structure or matrix effects this antibody binding could be affected.

An alternative to ELISA is provided by DNA-based methods. Several of such methods have recently been developed for the detection of peanut traces in food products [17–22]. The target molecules of these detection methods are not proteins but specific DNA sequences which are amplified by the polymerase chain reaction (PCR). This technique is commonly used in the detection of genetically modified organisms (GMO) and, when applied to allergen detection, it represents an indirect investigation of the allergenic ingredients. DNA target sequences can be selected within a gene coding for an allergenic protein or, within a species-specific region in the genome of the allergenic food. For peanut allergen detection, several real-

time PCR assays have been developed [17, 18, 22] that, like ELISA, have the potential to be applied for quantitative purposes. Although DNA-based methods target a different analyte compared to immunological methods like ELISA, food processing also affects the integrity and extractability of this target.

Consumers are almost solely exposed to heat-treated peanuts, since peanuts destined for consumption are usually roasted. Moreover, peanuts are widely used in different preparations like snacks and biscuits, and in the confectionary industry. This implies that they undergo additional (thermal) food processes. It is therefore of utmost importance to investigate the negative effects that food processes like thermal treatments exert on the detection of peanut-specific markers being either proteins or DNA.

Previously, several studies have reported on a qualitative comparison of ELISA kits and DNA methods for the analysis of processed food products [18, 21, 23], but such studies have not addressed the influence of food processing on either type of detection methodology. In this study, three real-time PCR assays all targeting DNA sequences coding for Ara h 3 [22] are used in parallel with two different ELISA kits for the analysis of raw and roasted peanut as well as on a heat-treated model food-matrix-containing peanut. The main focus of this study lays on the effect exerted by heat treatments on peanut kernels and on cookies containing peanut have on the detectability of peanut protein and DNA targets. The sensitivity of the different detection methods towards processing of allergenic foods, to which sensitised consumers are exposed to, was investigated. This allows an assessment of the possibility to confirm analytical results for the detection of peanut traces in processed food products obtained by ELISA with real-time PCR assays or vice versa.

Experimental

Food samples

Five varieties of peanut, identical to the five peanut components included in the IRMM-481 peanut test material (IRMM, Belgium), were analysed. Each component corresponds to a single peanut variety that underwent a specific-heat treatment, being blanching or roasting as listed in Table 1. The peanuts were obtained from IMKO (The Netherlands) and Duyvis (The Netherlands). In addition to the processed peanuts, raw peanuts were obtained from the same suppliers, except for peanut component **d**, for which only blanched peanut was available (blanching conditions: 20 minutes at 90 °C). The raw peanut component **c** was provided by Golden Peanuts (USA). In addition to the single varieties the mixture of

Table 1 List of the five peanut materials as included in the IRMM-481 test material

Variety	Origin	Heat treatment
(a) Runner	Argentina	Dry hot air roasting, 140 °C, 20 min
(b) Common Natal/Spanish	South Africa	Dry hot air roasting, 140 °C, 13 min
(c) Virginia	USA	Oil roasting, 145 °C, 25 min
(d) Virginia	China	Oil roasting, 140 °C, 9 min
(e) Jumbo Runner	USA	Blanching, 100 °C, 50 min

the 5 processed materials (component **f** of IRMM-481) was analysed.

A fine peanut powder of the mixture of the five peanut materials (component **f** of IRMM-481) had been used to spike cookies as described previously [22]. The peanut content in the cookies was approximately 10,000, 1,000, 100 and 10 mg kg⁻¹. All cookies were baked at 180 °C with a standard baking time of 16 min, or for different periods of time being 11, 21 or 26 min. All spiked cookies were ground, sieved on 0.7-mm sieves and aliquots of 15 or 30 g of the resulting powder were packed under vacuum in aluminium foil bags and stored at -20 °C until analysis.

DNA extraction and quantification

DNA extraction was performed combining a guanidine hydrochloride-based extraction and the Wizard® DNA Clean up system (Promega, Madison, USA) as described previously [22]. Samples of 2 g (±0.1 g) pure peanut and 5 g (±0.1 g) cookies were used for DNA extraction. Each sample was extracted in duplicate and the purified DNA from peanut and cookies was eluted in 100 and 130 µl TE buffer (10 mM Tris-HCl, 1 mM disodium EDTA, pH 8.0), respectively.

DNA quantification was carried out as described previously with Quant-iT™ PicoGreen® dsDNA reagent (Invitrogen Ltd, UK) [22].

Real-time PCR

PCR A, PCR B and PCR C assays all targeting Ara h 3 [22] have been applied.

For sample analysis, two separate DNA extractions were performed while two aliquots of 5-µl DNA extract were amplified in two separate reactions. In the case of the analysis of pure peanut extracts, DNA was diluted 1:10 and 5 µl of solution was added to each reaction. The dilution was taken into account for the expression of results.

For all analyses, the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, UK) was used with an automatic baseline setting and a threshold value of 0.15.

Each 96-well plate included three non-template controls (NTCs) and two positive control samples. For each sample, the final cycle threshold (Ct) values used for the calculation

of quantitative results refer to the mean value of four replicates (two replicates of extraction and two replicates of amplification). For quantification purposes, a cut-off Ct value of 40 was set, but higher Ct values up till 45 were considered acceptable for qualitative results.

ELISA methods

Two commercially available test kits targeting a specific peanut allergen were used: Biokits Peanut Assay kit (Tepnel BioSystems, UK), targeting Ara h 1; Peanut Residue ELISA kit (ELISA Systems, Australia), targeting Ara h 2. The ELISA test kits were used according to the manufacturer's instructions, by following the enclosed protocols. For sample analysis, two separate protein extractions were performed and analysed in two separate reactions.

Homogeneity test

The homogeneity of the cookie material was tested by employing the BioSystems peanut allergen test kit as well as the three PCR methods. Cookies of the two lowest spiking levels, containing either 10 or 100 mg peanut/kg cookie, that were baked for 16 min (standard baking time) were analysed for this purpose. Two samples from ten different bags of each concentration level were randomly taken and analysed in triplicate. The averages of the optical density (OD) or Ct values were subjected to analysis of variance (ANOVA) in order to assess the homogeneity of the cookie material.

Calibration model

The guidelines of Miller and Miller [24] for the identification of the most suitable calibration model were followed. The model equations obtained using spreadsheet software were confirmed by Statgraphics Plus (version 5.1; StatPoint, Inc, Virginia).

Calibration model with DNA dilutions (real-time PCR A, B, C)

DNA extracted from IRMM-481 component **f** (a mixture of five peanut materials) was used to prepare subsequent DNA

dilutions in water to establish a standard curve from 1 pg to 100 ng DNA (1 pg, 10 pg, 100 pg, 1 ng, 10 ng, 100 ng) for each PCR method. Three separate reactions were performed for each concentration level on two different days, using different stocks of PCR reagents. The equation of the calibration curve was generated by applying a weighted linear regression and plotting the Ct values against the logarithm of the corresponding DNA quantity. The weighting factors were calculated according to the following equation [24]:

$$w_i = s_i^{-2} / \sum_i s_i^{-2} / n$$

where

- w_i individual weights of each calibration point (DNA level)
- s_i standard deviation of the replicates per each calibration point (DNA level)
- n number of calibration points (DNA levels)

The curves obtained for each of the three PCR assays were used to interpolate unknown samples and to determine the amount of peanut DNA in the respective PCR reactions.

For the analysis of unknown samples, two DNA extracts were obtained from each material and two aliquots of both DNA extracts were taken for the analysis with each of the PCR assays. The average of those four values was interpolated in the respective PCR standard curve to define the peanut DNA content of the unknown samples.

Calibration model with cookies containing peanut (real-time PCR C)

A weighted linear regression was also applied to the Ct values obtained by amplifying DNA extracted from peanut-containing cookies that were baked for 16 min (standard baking time), using PCR C. For each of the four calibration points (peanut spiking levels of 10, 100, 1,000, 10,000 mg kg⁻¹), four PCR reactions were performed (two replicates per each of the two DNA extracts). The PCR reaction was then repeated twice on different days using different stocks of PCR reagents. The final set of data was composed of twelve PCR reactions for each calibration point. The curve was generated by plotting the Ct values against the logarithm of the corresponding peanut content. The weighting factors were calculated according to Miller and Miller [24] and the calibration equation was confirmed by Statgraphics Plus.

For the lowest and highest calibration points (10 and 10,000 mg kg⁻¹), the confidence intervals were calculated. Each 96-well plate analysed included a control of the calibration curve by the inclusion of two independent PCR reactions per DNA extract of the 10 and 10,000 mg kg⁻¹

calibration points. When the averages of the Ct values of both calibration points were within the confidence intervals, the analysis results of the plate were considered reliable. The calibration curves were updated every time the DNA of the calibration points ran out or when new extraction buffer stocks were used.

When the calibration curve was used to interpolated the Ct values obtained by the analysis of peanut-containing cookies baked for different periods of time, the variability of the method in predicting a concentration value from a certain measured Ct value (mean of four replicates) was calculated as the “*prediction limits*” with 95% confidence level, using Statgraphics Plus.

Calibration model with ELISA kits

The manual of the Biokits Peanut Assay kit suggests using a linear regression for quantitative purposes but, since the R^2 values obtained were always around 0.91, polynomial regression (third order) was applied in order to obtain a better fit ($R^2=0.99$). The peanut content (mg kg⁻¹) of the six calibration points (0, 1, 2, 5, 10, 20 mg kg⁻¹) was plotted against the mean (three replicates) of their respective ODs.

The manual of the Peanut Residue ELISA kit does not specify the use of any regression for quantification but only suggests comparing the ODs obtained from the unknown samples with the standards provided. In order to predict the peanut content of unknown samples, also in this case, polynomial regression (third order) was applied. The curve was based on six calibration points (0, 0.125, 0.25, 0.5, 1 and 2.5 mg kg⁻¹). The standards provided in the kit were 0, 1, 2.5 and 5 mg kg⁻¹ but since the 5 mg kg⁻¹ standard always returned saturated OD values, three lower concentrations were introduced by means of dilution to increase the data points for a calibration curve. The lower concentrations all returned OD values that were above the OD of the blank plus three times the standard deviation.

Regarding the analysis of unknown samples, two protein extracts were obtained from each sample material tested, and both protein extracts were analysed in duplicate. The concentration of peanut material in the sample was determined by interpolating the corresponding average of the four ODs with the calibration curve obtained with the standards provided in the kit.

Repeatability test

Ten samples from each concentration level of the cookie material (16 min baking time) were randomly selected. Three independent measurements per sample were performed with the BioSystems peanut allergen test kit and with PCR C. The average of each set of three measurements was considered

representative of the sample analysed. The relative standard deviations (or coefficients of variation) were calculated on the averages of the OD values for the ELISA method and on the Ct values for the real-time PCR method.

Results and discussion

Peanut detection by real-time PCR and ELISA

Prior to consumption, peanuts usually undergo heat treatments like blanching or roasting. The IRMM-481 peanut test material, which was used in this study, includes five peanut varieties from different geographical origins exposed to five different industrial heat treatments (Table 1). Therefore, it represents a material that might be comparable to the peanuts the consumer is exposed to. Each variety (component **a**, **b**, **c**, **d**, **e**) and the mixture of the five peanuts (component **f**) were analysed separately using the two ELISA kits targeting Ara h 1 and Ara h 2 and the three real-time PCR assays as quantitative methods. Component **f** of IRMM-481 that contains a mixture of different peanut varieties exposed to different heating processes was considered as the best suited material for the establishment of calibration curves for the PCR assays. Therefore, dilutions of the DNA extracted from this material were employed to generate calibration curves for the three real-time PCR assays as described in the “Experimental” section. For the data analysis and the generation of calibration curves, we followed the guidelines of Burns et al. [25]. Due to the heteroscedastic distribution of the data points, linear weighted regression was applied to describe the calibration models, moreover as suggested by Burns et al., individual Ct values collected for each calibration point, instead of their respective averages, were used to calculate the equation of the curves. The calibration curves obtained by the three PCR assays were characterised by R^2 values close to 1 (PCR A 0.998, PCR B 0.997, PCR C 0.997), and as determined from their individual slopes the efficiency of the PCR reactions ranged from 95% to 97%. The curves were generated by plotting Ct values against the logarithm of the amount of peanut DNA, aimed at the interpolation of unknown samples.

The ELISA test kits that were used were supplied with their own standards that were utilised to establish calibration curves for the interpolation of unknown samples.

To perform a comparative analysis with real-time PCR and ELISA, both sampling and the number of measurements performed were equal for both methodologies. However, different calibrants were utilised for either ELISA or PCR, and the final quantification of each peanut sample was differently expressed (in milli-

grammes peanut per kilogramme for ELISA, or nanograms peanut DNA for PCR). In order to evaluate and compare the data obtained from both detection methods, all results were normalised to the mixture component **f** of IRMM-481.

Effect of heating on peanut detection

In order to study the effect of the industrial processing as applied to the different components of IRMM-481 we compared the analysis of its components with their raw counterparts by means of ELISA and real-time PCR. In addition, the analysis of the five different raw peanut varieties allowed us to investigate the variability in the allergen detection as caused by differences in peanut variety.

The left part of Fig. 1 shows the results for the raw peanuts of all 5 varieties as determined by ELISA, while the left part of Fig. 2 shows the real-time PCR results for the raw peanuts. A clear variation in analytical response was apparent for both methodologies. Results obtained with the ELISA kit targeting Ara h 1 varied from 109% to 300% of the normalised value of component **f** of IRMM-481, while using the ELISA kit targeting Ara h 2 the variability was slightly lower ranging from 136% to 274%. Thus, the ELISA analysis of the different varieties can show a two- to threefold difference in signal.

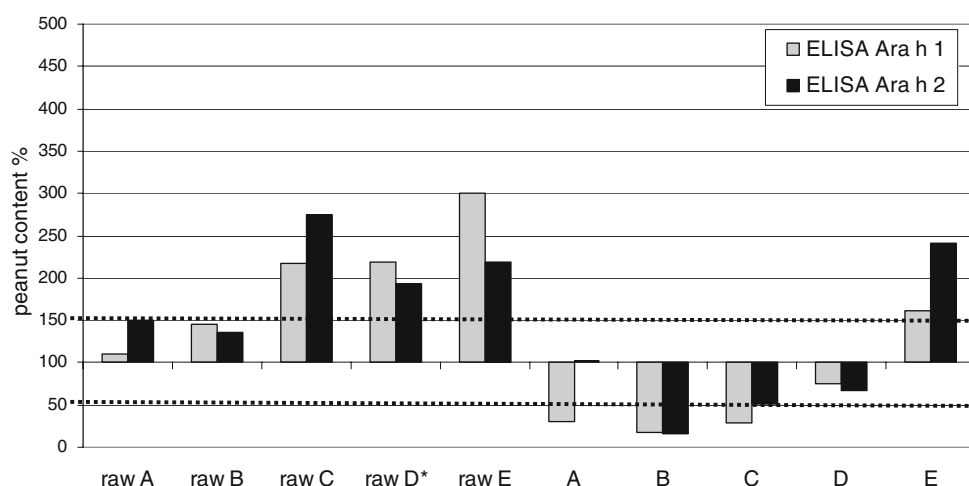
On the contrary, analysis of the same material with the three real-time PCR methods showed a reduced variability with signals differing by a factor 1.4 for PCR A, 1.7 for PCR B and 1.5 for PCR C. Those observations suggest that the concentration of the two proteins targeted by the ELISA kits varies considerably amongst the different varieties tested. In contrast to such variable protein levels, the DNA concentration is more similar among the different materials.

Interestingly, the three PCR methods showed a similar behaviour with all of them detecting the highest signals for raw E followed by raw A, raw B, or raw C and finally raw D. For ELISA, clear differences were observed with the highest Ara h 1 signal measured in raw E and the lowest in raw A while the highest Ara h 2 signal was measured in raw C and the lowest in raw B. Similar variabilities were reported previously in (validation) studies of ELISA kits targeting different proteins [26, 27].

The right part of both Figs. 1 and 2 shows the results obtained after analysis of the different industrially processed peanut varieties. A comparison with the left part of the figures visualises the effect of heat treatment, and it is apparent that the roasting processes dramatically reduces the ability to detect peanut when either ELISA kits or PCR methods are employed.

By comparing the ELISA results obtained with the mixture IRMM-481, which is composed of blanched and roasted peanuts, the concentration of the immunoreactive

Fig. 1 Estimation of the peanut content in different raw and roasted peanut varieties (listed in Table 1) as determined with an ELISA kit targeting Ara h 1 or with an ELISA kit targeting Ara h 2. All values are normalised to the peanut content determined in the mixture (IRMM-481 component f). The upper and lower dotted lines indicate respectively a peanut content 50 percent higher or 50 percent lower than that of the mixture (IRMM-481 component f). *This peanut material was blanched under mild conditions



material in raw kernels can be double or three times higher. This is consistent with other studies that have compared raw and roasted peanuts analysed by ELISA [13, 27] and seems to be largely caused by a difference in protein extraction efficiency [13, 15, 28]. Poms et al. and Chaissagne et al. observed that in roasted peanut the yield of extraction using commercial buffers can drop to 20% of the level of raw peanut, because of reduction in protein solubility [13, 15].

Looking at individual peanut varieties, Ara h 1 is more sensitive to heat treatment than Ara h 2. The signal of three out of five processed varieties fell below 50% of the value of the mixture for Ara h 1 (samples A, B and C); while in the case of Ara h 2 this happens only once (sample B). In addition to this, component e, that only underwent blanching, did not show a decrease, but a modest increase of Ara h 2 compared to its raw counterpart. A light heat treatment like blanching can probably induce cell breakage which facilitates protein extraction.

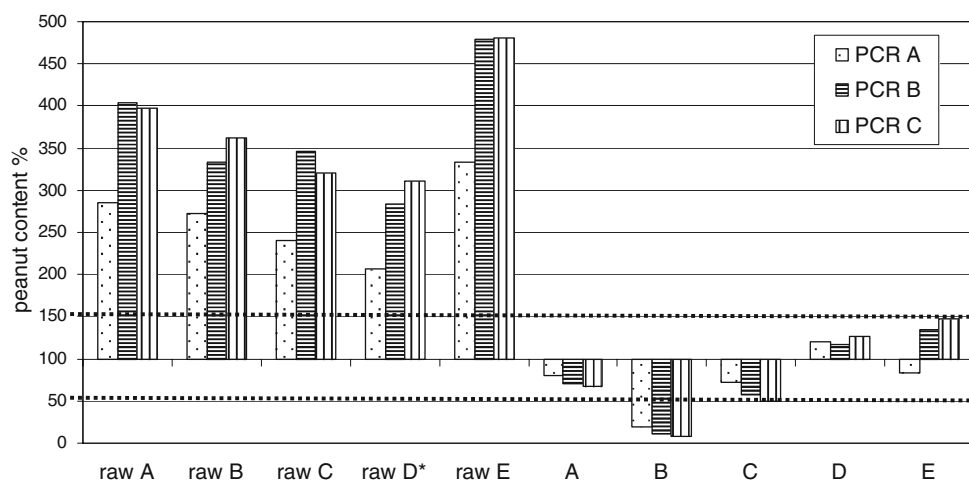
Figure 2 shows that the roasting process also resulted in a decrease of detectability when DNA was the target. Even though DNA, as a macromolecule, exhibits a relatively high

chemical stability, there is a broad spectrum of chemical and enzymatic reactions that cause DNA modification and/or degradation. Thermal treatments are known to contribute to such reactions [29–32]. Generally, the drop in peanut DNA detectability caused by processing is more evident than with protein detection by ELISA (Fig. 1). The PCR signals obtained after analysis of raw peanuts were two- to fivefold above those of the processed (standard) mix, while for ELISA detection this difference ranged only from 1.1- to threefold.

When the results of the individual processed varieties were compared, a lower variability was observed after PCR analysis compared to analysis with ELISA. This is apparent from the observation that after real-time PCR analysis only one processed variety (B) showed a more than 50% deviation from the standard, while after Ara h 2 detection 2 processed varieties (B and E) and after Ara h 1 detection even 4 (A, B, C and E) out of the 5 varieties showed a more than 50% deviation from the standard (dotted lines in Figs. 1 and 2).

We investigated the integrity of the DNA molecules as extracted from raw and processed peanuts. Raw, blanched

Fig. 2 Estimation of the peanut content in different raw and roasted peanut varieties (listed in Table 1) as determined with the real-time PCR A, B or C methods. All values are normalised to the peanut content determined in the mixture (IRMM-481 component f). The upper and lower dotted lines indicate respectively a peanut content 50% higher or 50% lower than that of the mixture (IRMM-481 component f). *This peanut material was blanched under mild conditions



(100 °C, 30 min), mild roasted (140 °C, 12 min) and strong roasted (140 °C, 20 min) peanuts from a single variety were analysed. Quantification of the DNA extracts from raw (177 ± 4 ng/ μ l), blanched (194 ± 3 ng/ μ l), mild roasted (76 ± 8 ng/ μ l) and strong roasted (48 ± 11 ng/ μ l) peanut indicated that the DNA extraction yield considerably decreases with increasing heat treatment. Therefore, lower amounts of template (extracted peanut DNA) are added to the PCR reactions of roasted samples where normally a fixed volume of DNA extract is used.

In addition to the yield, we investigated the quality or integrity of the extracted DNA by studying its amplifiability. For this purpose the DNA content was standardised before amplification by PCR. The amplification of 1 ng template DNA in each PCR reaction resulted in similar Ct values (28.4 ± 0.4) for all samples regardless of the heat treatment (Fig. 3). Therefore, we conclude that a major limiting factor in DNA detection as well as in protein detection is the extractability of peanut DNA or protein. This study also showed that DNA obtained from strong roasted peanuts was perfectly amplifiable by all three PCR assays with a comparable efficiency even at low concentrations, which shows that the extracted DNA is of good quality.

ELISA and real-time PCR analysis of a model food matrix

Accidental ingestion of peanut by an allergic consumer most likely occurs when peanut is hidden in a food matrix. Such peanut-containing foods can contain a variety of different ingredients and might have undergone a heat treatment. Therefore, we studied the detection of peanut in real food samples and compared DNA and protein based detection methods. For this purpose, we employed a processed food matrix (cookies), representative for bakery

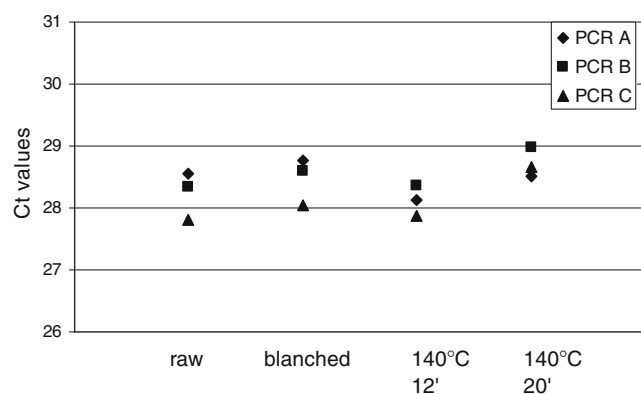


Fig. 3 Comparison of the amplification of DNA of raw, blanched (100 °C, 30 min), mild (140 °C, 12 min) and strong (140 °C, 20 min) roasted peanut (Runner) with the three real-time PCR assays (A, B, C). In each PCR reaction an aliquot of 1 ng of DNA was used for amplification

products, to which peanut was added before food processing (baking), as described previously [22].

All different cookies were analysed in duplicate with all three PCR assays and the two ELISA kits.

The peanut content in the cookie material was quantified with the ELISA kits as described in the “Experimental” section, whereby quantification was based on the protein standards provided in the kits. However, such standards were not available for quantification by means of the real-time PCR methods. For the proper comparison of ELISA and real-time PCR detection, all results were normalised to the mixture component f of IRMM-481. Previously, we have shown that analysis of this cookie material with the three PCR assays gave a linear response within a concentration range of 10 mg kg⁻¹ to 10,000 mg kg⁻¹ for cookies baked 16 min [22]; therefore, this material was used to establish calibration curves.

Due to its method of preparation the ground cookie material was likely to be homogeneous. This presumed homogeneity was tested by utilising the ELISA kit targeting Ara h 1 as well as the three PCR methods. For this purpose, cookies containing peanut at the two lowest levels (10 and 100 mg kg⁻¹) were analysed. ANOVA was applied to the values obtained with three PCR assays and the ELISA kit. The variability among the tested storage bags was not statistically different ($P > 0.15$) from the variability within (method precision), which implies that the cookie material can be considered homogeneous regardless whether DNA or protein was detected. PCR and ELISA methods gave similar results, which supports the applicability of the three real-time PCR assays for homogeneity testing.

Having assessed that the material was homogeneous, it was used for the establishment of a calibration curve for the real-time PCR C. The curve obtained by plotting Ct values against the logarithm of the concentration ($y = -3.275 \log(x) + 41.067$; PCR efficiency, 102%) was used to interpolate samples with an unknown peanut content and to estimate the peanut content in cookie material that underwent different periods of baking.

In contrast to PCR analysis, ELISA calibration curves are characterised by a very limited working range. This implies several dilutions of sample extracts from material with a high peanut content which likely affects the precision of the method. We therefore tested the repeatability of the different methods. For this purpose PCR C and the ELISA kit targeting Ara h 1 were utilised.

The repeatability of analytical methods like real-time PCR is an important parameter to consider as stated by the European Network of GMO laboratories [33]. It is defined as the standard deviation of test results obtained under repeatability conditions. In this study, the coefficient of variation (CV) was determined for both the real-time PCR and ELISA methods. Figure 4 shows a comparison of the

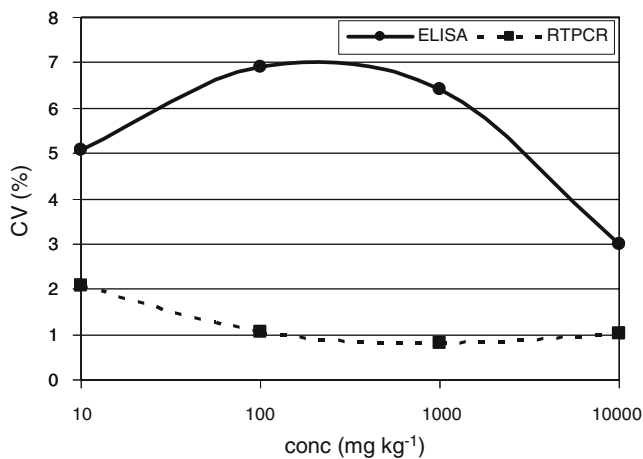


Fig. 4 Coefficient of variation (CV%) calculated from the Ct values obtained with real-time PCR C or from the ODs obtained with the ELISA kit targeting Ara h 1. Results are based on the analysis of ten cookie extracts per concentration level (standard baking time of 16 min)

calculated CVs and shows that a decrease in peanut content leads to an increase in measurement variability for the real-time PCR method. This confirms our observation that the real-time PCR calibration curves were characterised by a heteroscedastic distribution of data points, whereby the largest standard deviation corresponded to the lowest peanut level. The calculated CVs for the ELISA method show a similar trend although here the cookie material containing 10 mg kg⁻¹ of peanut returned a CV value that was lower than expected.

Despite the fact that the CV values generally follow the same trend for both methods (an increase at lower concentrations), for ELISA the CV values ranged from 3% to 5% while real-time PCR showed a better repeatability with CV values ranging from 1% to 2%. The latter CV values fall within the range of CV values reported by Píknova et al. [23] (1% < CV < 3%) who analysed subsequent dilutions of DNA by real-time PCR to detect hazelnut allergens.

The effect of heat treatment in a model food matrix

Food products that contain peanut as an ingredient, or in which peanut is unintentionally present (e.g. as a result of contamination during the production process) constitute a clear threat to the health of allergic consumers. Such food products often contain peanuts that have undergone two subsequent heat treatments, as exemplified by roasted peanuts used as ingredients for baked cookies. This is most dangerous for sensitised consumer since it can trigger allergic reactions while such a product might not be identified as peanut-containing (not listed on the label) and analytical detection of peanut fails due to the manufacturing processes applied.

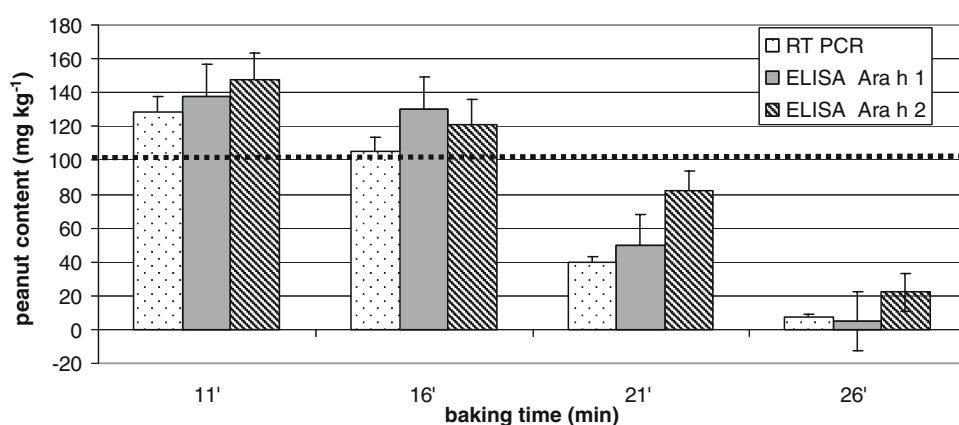
Up till now, only limited data are available about DNA or protein stability during processing in complex food matrices. In the case of DNA, most of the available studies are focussed on the detection of DNA sequences of genetically modified organisms in food and mainly focus on the degradation of the genomic DNA up to a certain amplifiable length [31, 34, 35]. Other studies highlight the effect of different parameters like pH, time and temperature on the extent of DNA degradation. Bauer et al. identified the heat treatment and acidic condition as the main parameters responsible for DNA degradation [29, 30].

In this study, we investigated the effect exerted by heat treatment of a complex food matrix on the detection of peanut. Cookies with different peanut contents that were baked for either 11, 16, 21 or 26 min were analysed by using PCR C and the two ELISA kits.

Figure 5 shows a comparison of the relative peanut contents measured over the range of baking times that were investigated. For the determination of the peanut content, the calibration curve based on the same cookie material baked for 16 min was applied. As expected, the detection of both the protein and DNA targets decreases with increased baking time. The standard material (baked at 180 °C for 16 min) showed a comparable quantification with all three methods, close to the true value of 100 mg kg⁻¹. Prolonging the baking time for 10 min resulted in a decrease of the estimated peanut content by a factor 10. Detection of the PCR target did not differ from detection of the Ara h 1 protein target, although the PCR detection was characterised by smaller prediction intervals. The ELISA kit targeting Ara h 2 generally followed the same trend, but returned higher estimations of peanut content after prolonged baking. This is in accordance with studies reporting a high heat stability of Ara h 2, and a better extraction efficiency of Ara h 2 from heat-roasted peanuts compared to Ara h 1 [13, 36].

The Ct values of the analysis of the cookies that underwent different times of heat exposure were investigated to study the sensitivity of the three PCR methods with regard to the heat treatment and to establish a limit of detection (LOD). Traditionally, the LOD is often derived from the mean of blanks and the standard deviation of the blank, which cannot be applied to quantitative PCR techniques as recently stated [37], since negative controls do not return a zero measurand result or a specific value, instead they are just defined as “undetermined”. An important parameter that needs to be fixed to define the LOD is the Ct cut-off value. It is important that the Ct cut-off value is not set too low in order not to omit true positive results but also not too high to include false positive results [37]. In this study, two Ct cut-off values were set, all Ct values below 40 were considered reliable for quantification purposes, and Ct values up till 45 were considered for

Fig. 5 Estimation of the peanut content (ppm = mg kg^{-1}) in cookies containing peanut at a level of 100 mg kg^{-1} and baked for 11, 16, 21 or 26 min. The measurements were performed with real-time PCR C and two ELISA kits (targeting Ara h 1 and Ara h 2). The error bars refer to the prediction intervals of each predicted value derived from the calibration curves



qualitative testing. When Ct values of 40 to 45 were obtained, the analytical result for the sample was expressed in terms of the number of PCR reactions that returned Ct values below 45 compared to the total number of analysis for that sample. Although the higher Ct values and therefore the estimated peanut content decreased with longer baking time all cookie material containing peanut at a level of 100 mg kg^{-1} or higher tested positive (data not shown). However, prolonging the baking time caused a drop in detection and resulted in several negative tests when the cookies containing peanut at a level of 10 mg kg^{-1} were analysed. Table 2 illustrates the results for this cookie material as analysed by the three PCR assays. Four separate reactions were carried out per method per baking time. The results show that at this low peanut content the prolonged heat treatment can prevent the detection of peanut traces in the cookies.

This represents a danger to erroneous interpretation. In the model matrix analysed, here all analyses of the blank

matrix samples returned without exception “undetermined” Ct values. Therefore, any positive test result cannot be assigned to the amplification of non-target DNA. Instead, the occurrence of positive as well as negative test results for the same sample points at the fact that low levels of target analyte are giving rise to stochastic effects of sample heterogeneity resulting in erroneous interpretation. The fact that material with prolonged baking times returned Ct values above and below 40 (but less than 45), and is characterised by a lack of repeatability may indicate that the DNA was degraded by heat treatment, or that the extraction of peanut (target) DNA is reduced after prolonged baking. Both explanations implicate that only a few copies of the template sequences were available for the amplification. Degradation of DNA during thermal processing is reported by Hiramoto et al. [38] who have shown that DNA fragmentation takes place during food processing which is caused by the Maillard reaction of glucose and amino acids in an aqueous system. However, our results on the effects of heat treatments on the quality and quantity of the DNA extracted from whole peanut (as reported above) suggest that the decreased detection in the material that had undergone prolonged baking most likely results from a decreased efficiency of extraction of the target DNA.

A different approach to define the limit of detection was applied to ELISAs. The ELISA kit targeting Ara h 1 sets its LOD at two times the value of the blank. Applying this LOD, all cookie samples containing peanut at a level of 10 mg kg^{-1} returned positive results, even at the higher baking times. The ELISA kit targeting Ara h 2 sets its LOD at a value equal to that of the chosen positive control sample. Since the positive control sample with the lowest peanut content is that of 1 mg kg^{-1} , this value was used as the LOD in our experiments. Applying this LOD, all cookie samples containing peanut at a level of 10 mg kg^{-1} returned positive results when the baking time was 11 min. However, after 16 min of baking only one out of four samples returned a positive result. On first instance, this

Table 2 The effect of baking time on the detection of peanut by means of the three PCR assays (A, B, C)

	10mg kg^{-1}		
	PCR A	PCR B	PCR C
11'	4/4	4/4	4/4
16'	4/4	4/4	4/4
21'	3/4 ^a	2/4 ^b	4/4 ^b
26'	3/4 ^a	2/4	1/4

The cookie material analysed contained peanut at a level of 10 mg kg^{-1} and was baked for 11, 16, 21 or 26 min. The results are presented as an index based on the number of positive results obtained after analysis of four samples. Positive results all had Ct values below 40, except when indicated with a footnote

^a Amongst the positive results one sample returned a Ct value above 40

^b Amongst the positive results two samples returned a Ct value above 40

might be unexpected since Ara h 2 shows higher heat stability than Ara h 1 but within peanut Ara h 1 is more abundant compared to Ara h 2 [39], which might explain our observations. Furthermore, our results are in line with those of an inter-laboratory validation study that included the same two ELISA kits as used in our current study. In the ELISA validation study, a higher number of false positive results were obtained with the Ara h 2 ELISA, compared to the Ara h 1 ELISA kit after analysis of cookies containing peanut at a level of 2 mg kg⁻¹ [40].

Conclusions

Recently, several studies have embarked upon a comparison of PCR and ELISA technologies designed to detect peanut traces in food products [18, 21, 23]. Those studies have reported qualitative comparisons, and have largely focussed on the analysis of market samples. The lack of reference materials for allergen detection has brought individual research groups to develop their own test material and their own experimental design, compromising a direct comparison of such studies.

In this study, we compared two different ELISA kits, one targeting the allergenic protein Ara h 1 and the other Ara h 2, with three real-time PCR methods. Our results show that despite the completely different nature of the target molecules, protein or DNA, the performance of the different detection methods was very similar. A comparison of five different peanut varieties confirmed that the level of DNA is more uniform among different peanut varieties than that of the proteins Ara h 1 or Ara h 2. As expected, roasting was found to strongly affect the detection of both target molecules, and our study shows that this decrease in detection follows a similar trend for both types of analytical methods. Despite this, we observed some variability which was higher between the two ELISA kits, compared to the variability between the PCR methods. The observed variability amongst ELISA kits is consistent with other studies [26] and is based on the fact that the ELISA kits target different proteins and therefore result in different estimations of peanut content, unlike the three PCR methods.

In addition, a model food matrix representative of bakery products has been utilised and both ELISA as well as real-time PCR methods could be employed to confirm the homogeneity of this test material. This material could be used to estimate the peanut content in cookies baked for different periods of time, to determine to which extent peanut DNA or protein detection is affected to the heat treatment in a complex food matrix. The two ELISA kits as well as the real-time PCR methods showed a similar trend, since prolongation of the baking time (26 min compared to

the standard 16 min) resulted in a five- to ten times decrease in detection.

Overall, we can conclude that the use of the three real-time PCR methods yields analytical results that are broadly comparable to those obtained with the commercial ELISA kits that were tested. With regard to the effects of roasting of peanuts, or baking in a cookie matrix it was interesting to note that one of the ELISA kits showed a behaviour that was more similar to the real-time PCR method than to that of the other ELISA kit.

Both ELISA- and PCR-based methods have disadvantages. The first might return false positive results due to cross-reactivities of the antibodies, while the latter detects DNA and therefore does not investigate the presence of allergenic proteins. Therefore, it is very important to be able to confirm a positive result obtained with one method with a different method. This study shows that ELISA kits and real-time PCR complement each other and that the one can indeed be used to confirm results obtained by the other.

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