



Analytical Methods

Towards comparability of ELISA results for peanut proteins in food:
A feasibility study

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ARTICLE INFO

Article history:

Received 22 November 2009

Received in revised form 4 May 2010

Accepted 6 June 2010

Keywords:

Peanut proteins

Quality control material

ELISA

Homogeneity

Stability

ABSTRACT

Results from different enzyme-linked immunosorbent assays (ELISAs) for the detection of peanut protein in food lack comparability due to different measurands, extraction procedures, antibodies, detection systems, and calibrants. Therefore, a feasibility study on a suitable quality control material (QCM) was conducted. A peanut powder and a non-de-fatted peanut homogenate were produced, and the materials properties including homogeneity and stability were assessed. Additionally, the influence of the extraction buffer on the protein yield and composition was investigated. A set of methods was applied including two ELISAs, SDS-PAGE, a “total soluble protein” assay, a “total fat” method, and a photometric assay to detect fat oxidation. Both preparations were sufficiently homogeneous for the intended use, and storage at 40 °C for up to 3 months did not reveal any material degradation. The extraction buffer influences the protein yield, but not the composition of the extracted proteins. The results constitute an important step towards the development of a peanut protein QCM.

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1. Introduction

Peanut allergy is one of the most severe and one of the most abundant food allergies in sensitised patients and therefore an important health issue (Hefle, Nordlee, & Taylor, 1996). Symptoms can range from itching and sneezing to nausea and anaphylactic shock. A main consideration is that several different peanut proteins, some of which are very abundant in the peanut (e.g. Ara h 1), have shown allergenic properties in sensitised patients, and that even trace amounts of these proteins in foodstuffs can induce an allergic reaction (Hourihane et al., 1997). Therefore avoidance is the main strategy for sensitised patients. Legislation in several countries all over the world, including the EU Member States, responded to that issue by introducing labelling requirements of foods that may contain sources of peanuts and other allergenic foodstuffs (Directives of the European Parliament and Council 2000/13/EC, 2000 and 2003/90/EC, 2003).

Reliable and accurate analytical methods are therefore needed to ensure effective consumer protection. A variety of techniques, which operate either at the protein or DNA level, are meanwhile available to detect trace amounts of peanuts and other known allergenic foods in food stuff: PCR-based methods, ELISAs including dip-stick rapid formats, and biosensors (Besler, 2001; Maier, Morgan, Lindner, & Pittner, 2008; Poms, Klein, & Anklam, 2004; Schubert-Ullrich et al., 2009). These methods are detecting either a

specific allergen, or a marker which reveals the presence of the offending food. In addition, LC-MS/MS methods become increasingly important for unambiguous identification and quantification (protein or peptide level) (Careri et al., 2007; Shefcheck & Musser, 2004). In routine analysis and for testing large amounts of samples in the food industry and control laboratories, however, ELISA is still the methodology of choice.

The measurement principle of an ELISA is based on the immunological recognition of one or several specific epitopes on extracted peanut proteins by the employed antibodies (usually a IgG sub-fraction isolated from animals immunized with the allergen in question, e.g. a pure dedicated peanut protein such as Ara h 1, or a mixture of extracted peanut proteins). Commercial kits are meanwhile available from several suppliers (Poms et al., 2005), and, in addition, several other ELISA methods for detection and quantitative determination of either dedicated peanut allergens or “total peanut proteins” have been published (Holzhauser & Vieths, 1999; Pomes et al., 2003; Schmitt, Cheng, Maleki, & Burks, 2004; Stephan & Vieths, 2004; Yeung & Collins, 1996). The ELISA methods differ in the extraction buffer used (usually neutral to slightly alkaline buffers, such as borate, bicarbonate or phosphate buffers), the sample preparation procedure (extraction modes such as turraxing and shaking, time, temperature, additives used), the set-up (e.g. sandwich ELISA, competitive ELISA), the antibodies employed (host animal, antibody class, mono- or polyclonal antibodies, specificity which itself depends on the antigen preparation; purified peanut protein, or “total soluble peanut extract” used for immunization), the detection system (enzyme, substrate, secondary antibody,

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etc.), and, very importantly, the calibrants used (whole peanut extracts or purified protein fractions such as Ara h 1 isoforms). In addition, the correlation factor from peanut protein to whole peanut is only approximately known and largely depends among other factors on the variety and the specific batch tested. Last but not the least, and not necessarily restricted to commercial kits, results are expressed differently (e.g. mg/kg “peanut” and ng/mL peanut protein in extract), and the exact calculation algorithm test-specific and not known to the user; therefore results only hold for a specific kit used under the provision that the respective working protocol is strictly followed. Consequently, different and non-comparable results are obtained when analysing a given sample with different ELISA tests, as they are targeting and detecting different measurands.

Several recent publications concerning peanut protein determination in foods focus on the development of ELISA methods (Kiening et al., 2005), on extractability of peanut proteins when using different extractions buffers (Poms, Capelletti, & Anklam, 2004), and on comparability among ELISA methods (Nogueira, McDonald, Westphal, Maleki, & Yeung, 2004) or between ELISA and PCR techniques (Stephan et al., 2004). In contrast, and despite clear statements in several papers requesting a reference material or quality control material to allow a better comparability of data and required for method validation purposes, only limited work has been published in this field. Poms et al. (2005) described the outcome of an interlaboratory comparison of five commercial ELISA kits for peanut protein determination in biscuits and dark chocolate, and Westphal, Pereira, Raybourne, and Williams (2004) reported on the application of a peanut butter suspension (SRM 2387 from NIST as described in Trucksess, Brewer, Williams, Westphal, & Heers, 2004) to compare the performance of three commercially available peanut ELISA kits. In some effort to close this gap, our laboratory investigated possibilities for the production and characterisation of a suitable quality control material (QCM), a first step towards the longer-term goal of having available a (certified) reference material, which would constitute an important tool for laboratories in method validation and therefore also highly requested in the field of allergens in food. For a reference material however, the important issue of the measurand definition (i.e. what exactly shall be certified) needs to be solved first.

In this article, we report on the production and characterisation of two peanut candidate quality control material preparations: a non-de-fatted peanut homogenate, subdivided into three particle-size fractions, and a de-fatted peanut powder. Material properties, especially homogeneity and stability, were evaluated. This was accomplished by assessing different parameters in the material, some of which are method-defined (e.g. Bradford assay for “total soluble protein” and extraction with hexane for the “total fat content”), whereas others are analysed by methods more specifically targeting a certain measurand (e.g. ELISA detecting conarachin). In addition, the influence of several extraction buffers on the peanut protein yield and the obtained protein pattern was tested.

2. Materials and methods

2.1. Peanuts

Light-roasted, non-oiled and unsalted peanuts (variety Virginia, imported from US) were obtained in a local store and stored at 4 °C until use. The shell and the outer pellicle were removed from each piece.

2.2. Preparation of de-fatted peanut powder

The peanuts were pre-ground with mortar and pestle. Then the sample was homogenised in a mixer (B-400, BUECHI, Q-Lab, Vilvoorde, Belgium) with 10 strokes of 1 s each. The obtained paste was

de-fatted as follows: 50 mL of hexane was added per 25 g of paste, and the sample was agitated on a horizontal shaker for 20 min, centrifuged (5 min, 3000 g) and the supernatant discarded. This procedure was repeated three more times. Finally, the resulting de-fatted sample was dispersed on a plate and left overnight at ambient conditions (evaporation of solvent residues). The resulting powder was again ground with a mortar and pestle. For the homogeneity study, a part of the produced powder (10 units with 5 g each) was bottled in amber glass vials preflushed with argon, and closed under argon atmosphere with rubber stoppers and aluminium crimp caps. For the stability study, 45 units with 2 g each were produced in the same way. Bottled and non-bottled powder was stored at –20 °C until use. The particle size distribution of the obtained powder was characterised by laser diffraction measurements using a Sympatec Helos laser light scattering instrument (Clausthal-Zellerfeld, Germany) and 2-propanol as dispersant.

2.3. Preparation of three particle-size fractions from peanut homogenate

Peanuts were put in liquid nitrogen for about 10 s, followed by grinding in a Moulinette S food chopper (Moulinex, Fleurus, Belgium) with two strokes of 3–4 s each. The resulting particles were manually passed through a 2 mm sieve (ALPINE, Augsburg Germany). Further sieving using 1 and 0.5 mm stainless steel sieves, respectively, was performed using a Russel Finex Industrial sieve (London, United Kingdom). A portion of the small particles passing the 1 mm sieve were again submersed in liquid nitrogen and subjected to grinding (six strokes of 4 s each), and passed through a 0.5 mm sieve. For the homogeneity study, a part of the produced homogenate (10 units with 5 g each) was bottled in amber glass vials preflushed with argon, and closed under argon atmosphere with rubber stoppers and aluminium crimp caps. For the stability study, 45 units with 2 g sample size (all three particle-size fractions) were produced in the same way. Bottled and non-bottled particle-size fractions were stored at –20 °C until use. Particle size distribution of each of the three fractions was assessed using a Hosokawa ALPINE sieve analyser (Augsburg, Germany) and nine sieves of mesh sizes between 120 and 2000 µm. Sieve fractions were weighed on a calibrated Mettler PM1200 balance with a resolution of 1 mg.

2.4. Preparation of extracts

Extracts were prepared by mixing 1 g of de-fatted peanut powder or peanut homogenate particle size fraction with 20 mL of the following buffers: 0.1 M ammonium bicarbonate pH 8.0; 20 mM Tris/HCl pH 8.2; 8 mM Tris, 25 mM Tricine pH 8.6; and 20 mM sodium phosphate pH 7.9 containing either 0, 0.2, 0.5, or 1 M sodium chloride. The samples were agitated on a horizontal shaker (IKA, HS 501, Q-lab, Vilvoorde, Belgium) for 2 h at 150 rpm at room temperature, followed by a centrifugation step (13,000g, 20 min). The resulting supernatant was filtered (0.45 µm filter, Millipore) and stored at –20 °C until use.

2.5. Total soluble protein assay (Bradford test; Bradford, 1976)

To 0.1 mL of sample, 1 mL reagent solution was added. That solution consisted of 0.1 g Coomassie Blue G250 (Sigma, Bornem, Belgium), 50 mL 96 v/v% ethanol, 100 mL ortho-phosphoric acid (85 v/v%), and water to yield 1 L. Whenever required, samples were diluted with 20 mM phosphate, pH 7.9 containing 1 M NaCl to concentrations in the working range of the calibration curve (0–0.35 mg/mL). The absorbance at 590 nm was measured after 10 min of incubation at room temperature. The blank was 20 mM phosphate pH 7.9 containing 1 M NaCl. Calibration curves were

prepared using bovine serum albumine (SIGMA, Bornem, Belgium) diluted in the same buffer. Photometric measurements were carried out on a UNICAM UV-500 spectrophotometer (Thermo Spectronic, Cambridge, GB).

2.6. Total fat determination

To 1 g of sample (particle size fraction), 20 g hexane was added. After turraxing the sample vigorously for 1 min, the slurry was left 15 min to sediment, filtered and the solvent collected. The sample was again extracted with 20 mL hexane. The pooled solvent phase was dried at 50 °C in a stream of nitrogen and the fat was weight out. The ratio of weighed fat to starting material was calculated to be the total fat content.

2.7. Monitoring of fat oxidation

Specific absorption at 232 nm of extracted fat dissolved in iso-octane can serve for detection of hydroperoxides and conjugated fatty acid dienes and trienes (Vieira & Regitano-d'Arce, 1999). An aliquot of the collected fat from the total fat determination was diluted in iso-octane to a concentration of about 3.5 mg/mL. The absorption of the sample was measured at 232 nm (iso-octane as blank) using a UNICAM UV-500 spectrophotometer. The obtained values were normalised to the exact sample weight. The obtained results were interpreted by comparing them with those from reference samples (stored at –20 °C) which were treated in the same way.

2.8. SDS-PAGE

Reducing SDS-PAGE was performed according to established procedures using a Mini Protean 3 Electrophoresis System (Bio-Rad, Nazareth Eke, Belgium) and self-casted gels (12 w/v% total monomer with 1 w/v% cross-linker). The NOVEX Mark12™ Wide Range Protein Standard was used as a molecular weight marker (Invitrogen, Merelbeke, Belgium). The SDS-PAGE sample buffer consisted of 31 mg dithioerythritol, 200 mg SDS, 5 mL stacking gel buffer (0.5 M Tris/HCl pH 6.8), 2.8 mL glycerol (87 w/v%), 0.01 w/v% bromophenol blue, and 13.2 mL water.

Proteins in the sample (extracts) were precipitated by adding a large excess of methanol, vortexing, and leaving the sample for 1 h at –20 °C. After centrifugation, the pellet was taken up in SDS-PAGE sample buffer as described above. Samples were heated to 95 °C for 5 min, and aliquots of 10 µL were applied per lane. Gels were run at 200 V for 45 min in electrophoresis running buffer (3.6 g/L Tris, 14.4 g/L glycine, 1 g/L SDS), fixed in 50 v/v% methanol/7 v/v% acetic acid for 30 min, stained with Coomassie blue (0.04 w/v% Coomassie brilliant blue G-250 in 3.5 v/v% perchloric acid) for at least 60 min, and finally de-stained in 7 v/v% acetic acid. Gels were photographed with a CCD camera in a Gene Genius gel documentation system (Westburg, Leusden, The Netherlands).

2.9. ELISA

Two commercial kits (Peanut assay kit, BioSystems Tepnel, Deeside Flintshire, GB, targeting conarachin; Veratox Quantitative Peanut Allergen test, Neogen, supplied by Biognost BVBA, Heule, Belgium, targeting total peanut protein) were employed according to the manufacturer's instructions except using diluted extracts prepared as described in Section 2.4.

2.10. Homogeneity studies

Four bottles were chosen randomly from the small batch produced ($n = 10$), and four independent sample preparations and

measurements were performed per bottle. All 16 measurements for each material were performed on one ELISA plate (repeatability conditions). Both the total protein assay and the conarachin ELISA (Tepnel kit) were applied for assessing possible in-homogeneity of the two materials. As for the peanut homogenate, only the 1–2 mm fraction was tested (worst-case scenario, as this fraction would most likely exhibit the largest in-homogeneity). Obtained data was technically scrutinised and subjected to a one-way ANOVA, and the within-bottle standard deviation (s_{wb}), the between-bottle standard deviation (s_{bb}), and the uncertainty due to possible in-homogeneity that can be hidden by the method repeatability (u_{bb}^*) was calculated as described elsewhere (van der Veen, Linsinger, & Pauwels, 2001).

2.11. Stability studies

Isochronous studies (Lamberty, Schimmel, & Pauwels, 1998) were performed. Samples (bottled 2 g portions, see 2.2 and 2.3) were stored at 4, 20, and 40 °C for 4, 8, and 12 weeks). The reference temperature was –20 °C. Three independent sub-samples for each of the following parameters were prepared and analysed for each time/temperature point: total protein content, conarachin content, total fat content, and fat oxidation determination (peanut homogenate), and total protein content as well as conarachin content (peanut powder). Measurement results were technically scrutinised and evaluated by applying linear regression, testing for significance of the slope, and calculating an uncertainty estimation due to possible instability of the material, as described elsewhere (Linsinger, Pauwels, Lamberty, Schimmel, & van der Veen, 2001).

3. Results and discussion

3.1. Processing of materials and basic properties

Two different peanut materials have been prepared, a ground non-de-fatted peanut homogenate subdivided into three different particle-size fractions, and a de-fatted peanut powder. The materials were filled in amber vials and closed under inert gas, a standard procedure at our premises to guarantee best possible conditions for long-term storage of our reference materials. The reason for producing different kinds of materials was to compare a material which can be conveniently stored (de-fatted powder) with a less processed material which more closely resembles a sample usually tested by laboratories (e.g. food containing peanut paste). The peanut homogenate was further processed to three particle-size fractions in order to see whether finer ground fractions would adversely affect product stability of such a material.

The quality control analysis comprised determination of the particle size distribution in the final products using sieve analysis for the three particle-size fractions, and laser diffraction spectrometry for the peanut powder. Supplementary Table S1 shows the results of the sieve analysis. It can be seen that for all three produced sub-fractions of the non-de-fatted peanut homogenate, particle sizes and distributions were close to the envisaged nominal values. Smaller particles than nominal were only present in low quantities, and larger particles (also relatively small abundances) can be explained by the fact that the final material contained various particle shapes, some of which do not behave like an “ideal spherical particle” in sieving, e.g. fibrous particles. The de-fatted powder was analysed by laser diffraction spectrometry, and showed to contain particles from 1 to 175 µm with a maximum in the distribution curve at 120 µm.

The production could be further optimised by applying different milling techniques, e.g. cryogenic milling. This would most likely result in an even narrower particle size distribution. However,

the analogy in behaviour of such even more homogeneous preparations with a typical food sample containing peanuts would have to be verified.

3.2. Influence of extraction buffer on protein yield

The peanut powder and the non-de-fatted homogenate (before sieving into sub-fractions) were extracted with seven different buffers to evaluate the obtainable protein yield. The buffers were selected on the basis of previous publications having reported efficient protein extraction from peanuts and/or development of ELISA test, and their use in commercially available ELISA tests (Basha & Cherry, 1976; Holzhauser & Vieths, 1999; Koppelman et al., 2001; Moutete et al., 1995; Poms, Capelletti, et al., 2004). With one of the buffers (20 mM phosphate pH 7.9), 4 buffer solutions containing 0, 0.2, 0.5 or 1 M NaCl were prepared for assessing the influence of the ionic strength on the extraction yield. Each extraction was performed 6 times (triplicates on two days). All extracts were subjected to the Bradford protein assay, and the extracts of the non-de-fatted material were tested for their total fat content. Table 1 summarises the results. Substantial differences up to a factor of about three in the soluble protein yield were obtained; however, triplicate extracts with a given sample and extraction buffer showed that extracts can be prepared reproducibly under the described conditions. Absolute values were quite comparable for a given extraction buffer between the two sample types (considering the ca. 47 m/m% total fat found in the peanut homogenate). In addition, the relative values (protein values for a given material normalised to the respective value obtained with Tris/HCl extraction buffer) agree well for all seven buffers when comparing peanut powder and the homogenate. Tris/HCl and Tris/Tricine buffers resulted in the highest protein concentrations. Interestingly, but not completely unexpected, the ionic strength in the buffer has an influence on the extraction efficiency, as could be shown with the series of phosphate buffers (0–1 M NaCl added). Surprisingly, highest yields were obtained with either no sodium chloride, or sodium chloride in high concentrations.

The total recovery of peanut protein (protein content in extract to total protein in peanut) was not checked in our laboratory, but was reported to be about 80% (Koppelman et al., 2001) when using Kjeldahl nitrogen as measurement method for both extract and whole peanut, and exactly the same extraction buffer, extraction conditions, and peanut variety and origin as used in our laboratory. The result of 5.7 mg/mL extractable protein using the Tris/Tricine buffer (Table 1), which equals 11.4% of extractable protein (1 g peanut paste corresponds to 20 mL of peanut extract, see Section 2.4) compares well with the one described by Stephan and Vieths (2004) who found 15.9% extractable protein when using ground

peanut paste, Tris/Tricine buffer containing gelatine, and comparable extraction conditions. Also, a generally good agreement was found between the obtained extraction yields and their repeatabilities with those published by Holzhauser and Vieths (1999). Finally, the results compare well with those reported by Poms et al. (2004) concerning obtainable extraction efficiencies.

It shall be noted that comparability of protein concentration in extracts and extraction yields with those published in the literature are often limited due to different extraction conditions (time/temperature), homogenisation state of sample, exact buffer used (ionic strength, pH, presence or absence of additives such as gelatine), peanut variety, and last but not least very importantly the protein determination method (discrepancies even among assays of the sample principle, e.g. Bradford, depending on the recipe – buffer, incubation time, etc.). However, most importantly and as stated earlier, we found relatively stable extraction recoveries (repeatability of some 5–10%, Table 2) when using the specified conditions; a suitable extraction protocol can therefore be established and used, for instance in the frame of a proficiency testing (PT) study using such materials.

In addition to the standard extraction procedure of 2 h at room temperature an alternative procedure (18 h at 4 °C) was investigated with the aim to verify that no material disruption occurs when using the standard procedure. This was done on sample triplicates of de-fatted powder and the 1–2 mm particle size fraction, and tested for three buffers (Tris/HCl, biocarbonate, phosphate with 1 M NaCl). No significant yield differences were obtained for any tested buffer when applying the two temperature/extraction duration combinations (details not shown). Finally, applying the standard extraction procedure and using the Tris/HCl buffer (sample triplicates), we compared the extraction yield of the homogenate sub-fractions. Highest values for the <0.5 mm fraction, and lowest values for the 1–2 mm fraction were obtained as expected (different particle surface-to-volume ratios). However, if the samples were turraxed after vortexing and before shaking, the same values were obtained for each size fraction (data not shown).

3.3. Investigation of protein profiles obtained from the materials using different extraction buffers

In a next step, the different extracts prepared from the two materials were analysed by SDS–PAGE in order to verify whether besides the abundance differences (total soluble protein) also qualitative differences (protein pattern) would be detected among samples.

Fig. 1 depicts two gels; on the left side (A), protein profiles of the peanut homogenate (before sieving) are shown, which were obtained from extracts with different buffers. On the right side (B),

Table 1

Total protein concentrations in the extracts prepared with seven different buffers as determined by the Bradford test. Average and standard deviations of six replicate samples, as well as relative averages are shown (normalised to the values obtained with buffer Tris/HCl).

Buffer	De-fatted powder, mg/mL protein means $\pm$ SD ^a	De-fatted powder, relative value in%	Non-de-fatted material, mg/mL protein means $\pm$ SD ^a	Non-de-fatted material, relative values in%	Ratio non-de-fatted: de-fatted sample
Ammonium bicarbonate	5.3 $\pm$ 0.4	50	2.8 $\pm$ 0.2	48	0.53
Tris	10.7 $\pm$ 0.8	100	5.8 $\pm$ 0.3	100	0.54
Tris–Tricine	10.8 $\pm$ 0.6	101	5.7 $\pm$ 0.4	98	0.53
Phosphate	8.8 $\pm$ 0.6	82	4.9 $\pm$ 0.3	84	0.56
Phosphate with 0.2 M NaCl	3.1 $\pm$ 0.3	29	1.6 $\pm$ 0.2	28	0.52
Phosphate with 0.5 M NaCl	5.5 $\pm$ 0.3	51	2.8 $\pm$ 0.3	48	0.51
Phosphate with 1 M NaCl	8.5 $\pm$ 0.5	79	4.5 $\pm$ 0.4	78	0.53

^a SD, standard deviation.

Table 2

ELISA results on the extracts prepared with seven different buffers. Average and standard deviations of six replicate samples, as well as relative averages are shown (normalised to the values obtained with buffer Tris/HCl).

Buffer	Ara h 1 ELISA				Total peanut protein ELISA			
	De-fatted powder		Non-de-fatted material		De-fatted powder		Non-de-fatted material	
	"ppm peanut" ^a means $\pm$ SD ^c	Relative value in%	"ppm peanut" ^a means $\pm$ SD ^c	Relative value in%	"ppm peanut" ^b means $\pm$ SD ^c	Relative value in%	"ppm peanut" ^b mean $\pm$ SD ^c	Relative value in%
Ammonium bicarbonate	10.0 $\pm$ 0.9	53	4.7 $\pm$ 0.6	42	9.7 $\pm$ 0.9	57	4.7 $\pm$ 0.3	59
Tris	18.8 $\pm$ 1.6	100	11.2 $\pm$ 1.1	100	17.1 $\pm$ 2.1	100	8.0 $\pm$ 0.9	100
Tris–Tricine	16.8 $\pm$ 1.8	89	10.7 $\pm$ 1.2	96	19.4 $\pm$ 1.5	113	7.9 $\pm$ 0.6	99
Phosphate	16.2 $\pm$ 1.3	86	8.9 $\pm$ 1.3	80	18.8 $\pm$ 2.6	110	7.4 $\pm$ 0.7	93
Phosphate with 0.2 M NaCl	4.8 $\pm$ 0.4	26	2.2 $\pm$ 0.3	20	8.0 $\pm$ 0.8	47	2.1 $\pm$ 0.3	26
Phosphate with 0.5 M NaCl	8.8 $\pm$ 0.9	47	4.2 $\pm$ 0.4	38	11.1 $\pm$ 1.6	65	5.5 $\pm$ 0.4	69
Phosphate with 1 M NaCl	12.6 $\pm$ 0.9	67	6.7 $\pm$ 0.8	60	19.0 $\pm$ 2.1	111	8.0 $\pm$ 0.9	100

^a Results as read out from the Tepnel assay; extracts (all 7 buffers) were diluted 1:500,000 prior to ELISA measurement.

^b Results as read out from the BIOGNOST assay; extracts (all 7 buffers) were diluted 1:250,000 prior to ELISA measurements.

^c Standard deviation.

the counterpart is presented with protein profiles of the peanut powder from extracts with those buffers. Hardly any difference can be detected visually among (a) extracts prepared with different extraction buffers on a given material (lanes 1–4 and 6–10 on gel A for the non-de-fatted fraction; lanes 11–13 and 15–18 on gel B for the de-fatted powder), but also (b) among materials for a given buffer (e.g. lanes 1 vs. 11, lanes 8 vs. lane 17, etc.). No difference in the protein profiles among the four different phosphate buffer extraction solutions (0–1 M NaCl; lanes 6–10, gel A) could be detected, nor were there differences in the profile when extracting for 2 h at room temperature or for 18 h at 4 °C (lanes 1–2, gel A). Abundance differences among lanes are due to differences in the amount of protein loaded per lane and the staining in-homogeneity of the gels. Densitometry was not applied as detection method for each lane, as it did not render added value compared to the visual inspection due to difficulties observed in suitable background subtraction, which is a quite subjective operation. Despite the fact that the separation efficiency and resolution on a 1-D-gel is somewhat limited and that more specific and sensitive methods would be required to detect minor differences in protein profiles among samples and/or differently prepared extracts from one sample, it can be concluded that – together with the quantitative ELISA results and their excellent correlation with the Bradford values on the same samples, see Section 3.4 – with both materials the same or at least very comparable extracts in qualitative terms are ob-

tained. Therefore, the data reveal that defatting has no or only a very minor influence on the obtained protein pattern of an extract from the powder material. Moreover, also the type of extraction buffer, the time/temperature combination of performing the extraction, and the ionic strength in a given buffer do not visibly change the protein profile.

3.4. Correlation of total protein and peanut protein (conarachin) abundance in the different extracts

To further substantiate the findings of Sections 3.2 and 3.3 (extracts from materials differ in the yield obtained, but not or only insignificantly in the composition), two different ELISA kits were applied on the various extracts from the two types of materials (Neogen Veratex, detecting "total soluble peanut protein"; Tepnel BioSystems, detecting "conarachin").

Prior to using the two ELISA methods to assess the analyte concentration in the different extracts, typical method parameters such as repeatability and intermediate precision of the two tests were examined by analysing a QC sample included in the kit, a de-fatted powder extracted with Tris/HCl buffer, and an extract (Tris/HCl buffer) of the 0.5–1 mm particle size fraction. Three independent samples were prepared per day, and the study was carried out on three different days (three different ELISA plates), with independent calibration per ELISA plate. Supplementary Table S2

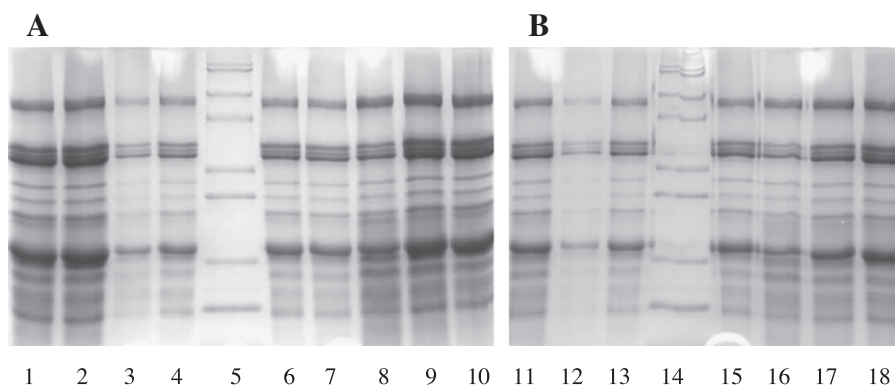


Fig. 1. SDS-PAGE of peanut extracts using different buffers. Aliquots of ca. 10–20 μ g protein were applied per lane. The gel was stained with Coomassie Blue. Gel A, non-de-fatted samples; lanes 1, NH_4HCO_3 (4 °C, 18 h); 2, NH_4HCO_3 (20 °C, 2 h); 3, Tris/HCl; 4, Tris/Tricine; 5, molecular weight marker; 6, phosphate; 7, phosphate/0.2 M NaCl; 8, phosphate/0.5 M NaCl; 9, phosphate/1.0 M NaCl; 10, same as 9. Gel B, de-fatted powder; lanes 11, NH_4HCO_3 ; 12, Tris/HCl; 13, Tris/Tricine; 14, molecular weight marker; 15, phosphate; 16, phosphate/0.2 M NaCl; 17, phosphate/0.5 M NaCl; 18, phosphate/1.0 M NaCl.

summarises the findings. It can be seen that the Tepnel test exhibited a somewhat better performance concerning repeatability and intermediate precision. The results show that the measurement itself and the plate-to-plate variation was the main contributor in the spread of results, whereas the sample preparation and the dilution steps are of minor influence on the variation.

Both ELISA tests were applied to investigate the correlation of results (quantified conarachin, quantified “total soluble peanut protein”) with those from the total soluble protein values (Bradford method), and the Tepnel assay was chosen as one test method in the subsequent homogeneity and stability studies (Section 3.5).

Table 2 shows the obtained ELISA results for the seven extracts of both materials (same samples as tested with the Bradford test as described in Section 3.2). Extracts were diluted appropriately so as to fit the working range of the ELISA tests. The extracts were diluted to a different extent for the two tests, but to the same extent applied to all seven buffer extracts for each ELISA test for direct comparability of these extracts. The absolute values, obtained in both tests in “ppm peanut”, are shown for allowing direct comparison between the seven different extracts for one material type. Typical relative standard deviations (three extracts each on two days/ELISA plates) were of 6–14%. In the vast majority of cases, relative values agreed well among extracts within a given combination of ELISA and extraction buffer, when comparing the two sample types (powder, non-de-fatted material). This again demonstrates that defatting has no adverse effect on the quantification of the measurands in question. In addition, relative values compare well among the two different ELISA test (for a given material and buffer), which demonstrates that despite the different ELISA setups and principles (“measurand”, calibrant, antibodies, ELISA design, etc.) comparable results (relative values) can be obtained. Last but not least, the relative values obtained with both tests are also in good correlation to the relative values obtained with the Bradford method (Table 3). This corroborates similar findings (Holzhäuser & Vieths, 1999; Stephan & Vieths, 2004).

Any further comparison of obtained “ppm peanut” values, especially those among different ELISA test, are meaningless, even if exactly the same sample (extract diluted to a certain extent) would be measured. This is attributed to the fact of different measured entities, calibrants (e.g. NIST SRM 2387, peanut butter, which is used in some cases), different varying extraction efficiencies (not included in our study as the same extracts were subjected to the two different ELISA methods), dilution steps, and calculation algorithms, especially referring to the conversion factor of an obtained ELISA raw data result to a mass fraction of “ppm peanut”. As stated earlier, additional methods need to be developed, validated, and applied in order to substantiate the findings presented here, and for a chance to detect possible minor differences in protein profiles. However, for the type of method still most frequently used in many laboratories, it was shown that the applied set of methods delivered a promising finding: for a given extraction buffer, extracts of the two types of materials looked the same both in quantitative and qualitative terms, and defatting does not impair the quality of the powder material. Consequently, both types of preparation can be used alternatively. To demonstrate their suitability as possible future QCMs, the homogeneity and stability of the materials was investigated.

3.5. Homogeneity studies

Homogeneity studies were performed on the peanut powder and one of the particle-size fractions of the homogenate (1–2 mm), whereby both total soluble protein (Bradford method) and conarachin (ELISA) values were investigated. Four independent measurements were performed for each of the 4 units randomly picked from the produced batches under repeatability conditions,

Table 3

Homogeneity study results for the de-fatted powder and one 1–2 mm particle size fraction. ELISA results were obtained using the Biosystems ELISA kit (Tepnel). See Section 3.5 for detailed explanations.

	Powder		1–2 mm fraction	
	Bradford	ELISA	Bradford	ELISA
RSD (%) ^a	2.48	5.99	3.48	5.56
s_{wb} (%) ^b	2.65	6.24	3.58	5.78
s_{bb} (%) ^c	n.c. ^d	n.c. ^d	n.c. ^d	n.c. ^d
u_{bb}^* (%) ^e	0.94	1.66	1.26	1.54
u_{bb} (%) ^f	0.94	1.66	1.26	1.54

^a Relative standard deviation of all measurements.

^b With-bottle standard deviation.

^c Between-bottle standard deviation.

^d n.c., not calculable because ANOVA result reveals that $MS_{between} < MS_{within}$.

^e Uncertainty of in-homogeneity that could be hidden by method repeatability.

^f Estimation of uncertainty due to possible material in-homogeneity.

and the obtained results were evaluated by one-way ANOVA and calculation of s_{wb} (within-bottle standard deviation), s_{bb} (between-bottle standard deviation), and u_{bb}^* (uncertainty due to possible in-homogeneity that can be hidden by the method repeatability) (van der Veen et al., 2001). Results are summarised in Table 3. Data are valid for a sample intake of 1 g for extract preparations. In both materials and for both methods applied, s_{wb} was larger than s_{bb} , indicating that the detection of a possible material in-homogeneity is limited by the measurement method. Estimated relative standard uncertainties of ca. 1.7% and 1.5%, respectively, for the powder and particle size fraction, when applying the conarachin ELISA fit well with the values of 0.9% and 1.3% obtained with the Bradford method for the two materials. It can also be seen that these values are in the same order of magnitude as the repeatability values (s_{wb}), and the repeatability values obtained in the initial performance evaluation of the conarachin ELISA (Supplementary Table S2). In conclusion, both peanut preparations can be regarded as sufficiently homogeneous for the intended use as QCMs.

3.6. Stability studies

Isochronous studies were performed, whereby the materials were stored at different temperature–time intervals and then kept at -20°C until measurements took place under repeatability conditions. The reference samples were stored at that temperature all the time. All four materials were tested, and the following parameters were evaluated for their stability: for the powder, the total soluble protein and the conarachin content were investigated, whereas for the three particle-size fractions of the homogenate in addition the total fat content and fat oxidation were monitored. Fig. 2 depicts the results obtained for the 1–2 mm peanut homogenate and the powder at a storage temperature of 40°C . Relative values to those obtained in the reference samples are shown for reasons of better comparability. It can be concluded that with the employed methods looking at a distinct analyte (Ara h 1 ELISA), at a group of analytes (total peanut protein content by Bradford), or at the integrity of the matrix (total fat, fat oxidation), no instability could be detected for a storage period at this elevated temperature up to 3 months, which holds for both de-fatted peanut powder as well as for non-de-fatted peanut homogenate particle-size fractions when bottled and stored appropriately (tight and inert gas atmosphere). Results for the 0.5–1 mm and the <0.5 mm fraction were similar (data not shown).

For both materials and storage temperatures of 4 and 20°C , similar results were obtained which confirm the findings of no detectable degradation (data not shown). Therefore it can be concluded that such material preparations could be dispatched with

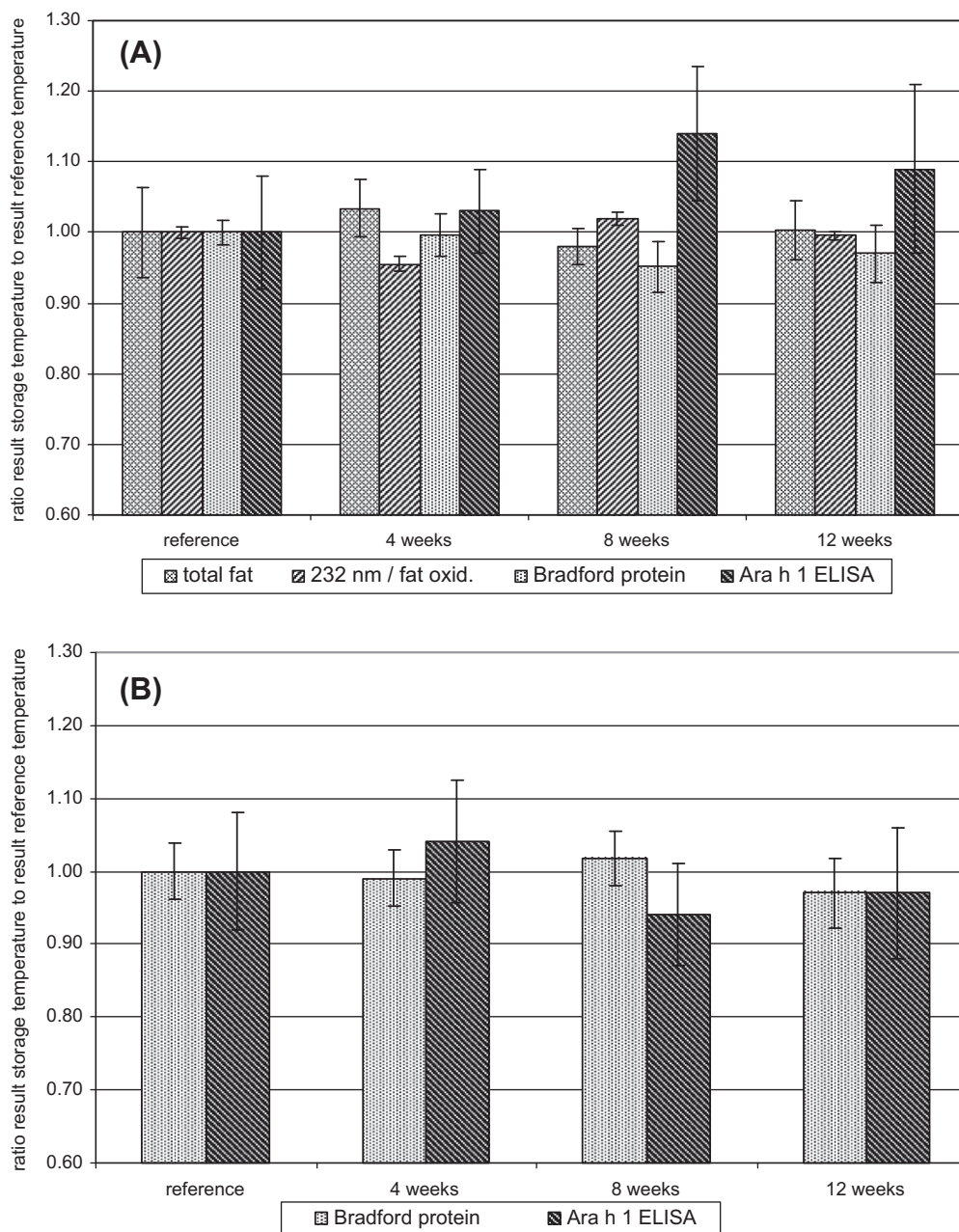


Fig. 2. Stability of (A) the 1–2 mm particle size fraction and (B) the de-fatted peanut powder at a temperature of +40 °C. Reference temperature was –20 °C. Relative values (i.e. obtained value for a given parameter and a given storage time divided by the value obtained for this parameter with the reference samples) are indicated. All values are mean values of three independent sub-samples.

cooling elements or even at room temperature without jeopardising the materials stability. Table 4 lists the figures calculated from

the performed regression analysis: relative slope per month, significance of obtained slopes, and the estimated uncertainty contribu-

Table 4

Results of the short-term stability study (3 months) using the total soluble protein assay (upper part of table) and the Ara h 1 ELISA (lower part of table).

Temperature	1–2 mm			0.5–1 mm			<0.5 mm			Powder
	4 °C	20 °C	40 °C	4 °C	20 °C	40 °C	4 °C	20 °C	40 °C	40 °C
Slope (%/month)	2.13	0.60	–1.28	–1.27	0.60	–0.01	1.76	–0.95	0.20	–0.18
Statistical significance (95% conf. interval)	No	No	No	No	No	No	No	No	No	No
u_{sts} (%/month) ^a	1.39	0.46	0.82	0.99	1.06	1.07	1.01	0.87	1.32	0.91
Slope (%/month)	2.61	1.15	3.62	0.70	–3.79	2.44	1.80	–2.03	–3.90	–2.47
Statistical significance (95% conf. interval)	No	No	No	No	No	No	No	No	No	No
u_{sts} (%/month) ^a	2.11	2.10	2.24	3.01	2.96	2.64	2.24	2.95	2.72	2.27

^a Uncertainty contribution of the short-term stability.

tions. Slopes were found to be indistinguishable from zero, and obtained uncertainty values are comparable among temperatures and among methods. Hence, no instability of the materials was detected at the tested storage conditions. Minor instabilities of certain protein analytes in the material which could possibly exist can only be detected by using highly specific, sensitive, and repeatable methods which need to be developed and validated (e.g. LC-MS method targeting a representative peptide of the protein analyte).

4. Concluding remarks and outlook

In the current work a set of analytical methods was applied to assess the properties of two types of peanut preparations, especially focussing on material integrity, homogeneity and stability. A thorough establishment of these parameters is a prerequisite towards the development of a respective QCM and later on a certified reference material. It was demonstrated that with the set of applied methods suitable judgements could be made about the material homogeneities and stabilities, but other properties could also be efficiently assessed, such as qualitative and quantitative composition of the protein profiles in the materials. It was shown that the produced materials exhibit suitable properties to be used as quality control materials. The development and application of methods with better repeatability, selectivity, and sensitivity would allow a further verification of these data, and might also allow producing a reference material with an unambiguously defined measurand that can be certified using validated methods. However, several key issues need first to be resolved (definition of a suitable measurand, availability and validation of suitable methods, calibrant issue, etc.).

Material preparations as described could be used for the following purposes: as a QCM in the laboratory (quality control chart using this material with a dedicated ELISA method); use of the materials as PT study materials to compare different peanut ELISAs with one another; and use of an extract prepared from such a material under defined conditions, which can then be used for spiking purposes or as calibration solution for quantitative methods ("pure peanut protein solution", to be quantified by e.g. Kjeldahl nitrogen method or amino acid analysis).

Possible next steps to proceed with could comprise (a) up-scaling and fine-tuning of the material preparation process, (b) extension of the study to different peanut varieties and/or differently processed peanuts such as raw/light roasted/dark roasted peanuts, (c) production of a mix of a peanut preparation with another food matrix, and (d) development, optimisation, and validation of appropriate methods (primarily LC-MS/MS on representative peanut protein peptides) which can be added to the toolbox of methods and can be used especially for homogeneity, stability, and – maybe later on – for a characterisation study.

Acknowledgements

The authors would like to thank Albert Oostra, Katharina Teipel, and Paul de Vos for assistance in the processing of the materials, and Stefan Harbeck for assistance in the preparation of extracts as well as for performing total protein and total fat measurements.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.foodchem.2010.06.018](https://doi.org/10.1016/j.foodchem.2010.06.018).

References

- Basha, S. M. M., & Cherry, J. P. (1976). Composition, solubility, and gel electrophoretic properties of proteins isolated from Florunner (*Arachis hypogaea*) peanut seeds. *Journal of Agricultural and Food Chemistry*, 24(2), 359–365.
- Besler, M. (2001). Determination of allergens in foods. *Trends in Analytical Chemistry*, 20(11), 662–672.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72(1–2), 248–254.
- Careri, M., Costa, A., Elviri, L., Lagos, J. B., Mangia, A., Terenghi, M., et al. (2007). Use of specific peptide biomarkers for quantitative confirmation of hidden allergenic peanut proteins Ara h 2 and Ara h 3/4 for food control by liquid chromatography–tandem mass spectrometry. *Analytical and Bioanalytical Chemistry*, 389(6), 1901–1907.
- Directive 2000/13/EC of the European Parliament and Council of 20 March 2000 on the approximation of the laws of the Member States relating to the labelling, presentation and advertising of foodstuffs. Official Journal of the European Union, L109, 29–42.
- Directive 2003/89/EC of the European Parliament and of the Council of 10 November 2003 amending Directive 2000/13/EC as regards indication of the ingredients present in foodstuffs. Official Journal of the European Union, L308, 15–18.
- Hefle, S., Nordlee, J. A., & Taylor, S. L. (1996). Allergenic foods. *Critical Reviews in Food Science and Nutrition*, 36(Suppl.), S69–S89.
- Holzhauser, T., & Vieths, S. (1999). Indirect competitive ELISA for determination of traces of peanut (*Arachis hypogaea* L.) protein in complex food matrices. *Journal of Agricultural and Food Chemistry*, 47(2), 603–611.
- Hourihane, J. O'B., Kilburn, S. A., Nordlee, J. A., Hefle, S., Warner, J. O., & Taylor, S. L. (1997). An evaluation of the sensitivity of subjects with peanut allergy to very low doses of peanut protein: A randomized, double-blind, placebo-controlled food challenge study. *Journal of Allergy and Clinical Immunology*, 100(5), 596–600.
- Kiening, M., Niessner, R., Drs, E., Baumgartner, S., Kraska, R., Bremer, M., et al. (2005). Sandwich immunoassays for the determination of peanut and hazelnut traces in foods. *Journal of Agricultural and Food Chemistry*, 53(9), 3321–3327.
- Koppelman, S. J., Vlooswijk, R. A. A., Knippels, L. M. J., Hessing, M., Knol, E. F., van Reijssen, F. C., et al. (2001). Quantification of major peanut allergen Ara h 1 and Ara h 2 in the peanut varieties Runner, Spanish, Virginia, and Valencia, bred in different parts of the world. *Allergy*, 56(2), 132–137.
- Lamberty, A., Schimmel, H., & Pauwels, J. (1998). The study of the stability of reference materials by isochronous measurements. *Fresenius Journal of Analytical Chemistry*, 360(3–4), 359–361.
- Linsinger, T., Pauwels, J., Lamberty, A., Schimmel, H., van der Veen, A. M. H., & Siekmann, L. (2001). Estimating the uncertainty of stability of matrix CRMs. *Fresenius Journal of Analytical Chemistry*, 370(2–3), 183–188.
- Maier, I., Morgan, M. R. A., Lindner, W., & Pittner, F. (2008). Optical resonance-enhanced absorption-based near-field immunochip biosensor for allergen detection. *Analytical Chemistry*, 80(8), 2694–2703.
- Moutete, H. F., Olszewski, A., Gastin, I., Namour, F., Moneret-Vautrin, D. A., & Gueant, J. L. (1995). Purification of allergenic proteins from peanut for preparative of the reactive solid phase of a specific IgE radioimmunoassay. *Journal of Chromatography B: Biomedical Sciences and Applications*, 664(1), 211–217.
- Nogueira, M. C. L., McDonald, R., Westphal, C., Maleki, S. J., & Yeung, J. M. (2004). Can commercial peanut assay kits detect peanut allergens? *Journal of AOAC International*, 87(6), 1480–1484.
- Pomes, A., Helm, R., Bannon, G., Burks, A., Tsay, A., & Chapman, M. (2003). Monitoring peanut allergen in food products by measuring Ara h 1. *Journal of Allergy and Clinical Immunology*, 111(3), 641–645.
- Poms, R. E., Capelletti, C., & Anklam, E. (2004a). Effect of roasting history and buffer composition on peanut protein extraction efficiency. *Molecular Nutrition and Food Research*, 48(6), 459–464.
- Poms, R. E., Klein, C. L., & Anklam, E. (2004b). Methods for allergen analysis in food: a review. *Food Additives and Contaminants*, 21(1), 1–31.
- Poms, R. E., Agazzi, M. E., Bau, A., Brohee, M., Capelletti, C., Nørgaard, J., et al. (2005). Inter-laboratory validation study of five commercial ELISA test kits for the determination of peanut proteins in biscuits and dark chocolate. *Food Additives and Contaminants*, 22(2), 104–112.
- Schmitt, D. A., Cheng, H., Maleki, S. J., & Burks, A. W. (2004). Competitive inhibition ELISA for quantification of Ara h 1 and Ara h 2, the major allergens of peanuts. *Journal of AOAC International*, 87(6), 1492–1497.
- Schubert-Ullrich, P., Rudolf, J., Ansari, P., Galler, B., Führer, M., Molinelli, A., et al. (2009). Commercialized rapid immunoanalytical tests for determination of allergenic food proteins: An overview. *Analytical and Bioanalytical Chemistry*, 395(1), 69–81.
- Shefcheck, K. J., & Musser, S. M. (2004). Confirmation of the allergenic peanut protein, Ara h 1, in a model food matrix using liquid chromatography/tandem mass spectrometry (LC/MS/MS). *Journal of Agricultural and Food Chemistry*, 52(10), 2785–2790.
- Stephan, O., & Vieths, S. (2004). Development of a real-time PCR and a sandwich ELISA for detection of potentially allergenic trace amounts of peanut (*Arachis hypogaea*) in processed foods. *Journal of Agricultural and Food Chemistry*, 52(12), 3754–3760.

- Stephan, O., Weisz, N., Vieths, S., Weiser, T., Rabe, B., & Vatterott, W. (2004). Protein quantification, sandwich ELISA, and real-time PCR used to monitor industrial cleaning procedures for contamination with peanut and celery allergens. *Journal of AOAC International*, 87(6), 1448–1457.
- Trucksess, M. W., Brewer, V. A., Williams, K. M., Westphal, C. D., & Heers, J. T. (2004). Preparation of peanut butter suspension for determination of peanut using enzyme-linked immunoassay kits. *Journal of AOAC International*, 87(2), 424–428.
- Westphal, C., Pereira, M. R., Raybourne, R. B., & Williams, K. M. (2004). Evaluation of extraction buffers using the current approach of detecting multiple allergenic and nonallergenic proteins in food. *Journal of AOAC International*, 87(6), 1458–1465.
- van der Veen, A. M. H., Linsinger, T. P. J., & Pauwels, J. (2001). Uncertainty calculations in the certification of reference materials. 2. Homogeneity study. *Accreditation and Quality Assurance*, 6(1), 26–30.
- Vieira, T. M. F. S., & Regitano-d'Arce, M. A. B. (1999). Ultraviolet spectrophotometric evaluation of corn oil oxidative stability during microwave heating and oven test. *Journal of Agricultural and Food Chemistry*, 47(6), 2203–2206.
- Yeung, J. M., & Collins, P. G. (1996). Enzyme immunoassay for determination of peanut proteins in food products. *Journal of AOAC International*, 79(6), 1411–1416.