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Effects of different extraction buffers on peanut protein detectability and lateral flow device (LFD) performance

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The accidental uptake of peanuts can cause severe health reactions in allergic individuals. Reliable determination of traces of peanuts in food products is required to support correct labelling and therefore minimise consumers' risk. The immunoanalytical detectability of potentially allergenic peanut proteins is dependent on previous heat treatment, the extraction capacity of the applied buffer and the specificity of the antibody. In this study a lateral flow device (LFD) for the detection of peanut protein was developed and the capacity of 30 different buffers to extract proteins from mildly and strongly roasted peanut samples as well as their influence on the test strip performance were investigated. Most of the tested buffers showed good extraction capacity for putative Ara h 1 from mildly roasted peanuts. Protein extraction from dark-roasted samples required denaturing additives, which were proven to be incompatible with LFD performance. High-pH buffers increased the protein yield but inhibited signal generation on the test strip. Overall, the best results were achieved using neutral phosphate buffers but equal detectability of differently altered proteins due to food processing cannot be assured yet for immunoanalytical methods.

Keywords: peanuts; allergens; extraction; immunoassays

Introduction

Food allergies are an increasing threat to human health. About 2–3% of adults and 3–6% of children suffer from adverse reactions to non-toxic food components (Pele et al. 2007; Wen et al. 2007; Kirsch et al. 2009). In most cases of food allergy proteins are responsible for IgE activation inducing more or less severe physical reactions ranging from mild urticaria and gastrointestinal disorders to anaphylactic shocks (Wen et al. 2007; Scaravelli et al. 2009). There is no NOEL (no observed effect level) or acceptable daily intake (ADI) for allergenic proteins because the amount of allergen triggering an allergic reaction is individually different. Peanut allergy is known to cause 10–47% of food-induced anaphylactic shocks and more than 50% of food allergy fatalities (Poms et al. 2004; Scaravelli et al. 2009). Highly allergic persons might react to milli- or even microgram amounts of peanut (Holzhauser and Vieths 1999; Trucksess et al. 2004). The incidence of peanut allergy does vary between the Eastern and Western worlds due to different traditions of food preparation and the allergic population. While boiling or cooking of peanuts, as it is mainly done in Asian countries, does not enhance allergenicity (Scaravelli et al. 2009), the process of

roasting, as peanuts are usually treated for cookies, snacks or chocolate production in Europe and America, seems to increase the epitope availability for IgE binding (Maleki et al. 2000; Poms et al. 2004). Research done by the Chinese Agricultural University revealed controversial findings showing that processing does not have an influence on the allergenicity of peanut proteins, but this might be due to a different genetic background and allergic population (Cong et al. 2007). Since there is currently no cure for allergic consumers the only safe way of food consumption is to avoid allergen-containing products completely. This requires exact and reliable labelling of food products. Within the European Union (European Commission 2007) there are now up to 14 food allergens that have to be declared on the labels of food regardless of their quantity if used as ingredient. In order to avoid severe health reactions due to unintentional contamination and to meet general product liability legislation, manufacturers often use labels such as 'may contain' or 'present in the environment' (Pele et al. 2007; Kirsch et al. 2009). Reliable detection methods for the determination of allergen traces in food matrices are required (Wen et al. 2007). Methods usually applied in the food industry are immunoanalytical tools such as

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ELISA and sometimes lateral flow devices (LFD). In the case of the detection of peanut residues these antibody-based methods can be directed against specific allergenic proteins, such as Ara h 1, Ara h 2, etc., a marker protein or a total protein extract, depending on the immunogen used for antibody production. Typical assays use polyclonal antibodies, but also monoclonal antibodies have been tested for assay development (Pomés et al. 2003). The standard or calibrators used in test kits should match the protein(s) used for immunisation. Furthermore, the applied extraction buffer plays a crucial role in the detection of allergenic contaminants (Freitas et al. 2000) as only successfully extracted proteins can be detected by the antibody. The extractability of peanut proteins with regard to processing history during food production is of great concern within the area of allergenic research (Nogueira et al. 2004; Poms et al. 2004; Westphal et al. 2004; Pomés et al. 2006; Chassaigne et al. 2007; Scaravelli et al. 2009). Different peanut varieties undergoing different heat treatments including boiling, oil roasting and dry roasting at different temperatures and for different times were tested for protein extractability. Extraction buffers from commercially available ELISA kits were applied (Scaravelli et al. 2009) as well as self-prepared buffers (Poms et al. 2004; Westphal et al. 2004) and one- and two-step (Chassaigne et al. 2007) extraction procedures. In most of the studies commercially available ELISA kits were used to determine the detectable amount of peanut extracted either from pure peanuts or from food products such as cookies or chocolate. Pomés et al. (2003, 2006) developed an Ara h 1-specific ELISA using monoclonal antibodies; and Pele et al. (2007) applied a commercially available LFD additionally to an ELISA. A comparison study of the handling, specificity and sensitivity of commercially available LFD was performed by Röder et al. (2009). The difficulty with commercially available test kits, when applied for scientific purposes, is that the complete information is not provided by the manufacturer (Holzhauser and Vieths 1999). For complete and reliable test results information about the antibody, the calibrators and the extraction buffer is essential, but because of intellectual property rights this information is usually not fully published.

The aim of this work was the comparison and optimisation of extraction buffers and running conditions for the detection of peanut protein using immunochromatographic strip tests. This included the development of LFDs for this application using polyclonal rabbit antibodies developed against total protein extract. Investigation of the effects of different buffers on the extractability of differently treated peanut sources as well as their influence on general test performance was carried out. As LFDs are in contrast to ELISA homogeneous assays, meaning that

there are no washing steps included in the test procedure, the impact of the buffers used can be expected to be higher than in a heterogeneous ELISA. We tried to identify whether there is one buffer with similar extraction capacities for light- and dark-roasted peanuts and which is furthermore compatible with LFD requirements.

Materials and methods

Peanut samples

Five different peanut samples were used for protein extraction and characterisation. In-shell-roasted peanut kernels (fat content of 50%) were purchased at local markets. Shells were removed and the kernels peeled and ground using a mixer (Osterizer, Sunbeam Products, Inc., Niles, IL, USA). Part of this peanut flour was defatted with pre-cooled acetone to a fat content of 6%. Light- and dark-roasted peanut flour with 12% fat content and light-roasted peanut flour with 28% fat content were purchased from Golden Peanut Company (Alpharetta, GA, USA). Total fat content was verified for all samples using the Soxhlet method (data not shown).

Food samples

Three different food samples were purchased at a local market: ice cream cones, ladyfingers and amaretti. None of the samples contained peanut and 'may contain' was not mentioned on the label. These samples were used to evaluate buffer effects under 'natural' conditions.

Extraction buffers

Buffers were prepared at 0.1 M from their corresponding sodium salts (Table 1). All salts and substances required for buffer preparation were purchased from

Table 1. Buffers and additives applied for peanut extraction.

Buffers
A – Phosphate pH 7.5
B – Carbonate pH 9.6
C – Tris/HCl pH 8.2
D – Citrate pH 5.0
E – Borate pH 8.0
F – Phosphate/citrate pH 7.0
Additives
1 – None
2 – 1 M NaCl
3 – 1% Tween
4 – 1% SDS
5 – 6 M urea

Sigma (Vienna, Austria) or Merck GesmbH (Vienna, Austria).

Sample preparation

Defatting

A total of 20–40 g ground peanuts were mixed with the ten-fold volume of pre-cooled acetone and mixed for 2×5 min with an analytical mill (Ultra Turrax T25, IKA-Labortechnik, Stauffen, Germany). The mixture was then vacuum-filtered using $0.6 \mu\text{m}$ glass microfiber filters MN 85/70 BF (Roth, Graz, Austria). The residue was washed twice with cooled acetone and left overnight for complete evaporation of acetone at room temperature under the fume hood.

Protein extraction

Depending on the fat content, between 0.54 and 1.00 g peanut flour were extracted with 10 ml buffer enabling a direct comparison of protein yield in the extract corresponding to an initial weight of 0.5 g fat-free peanut for each sample. Extraction was performed for 15 min at 60°C using a water bath, an extraction procedure which is commonly used in some commercially available test kits for the detection of peanut residues. Afterwards extracts were centrifuged at 4°C and 10 000g for 15 min, the supernatants were filtered through syringe filters ($0.45 \mu\text{m}$ cellulose acetate; Sartorius Stedim, Vienna, Austria) and protein concentration was determined using a BCA protein quantification kit (Pierce-Fisher Scientific, Vienna, Austria) using the included bovine serum albumin standard for calibration following the manufacturer's instructions. Extracts were stored until further use at -20°C . Food samples were extracted 1 g in 10 ml buffer and treated the same way as the pure peanut samples. Protein concentration was not determined.

Protein characterisation with LDS-PAGE

Lithium dodecyl sulfate-polyacrylamide gel electrophoresis was performed using the equipment and following the manufacturer's instructions from Invitrogen (Lofer, Austria). The protein amount was calculated based on the BCA measurements. About 5–6 μg protein mixed with 2.5 μl sample buffer per lane were loaded onto the gel (12% Bis-Tris, 1 mm $\times$ 15 well). Samples were heated before electrophoresis to 70°C for 10 min under non-reducing conditions. A total of 50 mM MES/Tris buffer pH 7.3 containing 0.1% (w/v) and 1 mM EDTA was used as a running buffer. Running conditions were set at 200 V for 75 min. The gel was stained with Coomassie Blue stain for about 1 h and washed for several hours or overnight.

Immunological detection using in-house produced LFD

Antibody–latex conjugation was performed following a slightly modified protocol of Brüning et al. (1999). A total of 100 μl latex microspheres (4%) were diluted in 9.9 ml coupling buffer (0.1 M borate buffer, pH 8.5), mixed thoroughly and centrifuged at 10 000g for 5 min. The supernatant was discarded and the pellet redissolved in 5 ml coupling buffer. A total of 5 ml analyte-specific antibody solution (0.05 mg ml^{-1} in coupling buffer) were added and the conjugation batch was incubated for 90 min at room temperature using an overhead shaker. Then 1 ml BSA, 1% (w/v) was added and the mixture incubated for another 45 min. After centrifugation (10 000g, 5 min) supernatant was again discarded and the pellet was redissolved in 10 ml borate buffer, 0.01 M, pH 8.5 and stored at 4°C until further use. Test strips were produced as described previously (Rudolf et al. 2009). Briefly, analyte-specific antibody (1 mg ml^{-1} , in-house produced rabbit anti-total peanut protein antibody) and species-specific antibody (0.5 mg ml^{-1} , goat-anti rabbit, Sigma R5506) were sprayed onto the nitrocellulose membrane, dried and assembled on an adhesive backing card, before strips of 4.5 mm width were cut. Equipment from BioDot (BioDot ZX1000 dispensing platform with Frontline contact tips and BioDot CM4000 guillotine cutter, Chichester, UK) was used. The protocol for the test strip procedure was varied slightly compared with the procedure described in Rudolf et al. (2009). Latex-labelled antibodies were diluted 40:60 in 0.1 M borate buffer containing 10% EtOH (v/v) and 10% Tween 20 (w/v). For the creation of a standard curve, defatted peanut flour extracted and diluted with 0.1 M PBS was used. The different peanut extracts were diluted in various extraction buffers to a calculated protein concentration within the measurement range (0.05 – $0.25 \mu\text{g ml}^{-1}$). A total of 50 μl of standard/extract were mixed with an equal volume of antibody–latex conjugate in running buffer. One strip was dipped into the reaction mixture and incubated for 3 min. Strips were measured using a scattered light reader (portable bench-top-sized XReader reflectance photometer; Romer Labs Singapore, Singapore) and the results were calculated from the signal intensity expressed in absorption units.

Results

Effect of extraction buffer on the measurable protein yield

First extraction experiments demonstrated clearly that proteins from in-shell-roasted peanuts could be extracted easily compared with the other peanut flour samples. No differences were observed between extracts of defatted and non-defatted peanuts (data

not shown), a fact that has also been observed by Zeleny and Schimmel (2010). Proteins from dark-roasted peanuts showed very poor extractability. For this reason these two samples (in-shell-roasted and defatted peanuts – I, and dark-roasted peanuts – II) were chosen for comparison of extraction buffers (Table 1). Phosphate and Tris/HCl buffers were selected since they are commonly used for extraction of samples to be analysed by immunoassays. Carbonate and borate buffers are used quite often as coating or coupling buffers in ELISA and LFD development and were applied to investigate the effect of higher pHs. Citrate buffer was chosen as a low-pH buffer in order to cover a wider pH range; finally citrate/phosphate buffer was the only buffer containing not only one, but two salts. Sodium chloride (NaCl) and Tween 20 are common additives for buffers, while urea and SDS are usually avoided in combination with immunoassays due to their denaturing effect. But they were nevertheless chosen to determine their extraction capacity for peanut proteins. Generally it can be stated that differences in between the extracts of dark-roasted peanuts were not as distinct as for in-shell-roasted samples (Figure 1a). Citrate buffer showed poor extraction properties for samples I and II, resulting in only 2 mg protein ml⁻¹, but it was the only buffer that obtained a higher extraction yield for dark-roasted peanuts than for in-shell-roasted ones. The phosphate-containing, neutral-pH buffers also did not reveal significant differences between the two samples, but they resulted in higher protein yield compared with the citrate buffer extracts (3–4 mg ml⁻¹). Much higher protein concentrations, but only for the in-shell-roasted sample, were obtained with high-pH buffers, especially carbonate buffer (8.6 mg ml⁻¹), which has already been described by Westphal et al. (2004), and Tris/HCl (8.2 mg ml⁻¹). The pH only slightly affected the protein yield of the dark-roasted sample and so did the addition of NaCl or Tween 20. Here the protein concentration did not exceed 3–4 mg ml⁻¹. By adding SDS or urea to the extraction buffer, the protein concentration was more than doubled for sample II. Also for sample I the denaturing agents increased the protein yield. Protein concentrations between 7.4 and 12.4 mg ml⁻¹ could be achieved using denaturing buffer additives (Table 2). For the in-shell-roasted sample the addition of NaCl to the low- and neutral-pH buffers increased the extractability of peanut proteins, while it was reduced in combination with high-pH buffers, especially carbonate and borate buffer (Figure 1b). As saline buffers usually improve the extractability of globulins, it can be assumed that NaCl-containing phosphate buffers increased Ara h 1 yield, but there is no explanation why this effect was somehow reversed for high-pH buffers. The addition of Tween 20 to the extraction buffer increased protein extractability slightly for borate and carbonate buffer

and did not affect the extraction capacity of the remaining buffers significantly.

Characterisation of protein patterns using LDS-PAGE

For the comparison of the protein profiles 5 µg protein of each extract were loaded onto a gel. Remarkable bands – at least in the sample I extracts (Figure 2a) – were detected at approximately 60, 35, 25 and below 14 kDa. The strongest protein band was at 60 kDa, which corresponds to the molecular weight of Ara h 1. The bands at 35 and 25 kDa can be explained by protein breakdown during roasting or sample preparation. Bands around 14 kDa were only slightly visible in buffers without additives and in those containing SDS more protein bands could be detected compared with the other buffer versions. Corresponding to the results of protein determination via BCA, bands in phosphate and phosphate/citrate buffer extracts were stronger when NaCl was added to the buffer. Non-denaturing citrate buffer extracts did not show any bands on the gel but the addition of SDS and urea overcame the extraction insufficiency of this low-pH buffer. Generally, the addition of SDS increased the extractability of proteins from peanut, more than did the addition of urea. This effect was especially visible for the extracts from dark-roasted peanuts (Figure 2b). The obtained protein patterns indicated a good extraction capacity of the applied buffers, except citrate buffer, for Ara h 1 from mildly heat-treated peanuts. This was not the case for Ara h 2, as no bands could be detected in the range of 17–19 kDa. As already mentioned by Zeleny and Schimmel (2010), overall protein profiles did not vary much between the different buffers. Although intensities of the bands were different, the general patterns were similar.

Immunochemical detection of peanut proteins using a sandwich-based LFD

Polyclonal antibodies were produced in rabbits and purified by affinity chromatography using Protein G, as previously published (Rudolf et al. 2009). Antibodies were tested for allergen specificity and were found to detect Ara h 1 as well as Ara h 2 and 6 (data not published). For immunisations in-shell-roasted and defatted peanuts were extracted with 0.1 M PBS pH 7.5, containing 0.18 M NaCl, as this was established as the standard procedure for immunogen preparation for antibody production. This peanut extract was also used to generate a standard curve against which results of extraction buffer comparison were evaluated. Linear measurement was achieved between 0.05 and 0.25 µg total peanut protein

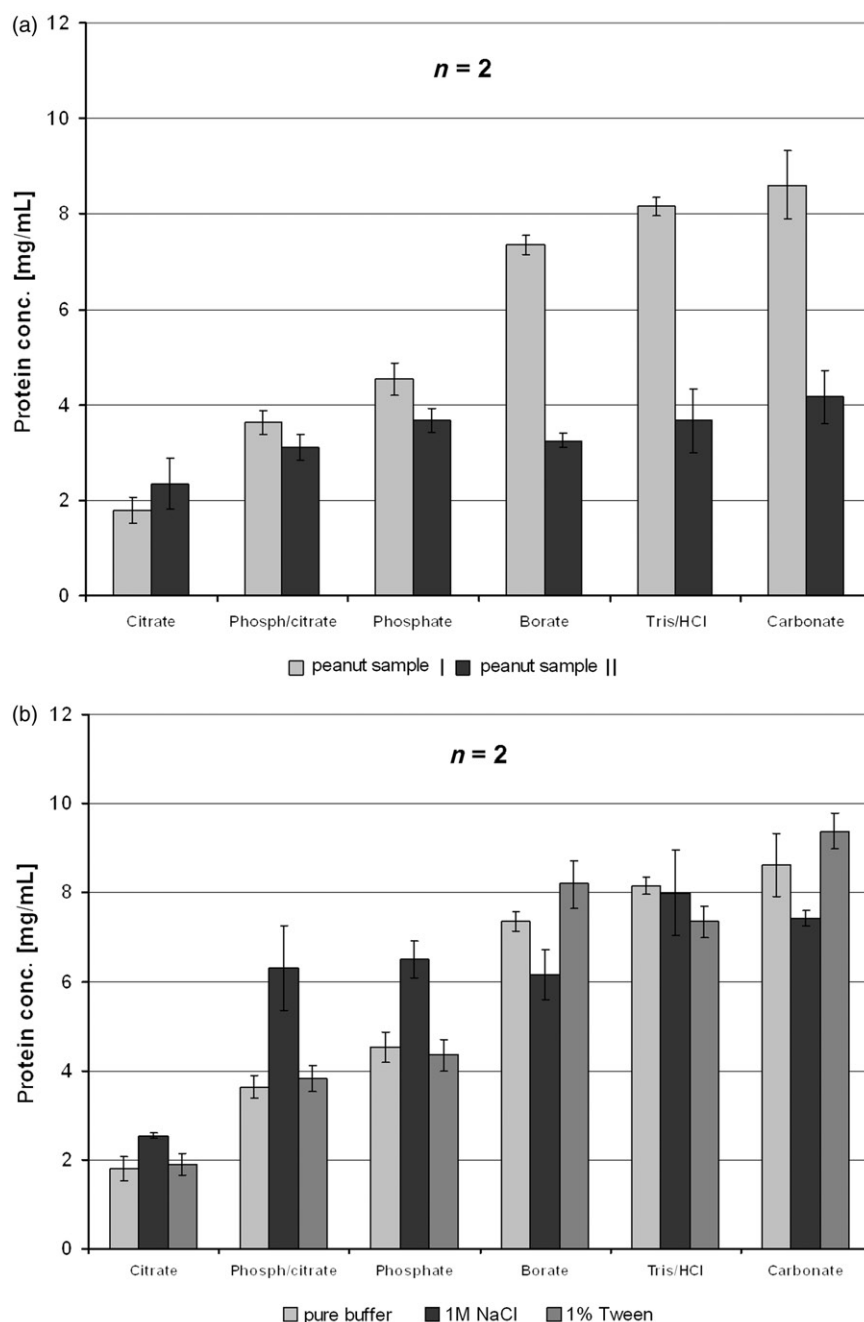


Figure 1. Extraction yield expressed as protein concentration of peanut samples I (in-shell-roasted peanuts, 6% fat) and II (dark-roasted peanut flour, 12% fat) extracted with different buffers (a) and the protein concentration of peanut sample I extracted with differently modified extraction buffers (b). The extraction of peanut flour samples was performed twice ($n = 2$); the protein amount was quantified in triplicate.

ml^{-1} . The results were evaluated according to the linear regression curve:

$$y = 928.56x + 29.4$$

with a regression coefficient $R^2 = 0.9872$ ($n = 4$).

For evaluation of the detectability of all peanut extracts, samples I and II were each extracted with 30 different buffers, diluted with 0.1 M PBS to a

protein concentration of $0.2 \mu\text{g ml}^{-1}$ and measured as described in the Materials and methods section. Results expressed in signal intensity produced by a scattered light reader were calculated to $\mu\text{g protein ml}^{-1}$ using the above equation resulting from the standard curve. Finally the actual value was divided through the theoretical one to gain an independent comparison expressed in percentage terms. Although all samples were diluted to the same protein

Table 2. Protein concentration of peanut samples I and II extracted with 30 different buffers determined with BCA-protein quantification kit.

	Citrate pH5.0	Phosph/Citrate pH 7.0	Phosphate pH 7.5	Borate pH8.0	Tris/HCl pH8.2	Carbonate pH 9.6
Sample I						
Pure	1.8+/-0.3	3.6+/-0.3	4.5+/-0.3	7.4+/-0.2	8.2+/-0.2	8.6+/-0.7
1 M NaCl	2.5+/-0.1	6.3+/-1.0	6.5+/-0.4	6.2+/-0.6	8.0+/-1.0	7.4+/-0.2
1% Tween	1.9+/-0.3	3.8+/-0.3	4.4+/-0.4	8.2+/-0.5	7.4+/-0.4	9.4+/-0.4
1% SDS	8.5+/-0.3	7.0+/-1.0	8.3+/-0.4	10.8+/-1.2	9.4+/-0.2	9.4+/-0.5
6 M urea	10.8+/-1.1	10.9+/-1.0	11.2+/-0.6	10.0+/-0.8	12.0+/-0.9	12.3+/-0.2
Sample II						
pure	2.4+/-0.5	3.1+/-0.3	3.7+/-0.3	3.3+/-0.2	3.7+/-0.7	4.2+/-0.6
1 M NaCl	2.4+/-0.1	2.7+/-0.1	2.8+/-0.4	3.0+/-0.4	2.8+/-0.5	3.1+/-0.8
1% Tween	2.8+/-0.2	3.7+/-0.2	3.6+/-0.4	2.9+/-0.4	3.8+/-0.8	4.0+/-1.1
1% SDS	4.9+/-0.6	8.2+/-0.3	8.3+/-0.3	10.6+/-1.4	10.5+/-1.6	10.0+/-1.2
6 M urea	7.4+/-0.6	9.9+/-1.2	9.1+/-1.1	9.8+/-0.2	9.2+/-0.7	10.2+/-0.1

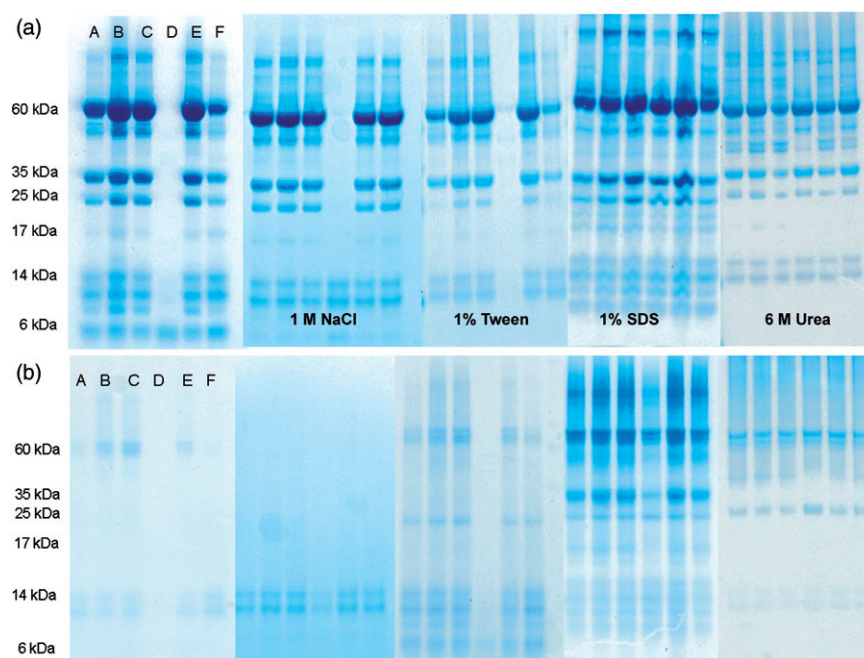


Figure 2. Protein patterns of differently extracted peanut samples I (a) and II (b). A=phosphate, B=carbonate, C=Tris/HCl, D=citrate, E=borate, and F=phosphate/citrate buffer. Buffer additives indicated in (a) refer also to (b). The order of the application of samples was kept constant on each gel.

concentration before measurement, the developed LFD did not detect the same peanut protein concentrations. None of the dark roast extracts could be detected with the in-house developed LFD, although protein concentrations up to 2.5 and 5.0 $\mu\text{g ml}^{-1}$ were applied. This is, on the one hand, likely because rabbit antibodies were raised against proteins from mildly roasted peanuts and therefore were not able to detect peanut proteins that have been altered by heat treatment. On the other hand, it can be explained by the incapability of the applied extraction buffers to extract those specific proteins such as putative Aha h 1 from dark-roasted peanuts which this antibody would be able to detect. The LFD results of the in-shell-roasted

peanut sample were more or less in agreement with the results of the protein quantification and the protein patterns obtained on the LDS-PAGE gel. High signals were reached for samples extracted with carbonate and borate buffer as well as with Tris/HCl. Phosphate buffer extracts showed clearly lower signals and peanut extracted with phosphate/citrate buffer gave only reasonable results in combination with 1 M NaCl added to the extraction buffer. Samples extracted with citrate buffer did not provide any signals at all. Also, no signals could be produced with extracts from denaturing extraction buffers. SDS and urea concentrations during test performance were low enough (approximately 1:50 000 dilution, depending on the

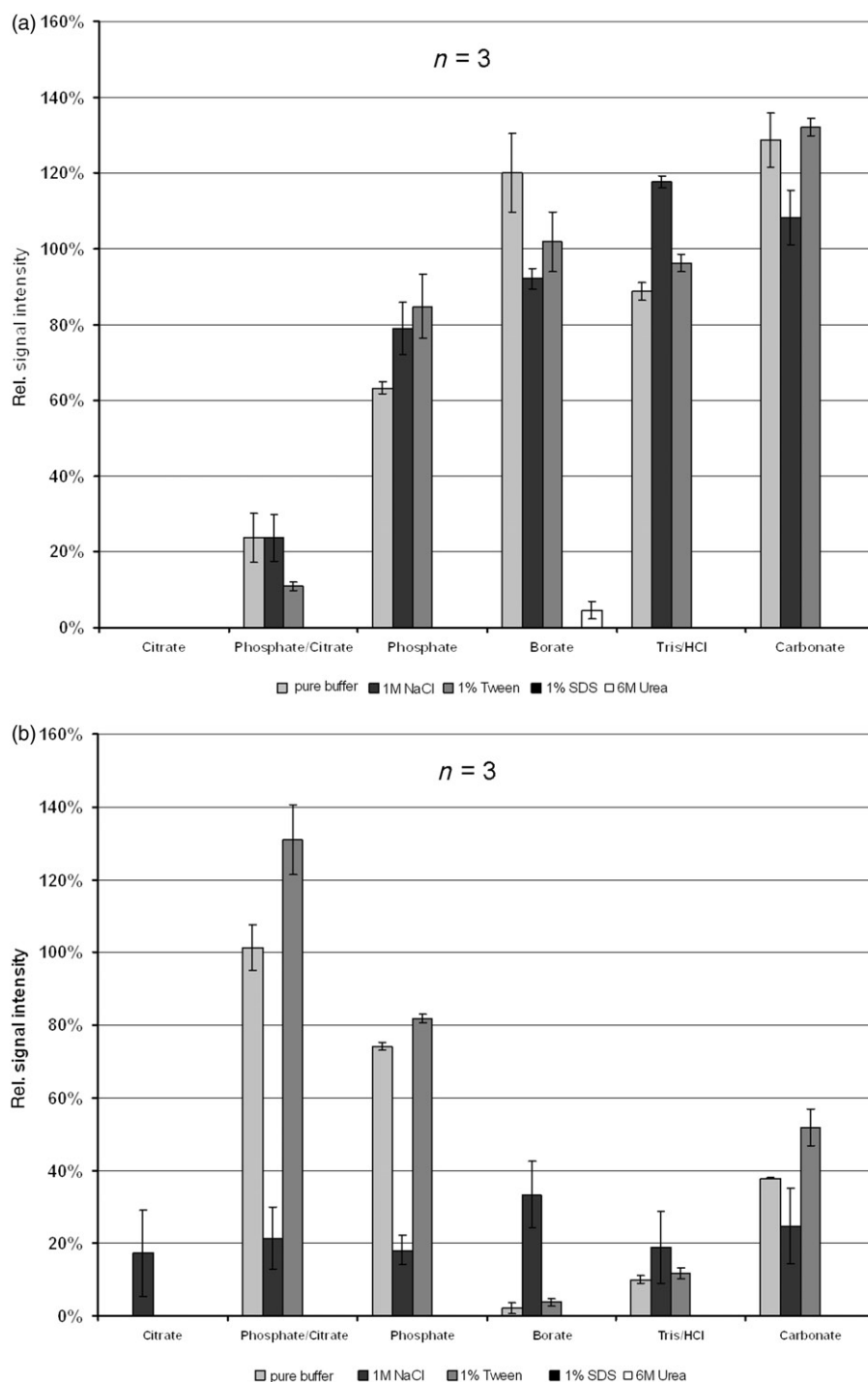


Figure 3. Relative signal intensity of different peanut extracts diluted with 0.1 M PBS (a) and peanut standard extract diluted with different extraction buffers (b). Peanut standard extract diluted with 0.1 M PBS was used as a benchmark (100%).

original protein concentration) to eliminate inhibiting effects on the antibody. Therefore, it has to be concluded that the denaturing buffer additives are indeed capable of extracting higher protein amounts than their non-denaturing pendants, but also they seem to alter irreversibly the epitopes responsible for antigen-antibody recognition (Figure 3a). As all extracts were diluted with the same buffer and dilutions were supposed to be high enough to avoid influences of the

original extraction buffers, this first comparison study was designed to identify differences in the extraction capacity of the various buffers. High-pH buffers can extract more protein than neutral-pH buffers, and citrate buffer pH 5 is not suitable for peanut protein extraction at all. In general, the extractability of heat-processed dark-roasted samples with the applied buffers was lower than for the light-processed in-shell-roasted samples.

Effect of extraction buffer on the strip test performance

As this study was carried out using pure peanut flour and not samples containing only peanut traces, extracts had to be diluted before measurement in order to fit into the linear measurement range of the antibody. In the case of real sample extraction for the detection of allergenic contamination peanut concentrations can be very low. If no dilution is necessary, extracts can be directly measured and lower peanut concentration can be detected. Therefore, the used extraction buffer should not have a negative effect in LFD measurements. Hence, the applied extraction buffers were also tested for their direct effect on the LFD performance. The peanut standard, which was used for immunisation as well as for generation of the standard curve, diluted with 0.1 M PBS, was now diluted with each of the 30 extraction buffers to a peanut protein concentration of $0.2 \mu\text{g ml}^{-1}$ and tested for signal generation in LFD. Also, general test performance and background intensity were monitored. Results were evaluated using the above explained standard equation and expressed as percentage 'recovery', comparing the calculated with the measured protein concentration. As presented in Figure 3b, most extraction buffers showed inhibiting effects on LFD performance compared with 0.1 M PBS. Buffers that showed good extraction capacities, such as carbonate, Tris/HCl, and borate buffer did not show satisfying results during assay performance. Carbonate buffer containing Tween inhibited partially (51.9%) and borate buffer inhibited totally (2.0%) the signal generation on the test strip. The addition of NaCl to phosphate-containing buffers, which had a positive effect on protein extraction, reduced the signal compared with the 0.1 M PBS diluted peanut extract to approximately 20%. Proteins of the globulin family such as Ara h 1 were better extracted in saline solutions, but a high NaCl concentration as well as generally higher molarity buffers (>0.1 M PBS, data not shown) seemed to interfere with antibody-antigen binding due to ionic strengths. Citrate buffer as well as all denaturing agent-containing buffer versions turned out to be totally incompatible with LFD requirements. The low pH of the citrate buffer caused destabilisation of the antibody-latex conjugate, therefore no migration on the strip test could be monitored. In this case the addition of NaCl showed a partially protective effect, resulting in – at least – a weak development of test and control line. SDS and urea, as expected, caused denaturation of the antibodies, i.e. the detection antibody coupled to latex as well as the capture-and-control antibody immobilised on the strip test. Antibody-antigen binding was hence inhibited, which resulted in neither test nor control line development. The only

buffers delivering reasonable results were phosphate and phosphate/citrate buffer either unaltered or containing Tween 20. Generally, the phosphate/citrate buffer obtained higher signals than the phosphate buffer 'only'.

Total buffer performance

In the previous experiments buffers were checked separately for extraction capacity and LFD compatibility. For completeness of the study and determination of the total suitability of buffers for the detection of peanut proteins in an LFD format, extracts were diluted with the corresponding buffer and measured as stated above (Figure 4). Since citrate buffer and all buffers containing either SDS or urea did not show satisfying results for protein extraction and LFD compatibility, these buffers were excluded from this last comparison. Buffers that gave good results in the extraction buffer comparison or LFD compatibility could not compensate for an insufficient performance in the corresponding experiment. Carbonate and borate buffer, which showed good extraction capacities, but were not compatible with LFD requirements, also gave weak signals when the same buffer was used for extraction and dilution. A similar effect was monitored for buffers that only insufficiently extracted proteins from peanuts, e.g. Tween 20 containing phosphate/citrate buffer, but strongly enhanced the signal of the peanut protein standard when diluted with the respective buffer. Finally, the best results were obtained with phosphate buffer without additives and with 1% Tween 20. The latter showed the same suitability as the originally used 0.1 M PBS.

Matrix effects

The purchased food samples were tested with six different extraction buffers: phosphate and phosphate/citrate buffer without additives and with 1% Tween 20, carbonate buffer with 1% Tween 20 and 0.1 M PBS. Those were the buffers that achieved overall the best results (Figure 4). A total of 1 g of grinded sample was extracted in 10 ml buffer following the procedure described above. These blank extracts were not diluted any further and were all tested negatively according to the corresponding buffer blanks. For ice cream cones strong matrix effects were observed with all buffers except phosphate/citrate and carbonate buffer containing 1% Tween 20. The combination of the food matrix of ice cream cones and those four buffers inhibited a continuous migration along the membrane – most of the conjugate-sample mixture did not reach the wick pad of the test strip. For the other two samples no background affecting the buffer-matrix interaction was observed. Although no positive

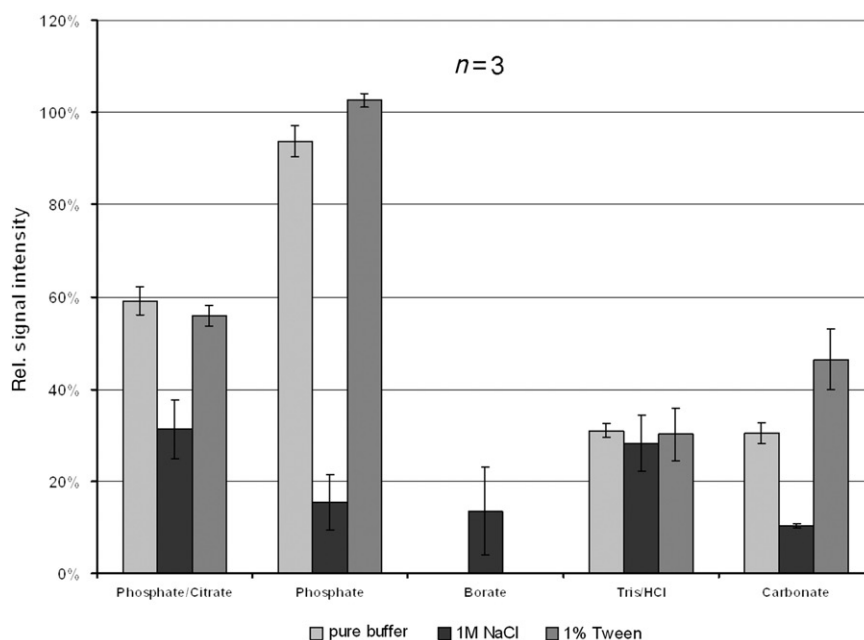


Figure 4. Relative signal intensity of selected peanut extracts diluted with the corresponding buffer. Peanut standard extract diluted with 0.1 M PBS was used as a benchmark (100%).

or spiked samples were tested, these results demonstrate that buffers containing Tween 20 might be more adequate for application in LFD as they are more unlikely to interfere with matrix substances of some products.

Discussion

We developed an LFD for the detection of peanut protein and compared buffers for their extraction capacity for differently heat treated peanut samples and their effect on LFD performance. The crucial point of the study was to compare buffers on strip tests and not ELISA as buffers would exhibit a greater impact on a homogeneous assay. LFDs compared with ELISA usually show lower sensitivity (Poms et al. 2005; van Hengel et al. 2006), but application on-site can be handled easily and carried out by lesser trained personnel. A second goal was to obtain all information about the produced LFD, the antibodies and the extraction buffers, which is usually only possible in the case of in-house production. Only the exact heat treatment procedures of the used peanut samples were not known. The results showed that the used extraction buffers were able to extract putative Ara h 1, but as expected not from dark-roasted peanuts. Processing alters the solubility of peanut proteins (Koch et al. 2003; Chassaing et al. 2007; Cong et al. 2007) and the extractability of Ara h 1 was reported to be most efficient from mildly roasted peanuts (Pomés et al.

2006). This corresponds to the results obtained with in-shell-roasted peanuts used for standard preparation. Buffers showed best comparability with this peanut sample. High-pH buffers did exhibit good extraction capacities but showed weak performance in LFD tests. The only low-pH buffer used worked neither for extraction nor for the dipstick procedure. It can therefore be assumed that peanut proteins, especially Ara h 1 with a pI of 4.5 (Burks et al. 1991; Pastorello and Trambaioli 2001) can be better extracted when highly negatively charged, which is the case in higher-pH buffers. It can be conjectured that with heat treatment hydrophobic groups of the protein might be turned outside (Nogueira et al. 2004) and hence aqueous or saline buffers lose their ability to solubilise peanut proteins. The denaturing substances used such as SDS and urea showed, as expected, good extraction capacities, also for strongly heat processed peanut proteins, but had a destructive effect on the antibodies used in LFD assembly. Interestingly also SDS and urea protein extracts highly diluted with non-denaturing buffer could not be detected, leading to the conclusion that those additives induce the alteration of epitopes in peanut proteins. Finally, it can be stated that best compatibility between protein extraction and LFD performance was achieved with neutral-pH phosphate buffers. Additional results from blank food matrices showed that the addition of Tween 20 to the extraction buffer can prevent undesired matrix effects. The achieved results gave a good overview where difficulties in peanut detection by LFD can be found.

Conclusions

Several studies concerning peanut protein extractability with regard to heat treatment have been carried out and published in recent years (Pomés et al. 2003, 2006; Koch et al. 2003; Nogueira et al. 2004; Poms et al. 2004; Westphal et al. 2004; Kiening et al. 2005; Scaravelli et al. 2009). These studies focused either on the comparison of commercially available ELISA kits (Koch et al. 2003) or on the analysis of locally purchased food samples (Kiening et al. 2005). Different extraction buffers and procedures were evaluated. Overall, the results showed that most of ELISA kits can detect at least qualitatively purified peanut proteins/allergens (Nogueira et al. 2004), but they are usually not intercomparable (Koch et al. 2003). Depending on the food matrix, a certain number of samples without peanut declaration on the label were tested positive using commercially available ELISA and dipsticks (Pele et al. 2007). It cannot be clearly stated if these samples actually contained peanut traces or if another interference factor caused the positive results. One of the great concerns in allergen research is that there is no certified reference material available, neither a pure peanut standard nor a representative food sample with a defined amount of peanut incorporated. Although there is test material (IRMM – 481 Peanut Test Material Kit) available, a universal standard for all applications does not exist. This is not due to a 'lack' in scientific development – much effort is taken in order to develop such materials – but there is still a question about what is to be detected and how should it be extracted, purified and validated. This challenge of standard material leads further to the question of antibody specificity. On the one hand, applied antibodies should be able to detect proteins from light- and dark-roasted peanuts – ideally independent from previous heat treatment, as production procedures of food products are usually not known completely. On the other hand, antibodies applied in a rapid test can only detect proteins that have been efficiently extracted. Furthermore, antibodies can only detect antigens that have been used for immunisation and therefore have been isolated sufficiently from the corresponding food source. This leads back to the challenge of the optimal extraction buffer. The perfect buffer should be able to extract proteins from peanuts, which are detectable by the applied antibody, do not have denaturing or inhibiting effects on the proteins, neither the potential allergens, nor the antibodies, and furthermore positively influence LFD performance concerning background, signal intensity and migration properties. The results showed that this 'all-in-one' buffer will take much effort to be realised. Due to changes in protein solubility caused by heat treatment, it appears very challenging to find one buffer with the same extraction capacity for native as well as for

heat-altered proteins. Moreover, it is necessary to develop antibodies against those differently heat-treated peanut proteins and it can finally not be predicted if proteins extracted from harshly heated peanuts are equally suitable as immunogens as from mildly roasted kernels. Achieving this goal will be a time-consuming approach as antibody development and application for LFD does provide its own challenges (Rudolf et al. 2009).

In conclusion, it can be stated that the quantitative determination of peanut traces in food products independent from previous treatment cannot yet be assured. This study summarises some important factors that have to be considered during LFD development for peanut detection. Having one generic buffer formulation applicable for different food matrices and food allergens would facilitate allergen detection. From our point of view this general request is not (yet) realisable according to the discussed challenges and requirements associated with analysis of such heterogeneous material as foodstuff.

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