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Improved extraction of peanut residues from a wheat flour matrix for immunochemical detection

Shyamali Jayasena, Subhashinee S.K. Wijeratne, Steve L. Taylor, Joseph L. Baumert*

Food Allergy Research and Resource Program, Department of Food Science & Technology, University of Nebraska, 279 Food Innovation Center, Lincoln, NE 68588-6207, USA

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ABSTRACT

The efficacy of different buffers in extracting peanut from a solid model food incurred with peanut and subjected to processing was evaluated using two commercial ELISA kits: Veratox® for peanut allergen and peanut ELISA from Morinaga. Average percentage recoveries of peanut from unprocessed samples using the kit supplied buffers were 46 ± 5 and 28 ± 2 with the Veratox and Morinaga kits, respectively. However, Na_2CO_3, pH 9.6 and PBS containing 1 M GuHCl recovered 65% ± 4% and 77% ± 10% of peanut, respectively from unprocessed samples with the Veratox kit. These two buffers also performed better than the Veratox buffer with fried, high pressure processed, and baked samples. PBS containing SDS and β-ME, performed significantly better than the Morinaga buffer in recovering peanut from unprocessed, boiled and fried samples. Thus, the use of alternative extraction buffers provides better recovery of peanut residues from a processed solid food matrix.

1. Introduction

In the USA, almost 5% of adults and approximately 8% of children are affected by food allergies, the majority of which are caused by the 'big eight' food groups; milk, eggs, tree nuts, peanuts, soybeans, fish, crustaceans, and wheat (Sampson, 2004; Sicherer & Sampson, 2014). Additionally, some of the most severe adverse allergic reactions are caused by the ingestion of tree nuts and peanuts by sensitive individuals. Approximately 1.3% of adults and 2.1% of children are affected by tree nut or peanut allergies, while peanut allergies alone affect 0.6% of adults and 1–2% of children in the USA (Gupta et al., 2011; Sicherer, Munoz-Furlong, Godbold, & Sampson, 2010).

With a high protein (25%) and fat content (50%), peanuts are of notable nutritive value and are widely consumed worldwide (Oerise, Lau, Ritchey, & Murphy, 1974). The yearly per capita consumption of peanuts and peanut products in the USA is nearly six pounds of which more than 40% is consumed as peanut butter. Additionally, peanuts are incorporated into a wide range of food products including breakfast cereals, candy, confectionaries, ice creams, syrups, soups, sauces, etc. (Saavedra-Delgado, 1989). Consequently, peanut allergenic individuals are at risk of unintentional exposure to peanuts through the use of raw materials contaminated with peanut, cross-contamination during processing, carry-over from shared equipment, or use of allergen containing rework (Huggett & Hischenhuber, 1998). Therefore, in order to protect the allergenic consumers from unintentional exposure to peanuts, sensitive and specific detection methods should be in place within the food industry. Food manufacturers need sufficiently sensitive assays to detect peanut residues in a variety of food matrices as well as on equipment surfaces. Currently, one of the more commonly used methods of detection is the enzyme linked immunosorbent assay (ELISA), using either a sandwich or competitive format that has been developed with monoclonal or polyclonal antibodies raised against specifically targeted proteins (Poms, Klein, & Anklam, 2004; Schubert-Ullrich et al., 2009).

A number of such commercial peanut ELISA kits are already available on the market, and a majority of these have been shown to perform quite well with most food matrices in detecting peanut residues. However, with certain food matrices (e.g. dark chocolate) and/or with heat processing (e.g. baked cookies), these same kits underestimate the presence of peanuts (Fu & Maks, 2013; Hurst, Krout, & Burks, 2002; Koch et al., 2003; Poms et al., 2005; Scaravelli, Brohee, Marchelli, & van Hengel, 2009; Whitaker, Williams, Trucksess, & Slate, 2005). Moreover, as shown by a number of studies, the effects of thermal treatment differ between different peanut proteins (Fu & Maks, 2013; Koppelman, Bruijnzeel-Koomen, Hessing, & de Jongh, 1999; Schmitt, Nesbit, Hurlburt, Cheng, & Maleki, 2010). As a result, based on the protein(s) targeted by a given ELISA kit, their performance will also vary. Likewise, with heat processing, proteins tend to aggregate as well

* Corresponding author.
E-mail address: jbaumert2@unl.edu (J.L. Baumert).

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as lose their native conformation, thereby affecting both solubility and immunoreactivity of the proteins that are targeted by ELISA. As an example, total protein that is extracted from roasted peanuts can be 50–80% lower compared to the yield from raw peanuts, indicating a significant reduction in extraction efficiency due to thermal processing (Poms, Capelletti, & Anklam, 2004). Consequently, this results in lower recovery and detection of peanut residues with ELISA when analyzing heat-processed foods. Thus, the likelihood of false negative results is higher, thereby posing a risk to peanut sensitive individuals (Chassaigne, Brohée, Nørgaard, & van Hengel, 2007; Poms et al., 2004; Scaravelli et al., 2009; Schmitt et al., 2010). Nevertheless, by using alternate extraction methods, some of the proteins that aggregate during heat processing can be potentially re-solubilized thereby improving recovery with ELISA.

Therefore, there is a need to evaluate alternate extraction methods that will result in better detection of peanut residues from heat-processed samples. In the current study, several extraction buffers were evaluated for their efficacy in extracting peanut proteins from a simple solid food matrix incurred with different levels of peanut and subjected to different processing conditions. The total peanut recovered with these different buffer systems was evaluated using two commercial ELISA kits, Veratox® peanut allergen kit from Neogen and the peanut ELISA kit from Morinaga.

2. Materials and methods

2.1. Commercial peanut ELISA kits

Two commercial ELISA kits were used in the current study: Veratox® for peanut allergen from Neogen, Lansing, MI, USA and Peanut ELISA kit from Morinaga Institute of Biological Science, Inc. Japan.

2.2. Preparation of the solid food matrix

A solid model food system of pastry dough squares was prepared as described previously (Downs & Taylor, 2010). Partially defatted light roast peanut flour containing 12% fat and 50% protein (Golden Peanut and Tree Nuts, Georgia, USA) was used to prepare a concentrated spike of 10 000 ppm of peanut flour (5000 ppm peanut protein) in wheat flour. Pastry dough containing different levels of peanut flour (0–250 ppm of peanut flour) was prepared by replacing an appropriate amount of flour in the dough formulation with the spike concentrate. The pastry dough was cut into 2 × 2 cm squares of equal thickness (3 mm) and subjected to four different processing conditions as described below.

Pastry dough squares containing 0, 10, 25, 50, 100 and 250 ppm of peanut flour (100 g of each concentration) were subjected to boiling, baking, frying or high pressure processing (HPP). Pastry squares were either boiled in 1.5 L of water at 100 °C for 2 min and allowed to drain; baked in a conventional oven at 190 °C for 10, 20 or 30 min; or fried in soybean oil at 190 °C for 2 min followed by draining the oil on absorbent paper. For HPP, pastry squares were vacuum packaged and processed under a pressure of 600 MPa for 5 or 30 min in a 2 L high pressure processing system (HP ISOLAB System, Stansted Fluid Power Ltd., Essex, UK) while maintaining the temperature between 26 and 42 °C with the aid of a chilling unit connected to the high pressure system. Both raw unprocessed pastry dough squares and processed pastry squares were homogenized using an Osterizer blender (Model 6640, Sunbeam Products, Inc. Florida, USA) and stored at −20 °C until further use.

2.3. Extraction buffers

Five different buffer systems were evaluated for their efficacy in extracting peanut from the unprocessed pastry dough squares containing 0, 25 or 250 ppm peanut flour. Buffers 0.1 M phosphate citrate (pH 7.0), 0.1 M sodium borate (pH 8.0), 0.1 M Tris-HCl (pH 8.2), 0.1 M sodium carbonate (pH 9.6 and pH 11) and 0.01 M phosphate buffered saline (PBS) containing 0.85% NaCl (pH 7.2) were prepared both in the presence and absence of different additives (salts, detergents, denaturants and reducing agents). The additives were incorporated to the buffers at concentrations of 1 M NaCl, 1% Tween 20, 1% fish gelatin, 1% SDS and/or 6 M urea. Guanidine hydrochloride (GuHCl) and β-mercaptoethanol (β-ME) were added to PBS at concentrations ranging from 0.125 to 2 M and 12.5–250 mM, respectively either in combination or separately.

2.4. Protein extraction and ELISA analysis

1 g of unprocessed pastry dough was mixed with 19 mL (Morinaga) or 25 mL (Veratox) of preheated extraction buffer and samples were extracted at 60 °C for 15 min. For sample extracts that were being analyzed using the Veratox kit, 0.82 g of extraction additive supplied with the kit was added to the extraction mixture. Pastry dough was extracted using the kit supplied buffers (Veratox and Morinaga extraction buffers) as well as with the buffers listed above. With high pH buffers (sodium carbonate buffer at pH 9.6 and pH 11), following extraction samples were allowed to reach RT and the pH of the extracts were adjusted to neutral pH (pH 7–8) with 1 N HCl using a calibrated pH meter under constant magnetic stirring to minimize protein aggregation. An aliquot of all extracts was centrifuged at 13 000 rpm for 10 min and the resulting supernatant was tested by ELISA. The supernatants of each sample extract were tested in triplicate with both ELISA kits adhering to the protocols outlined by each respective kit. Samples that required further dilutions prior to being tested by ELISA were diluted using the respective buffers that were used for extraction but in the absence of any additives. Sample extracts containing denaturants or reducing agents were diluted at least 20 fold in each respective buffer containing no additives prior to being used for ELISA so as to minimize any effects these additives may have on the ELISA format. When high pH buffers were used for sample extraction, samples that required further dilution were diluted using the buffers that were supplied with each respective kit. The peanut concentration in each sample was interpolated from the standard curves generated using the software supplied by the kit manufacturer (Veratox) or using the GraphPad Prism 4 software (Morinaga). The total peanut recovered with each respective kit for each of the extraction buffers was calculated as a percentage relative to expected recovery. Only the buffers that resulted in significantly higher recovery of peanut from the unprocessed pastry samples compared to the kit supplied buffers were applied to extract peanut from the processed pastry samples.

2.5. SDS-PAGE analysis

1 g of light roast peanut flour was mixed with 100 mL of each of the extraction buffers and samples were extracted at 60 °C for 15 min to prepare extracts of 10 000 ppm of peanut flour. An aliquot of each of the extracts were centrifuged and the soluble protein concentration of the resulting supernatants was determined using the Lowry, Rosebrough, Farr, and Randall (1951), method of protein determination. Samples were analyzed under reducing conditions in Mini-Protean TGX Precast 4–20% gradient gels (Bio-Rad, USA). Gels were run for 30–45 min at 200 V and the proteins were visualized by staining with Coomassie Brilliant Blue R-250 (Bio-Rad, USA). Precision Plus Protein Dual Xtra Standards (Bio-Rad, USA) were used as molecular weight (MW) markers.

2.6. Statistical analysis

All sample extractions were performed in duplicate and the resulting extracts were analyzed in triplicate with each of the ELISA kits.

Dunnett's test was used to determine statistical significance of the mean recovery of peanut with each buffer with that of the kit supplied buffer. All statistical analyses were performed using the SAS 9.2 software package and the level of significance was set at $p \leq 0.05$.

3. Results

3.1. Recovery of peanut from unprocessed pastry dough

The recovery of peanut from raw pastry dough containing 0, 25 and 250 ppm of peanut flour was initially determined with the Veratox and Morinaga ELISA kits using the extraction buffers supplied with each respective kit. With the Veratox ELISA kit, the percentage recovery of peanut was 42 ± 3 and 49 ± 3 from pastry doughs containing 25 and 250 ppm peanut flour, respectively. In comparison, the recovery was lower with the Morinaga ELISA kit where only 26 ± 1% and 29 ± 1% of peanut was recovered from pastry doughs containing 25 and 250 ppm of peanut flour, respectively. Each of these two ELISA kits was used to quantify the recovery of peanut from raw pastry dough following extraction with the different buffer systems. As expected, the percentage recovery of peanut showed variation with different buffers as well as between the two ELISA kits. Although some of the buffers underperformed compared to the extraction buffers supplied with the ELISA kits, several buffers recovered significantly higher amounts of peanut ($p < 0.05$) compared to the kit supplied buffers.

With the Veratox ELISA kit, both phosphate citrate and sodium borate buffers were comparable to the Veratox extraction buffer in recovering peanut, except in the presence of 1 M NaCl when recovery significantly decreased (Fig. 1A and B). In contrast, recovery was considerably lower compared to the kit supplied extraction buffer ($p < 0.05$) when Tris-HCl buffer was used for extraction, either by itself or in the presence of most additives (Fig. 1A and B) and also with PBS containing varying levels of β-ME (Fig. 2A). However, recovery of peanut with Tris-HCl containing 1% Tween 20 and PBS without any additives was similar to the Veratox extraction buffer ($p > 0.05$) which is also 0.01 M PBS (pH 7.4). Notably, when PBS containing 0.125, 0.5, 1 and 2 M GuHCl or sodium carbonate buffers (both at pH 9.6 and pH 11) were used for sample extraction, ≥50% of peanut was recovered which is a significant improvement compared to the other buffers that were evaluated using the Veratox ELISA kit (Fig. 1A, 1B and 2A). Indeed, when PBS containing 1 M GuHCl was used for extraction, percentage recovery of peanut was as high as 70 ± 5 and 84 ± 4 from pastry doughs containing 25 and 250 ppm of peanut flour, respectively.

Unlike with the Veratox kit, most of the pastry dough extracts in different buffers that were evaluated using the Morinaga ELISA kit resulted in significantly lower ($p < 0.05$) recovery compared to the Morinaga extraction buffer. These included PBS with GuHCl or β-ME, phosphate citrate, sodium borate and Tris-HCl buffers either in the presence of absence of different additives. On the other hand, when high pH buffers, sodium carbonate at pH 9.6 and pH 11 were used for sample extraction, recovery of peanut was equivalent to the Morinaga extraction buffer ($p > 0.05$). Only a single buffer performed considerably better than the Morinaga extraction buffer ($p < 0.05$), in recovering peanut from raw pastry dough; PBS containing 250 mM β-ME and 1% SDS (Fig. 1C, 1D and 2B).

The SDS-PAGE protein profiles of light roast peanut flour extracts in

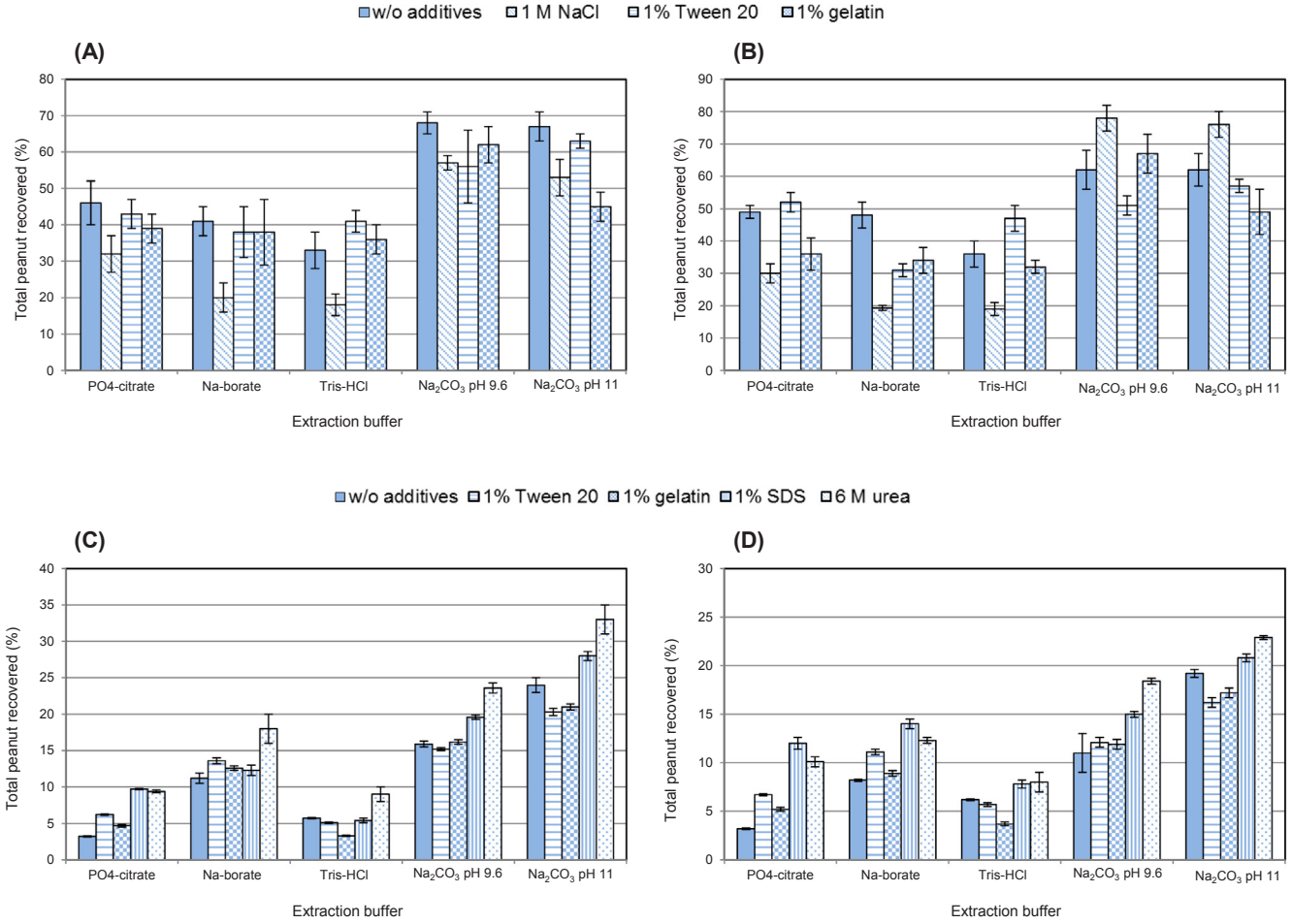

Fig. 1. Efficacy of different buffer systems in recovering peanut from unprocessed pastry dough samples containing 25 (A, C) and 250 ppm (B, D) of peanut flour using the Veratox (A, B) and Morinaga (C, D) ELISA kits. Data are expressed as mean ± standard deviation (n = 2, each analyzed in triplicate).

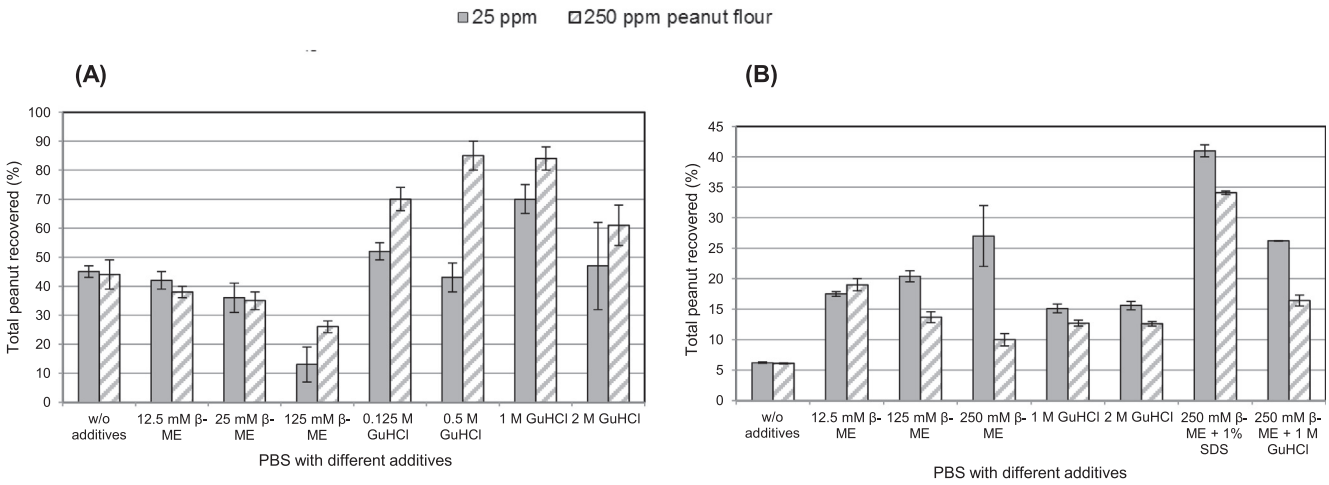

Fig. 2. Extraction efficacy of PBS in the presence of different additives in recovering peanut from unprocessed pastry dough samples containing 25 and 250 ppm of peanut flour using the Veratox (A) and Morinaga (B) ELISA kits. Data are expressed as mean ± standard deviation (n = 2, each analyzed in triplicate).

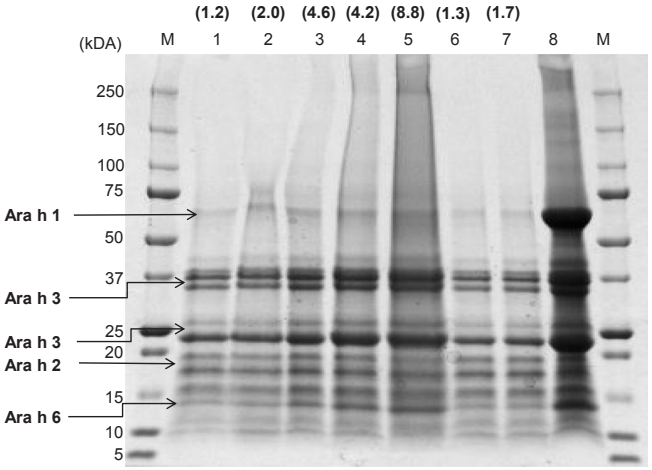

Fig. 3. SDS-PAGE analysis under reducing conditions of light roast peanut flour extracted using different buffers. Samples diluted 1:6 in SDS sample buffer, load per well 10 µL. M; standard protein marker (indicated in kDa for each standard in the left margin), 1–8 represents buffers phosphate citrate, sodium borate, Tris-HCl, sodium carbonate pH 9.6, sodium carbonate pH 11, PBS, Veratox kit buffer and Morinaga kit buffer, respectively. Values above each lane number represent the protein concentration (mg/mL) of each extract as determined by the Lowry method. The major peanut allergens are indicated by arrows.

different buffer systems are mostly similar, although there are differences in the protein yield and banding intensities with different buffers. Higher band intensities of most peanut proteins are attained with the Morinaga extraction buffer compared to the other buffers, indicating better extraction efficiency (Fig. 3). The protein band intensities and the soluble protein concentrations as determined by the Lowry method for light roast peanut flour extracts in phosphate citrate, sodium borate, PBS and Veratox extraction buffers are more or less similar (Fig. 3). Similarly, equivalent amounts of peanut were recovered with the Veratox kit when these four buffers were used to extract proteins from unprocessed pastry dough. Although the soluble protein concentration of the peanut flour extract in Tris-HCl buffer was 4.6 mg/mL, considerably lower amounts of protein was detected from the pastry dough extract by ELISA, indicating possibly lower efficiency of peanut protein extraction from the dough matrix. The soluble protein concentration of peanut flour extracts in the sodium carbonate buffers at pH 9.6 and pH 11 was also high, as was the band intensity of several proteins, particularly Ara h 3 (Fig. 3). Likewise, these two buffers performed equally or significantly better than both of the kit supplied buffers with each respective kit with the unprocessed pastry dough as well.

3.2. Recovery of peanut from processed pastry samples

The buffers that performed significantly better than the kit supplied buffers in recovering peanut from unprocessed pastry dough were evaluated for their efficacy in recovering peanut from pastry squares that had been subjected to different processing conditions. Thus, sample extracts of the two buffers, 0.1 M sodium carbonate (pH 9.6) and 0.01 M PBS containing 1 M GuHCl (pH 7.2), were evaluated using the Veratox kit while sample extracts of a single buffer, 0.01 M PBS containing 250 mM β-ME and 1% SDS were evaluated using the Morinaga kit. The percentage recovery of peanut as determined using ELISA for each of these buffer extracts were compared with that of each respective kit supplied buffer extracts with the processed pastry samples.

The extraction efficiency of these buffers differed from one to another and also showed differences based on the type of processing condition applied to the samples. With both ELISA kits, recovery was least affected with pastry samples baked for 10 min, where the average percentage recoveries of peanut were 44 ± 5 and 30 ± 2, respectively with the Veratox and Morinaga extraction buffers, respectively. Comparatively similar amounts of peanut were recovered with the Veratox and Morinaga extraction buffers from pastry samples that had been subjected to boiling, frying and HPP (5 min). However, with pastry squares subjected to HPP for 30 min, average recovery of peanut was more than two fold higher with the Morinaga kit (28 ± 1%) compared to the Veratox kit (11 ± 3%) using the kit supplied buffers of each respective kit. A significant difference in mean recovery between the two kit supplied buffers were also observed with samples baked for 20 min with the Morinaga kit (20 ± 3%) outperforming the Veratox kit (12 ± 3%). Thus, these observations suggest that the Morinaga kit is better in the detection of peanut from pastry samples that have undergone more severe processing conditions, while, with minimally processed or unprocessed pastry samples, the Veratox kit outperforms the Morinaga kit.

Of the two buffers that were compared using the Veratox ELISA kit, when pastry squares were subjected to boiling and frying, both PBS containing GuHCl and sodium carbonate buffers were more or less comparable to the kit supplied buffer in recovering peanut, although both these buffers performed significantly better than the kit supplied buffer ($p < 0.05$) in recovering peanut from fried pastry samples containing 10 ppm of peanut flour (Fig. 4A and D). On the other hand, both these buffers performed significantly better than the Veratox buffer in recovering peanut from pastry samples that were subjected to HPP for 5 min at almost all concentrations of peanut flour (Fig. 4B).

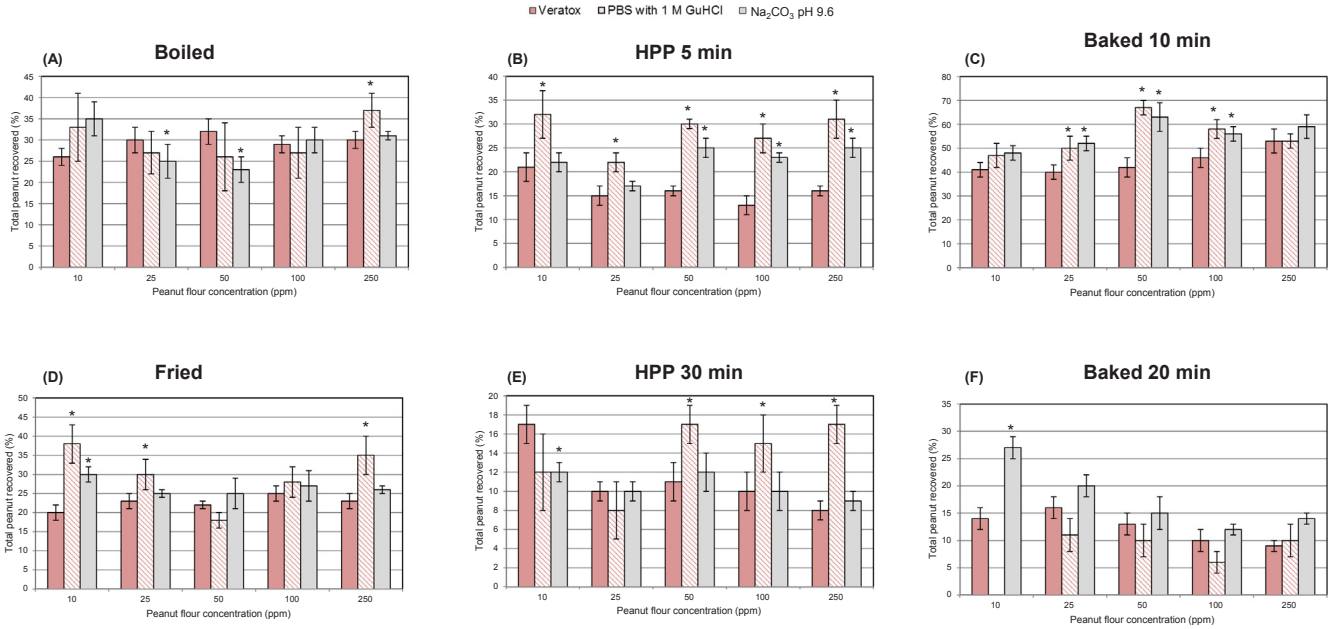

Fig. 4. Comparison of the percentage recovery of total peanut determined using the Veratox ELISA kit from pastry squares incurred with 10–250 ppm of peanut flour and subjected to boiling (A), HPP for 5 min (B), baking for 10 min (C), frying (D), HPP for 30 min (E) and baking for 20 min (F). Data are expressed as mean ± standard deviation (n = 2, each analyzed in triplicate). *; mean recovery significantly different ($p < 0.05$) compared to the kit supplied buffer for each respective concentration.

Even under extreme conditions of HPP (30 min), with the exception of the 10 ppm samples, recovery of peanut using the sodium carbonate buffer was comparable to that of the Veratox buffer. When PBS containing 1 M GuHCl was used, it had a significant advantage ($p < 0.05$) over the kit supplied buffer in recovering peanut from HPP (30 min) samples incurred with ≥50 ppm of peanut flour (Fig. 4E). However, when pastry samples were subjected to extreme conditions of baking (190 °C for 30 min), neither the Veratox extraction buffer nor the other two buffers evaluated using the Veratox kit were able to detect any peanut from these samples, which is not unduly surprising given that the pastry squares were 2 × 2 cm and only of 3 mm thickness. Even when the baking time was limited to 20 min, 10 ppm sample extracts of PBS with GuHCl were not detected by the Veratox kit, although recovery was comparable to the kit supplied buffer at peanut flour concentrations of > 10 ppm as were the sample extracts of sodium carbonate buffer (Fig. 4F). Nonetheless, following 10 min of baking, both of these buffers recovered significantly higher ($p < 0.05$) levels of peanut ranging from 50 to 67% compared to the Veratox buffer (40–46% recovery) from pastry squares incurred with 25, 50 and 100 ppm of peanut flour (Fig. 4C).

Compared to the Morinaga extraction buffer, PBS containing SDS and β-ME performed significantly better in recovering peanut from boiled and fried samples at several concentrations of peanut flour (Fig. 5A and B). However, with HPP samples (5 min), the efficacy of this buffer was mostly similar to that of the Morinaga extraction buffer (Fig. 5C), while under extreme conditions of HPP (30 min), the Morinaga buffer performed notably better than this buffer ($p < 0.05$) in recovering peanut at all sample concentrations (Fig. 5D). The efficacy of this buffer was also limited with the baked samples (20 min) where the Morinaga extraction buffer outperformed this buffer at most sample concentrations, although recovery was comparable for these two buffers with samples subjected to only 10 min of baking. However, it should be noted that unlike with the Veratox kit, which did not detect any protein from pastry squares baked for 30 min, the Morinaga kit was able to detect peanut in sample extracts of both the Morinaga buffer and PBS buffer with SDS and β-ME at ≥50 ppm of peanut flour, although percentage recovery was extremely low at < 5% (Fig. 6).

Thus, in summary, PBS containing 1 M GuHCl recovered significantly higher amounts of peanut from raw and HPP (5 min) samples at all peanut concentrations as well as from fried, HPP (30 min) and baked (10 min) pastry squares at several peanut concentrations compared to the Veratox extraction buffer. Sodium carbonate (pH 9.6) outperformed the Veratox buffer only with raw, baked (10 min) and HPP (5 min) samples at a number of sample concentrations. With the Morinaga kit, PBS containing β-ME and SDS recovered significantly higher amounts of peanut mostly from unprocessed, boiled and fried pastry squares. These results indicate that selected buffers are significantly more effective than the kit supplied buffers in recovering peanut from certain processed solid food matrices.

4. Discussion

In this study, we evaluated a wide selection of extraction buffers for their efficacy in recovering peanut protein from a solid food matrix, so as to facilitate better recovery by ELISA. Two peanut ELISA kits, Veratox and Morinaga each of which predominantly detects distinct peanut allergens, Ara h 3 and Ara h 2/h 6, respectively (Jayasena et al., 2015) were used to quantify peanut proteins extracted from a pastry matrix with each of the different buffers. Of the different buffers tested, two were identified as performing significantly better than the kit supplied buffer of the Veratox kit (sodium carbonate and PBS with GuHCl) while a single buffer (PBS with β-ME and SDS) performed significantly better than the Morinaga extraction buffer in recovering peanut from unprocessed pastry dough as assessed by each respective ELISA kit. Consequently, these three buffers were used to extract proteins from pastry squares that had been subjected to different processing conditions. Sample extraction was carried out at 60 °C for optimal extraction efficacy (Albillos, Al-Taher, & Maks, 2011) which incidentally is the temperature used for extraction by most commercial ELISA kits including the Veratox kit.

The recovery of peanut from unprocessed pastry dough ranged between 25 and 50% with the kit supplied buffers, while higher recoveries approaching 80–90% were achieved with select buffers. Besides the differences in the extraction efficacies of different buffers, food matrix effects also likely had an effect on recovery as observed in a similar study with pastry dough incurred with milk (Downs & Taylor, 2010). Of

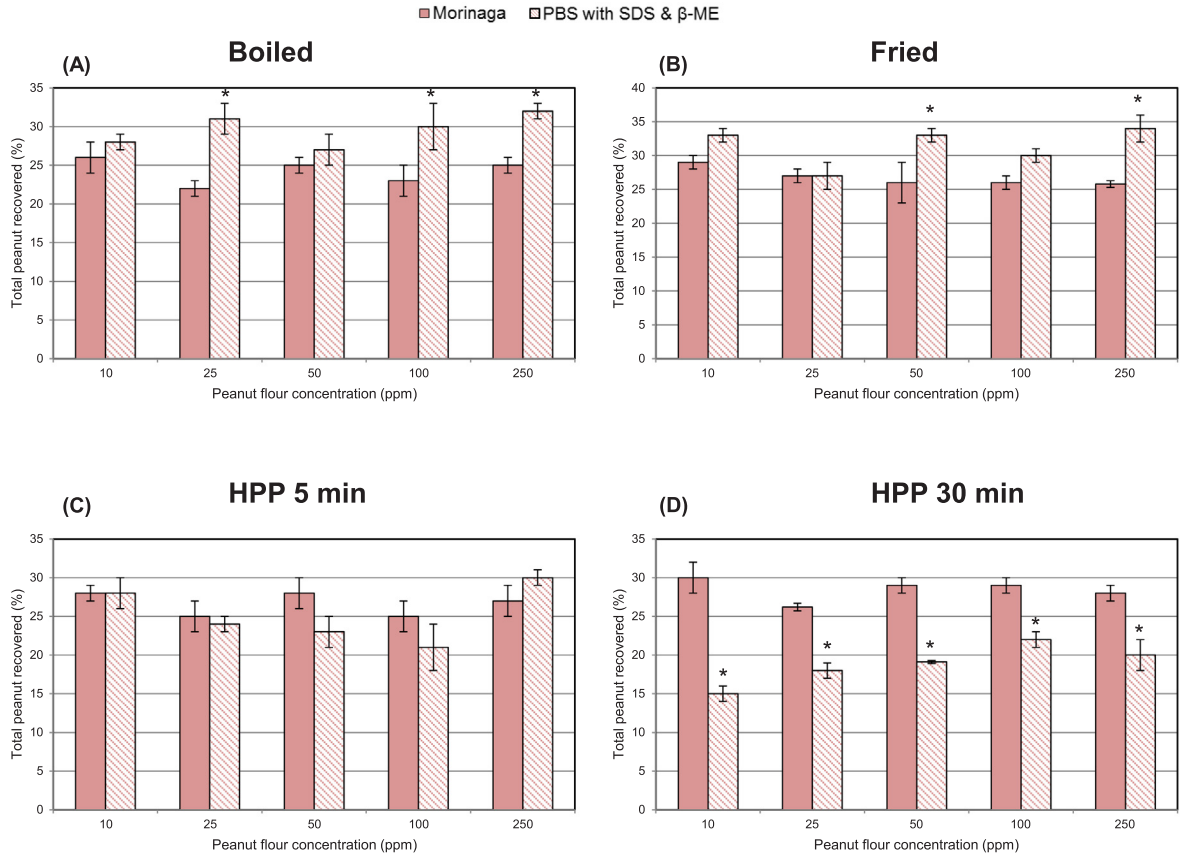

Fig. 5. Comparison of the percentage recovery of total peanut using the Morinaga ELISA kit from pastry squares incurred with 10–250 ppm of peanut flour and subjected to boiling (A), frying (B), HPP for 5 min (C) and HPP for 30 min (D). Data are expressed as mean ± standard deviation (n = 2, each analyzed in triplicate). *; mean recovery significantly different ($p < 0.05$) compared to the kit supplied buffer for each respective concentration.

the different processing conditions that were evaluated in the current study, recovery was least affected by baking for 10 min, followed by boiling where recoveries ranged from ~40–70% and 20–40% from baked (10 min) and boiled samples, respectively with the Veratox kit (Fig. 4). Fu and Maks (2013) have reported a similar observation, where they reported a 50% decrease in recovery of peanut from peanut flours subjected to boiling while dry heating up to 120 °C (10 min) did not significantly affect recovery. It should be also noted that the Ara h 1 content in peanuts is decreased more by boiling and frying than roasting (Beyer et al., 2001). Thus, a lower yield of Ara h 1 will translate to lower detection by ELISA especially with the Veratox kit with a higher sensitivity to Ara h 1 compared to the Morinaga kit (Jayasena et al., 2015). Thus, the effects of boiling and frying on Ara h 1 may be partly responsible for recovering less peanut from boiled and fried pastry compared to the baked (10 min) samples especially with the Veratox kit.

However, when the baking time was increased to 20 or 30 min, a significant decrease in the recovery of peanut was observed with both ELISA kits. Indeed, when pastry squares were baked for 30 min, no peanut was detected with the Veratox kit while < 5% was recovered with the Morinaga kit. Furthermore, an almost four-fold reduction in the average recovery of peanut between 10 and 20 min of baking was observed with the Veratox kit (Fig. 4), while, with the Morinaga kit, this decrease was less than two fold (Fig. 6). This is not surprising given that the Morinaga kit predominantly recognizes the more heat stable proteins, Ara h 2 and Ara h 6, while the Veratox kit shows higher sensitivity to the more heat labile protein, Ara h 3 (Jayasena et al., 2015). These observations are in agreement with several other studies carried out using different food matrices. Khuda et al. (2012) illustrated that the recovery of multiple allergens decreased significantly following baking of cookies incurred simultaneously with peanut, egg and milk. Similar to our observations, they report that the decrease in recovery following baking is not as significant with the Morinaga kit as with the other kits, although initial recovery is low for this kit. A similar decrease in the detection of peanut from baked cookies has been reported using both ELISA and PCR, where the authors report recoveries of ~50–80% for cookies baked at 180 °C for 21 min, and recoveries of only 10–20% when the baking time is increased to 26 min (Scaravelli et al., 2009). Likewise, when the baking temperature is increased, a significant decrease in the detection of peanut by ELISA has been reported for peanut flours dry heated to ≥232 °C for 10 min (Fu & Maks, 2013) as well as for whole peanuts either dry or oil roasted (Poms et al., 2004). In contrast, recoveries approaching > 90% with the Veratox kit have been reported when peanut free cookies were spiked with different levels of roasted peanut (Koch et al., 2003). Likewise, a study evaluating the performance of four commercial peanut ELISA kits, showed that the recovery of peanut was higher with four different food matrices spiked with peanut compared to these same food matrices incurred with peanut (Whitaker et al., 2005). These results demonstrate that the use of incurred samples is preferable to spike and recovery approaches when evaluating extraction efficiency. Thus, it should be noted that spiking a food matrix would only give a representation of the matrix effects and extraction efficacy on the recovery of the target allergen, while incurring a food matrix will reveal the effects of processing on recovery and detection by ELISA (Lacorn & Immer, 2009).

When peanuts are boiled, low molecular weight proteins, including Ara h 2 leach into the cooking water (Mondoulet et al., 2005). Since the

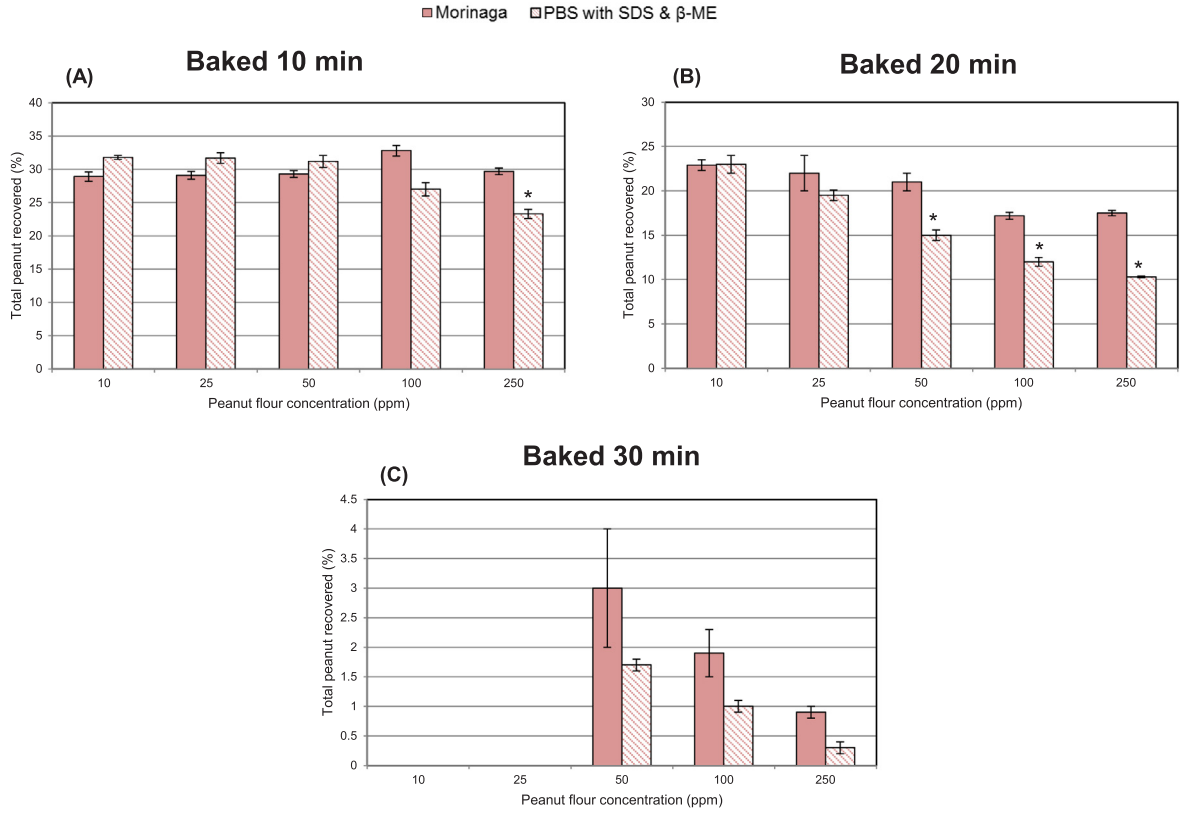

Fig. 6. Percentage recovery of total peanut determined using the Morinaga ELISA kit from pastry squares incurred with 10–250 ppm of peanut flour and baked for 10 (A) 20 (B) and 30 min (C). Data are expressed as mean ± standard deviation (n = 2, each analyzed in triplicate). *; mean recovery significantly different ($p < 0.05$) compared to the kit supplied buffer for each respective concentration.

Morinaga kit predominantly detects Ara h 2 and Ara h 6 (Jayasena et al., 2015), a lower recovery could be expected with the Morinaga kit for boiled samples. However, we did not observe a significant decrease in the recovery of peanut from boiled pastry squares in comparison to the other processing conditions nor were peanut proteins detected in the boiled water with the Morinaga ELISA. Nonetheless, in the current study, samples were boiled for only 2 min, compared to a boiling time of 30 min in the study carried out by Mondoulet et al. (2005), and thus lower amounts of Ara h 2 and Ara h 6 may have leached into the water, thereby having a lower effect on the detection of peanut with the Morinaga kit. Additionally, the leaching of Ara h 2 may differ for the wheat flour pastry dough matrix in comparison to whole peanuts.

A significant difference in the average percentage recovery of peanuts was not observed between the Veratox (22%) and Morinaga (26%) ELISA kits from samples subjected to HPP for 5 min. However, with HPP for 30 min, average percentage recovery of peanut was only 12% with the Veratox kit, while a longer processing time did not have a significant impact on recovery with the Morinaga kit (24% recovery). The structures of both Ara h 2 and Ara h 6 are not significantly affected by HPP (Johnson et al., 2010) which is in agreement with our observed results for HPP pastry samples between the two ELISA kits. It is more likely that the heat labile Ara h 3 is affected more by HPP resulting in lower recovery with the Veratox kit.

Other than processing conditions, buffer composition also has an effect on the protein yield as indicated by both the gel profile and Lowry protein estimations (Fig. 3). Of the different buffers evaluated for their efficacy in detecting peanut proteins by ELISA, one buffer system that performed either equally or better than both of the kit-supplied buffers was sodium carbonate buffer at both pH 9.6 and pH 11. The superior extraction efficiency of high pH buffers compared to more neutral buffers has been well documented (Khuda, Jackson, Fu, & Williams, 2015; Poms et al., 2004; Rudolf, Ansari, Kern, Ludwig, & Baumgartner, 2012). However, a major disadvantage with high pH buffer extracts is that such extracts are not always compatible with ELISA formats (Poms et al., 2004; Rudolf et al., 2012) unless they are diluted in neutral buffers prior to being tested by ELISA (Westphal, Pereira, Raybourne, & Williams, 2004). In the current study following the adjustment of the extracts to near neutral pH, we were able to overcome any adverse effects possibly associated with the high pH.

The addition of NaCl has been shown to enhance protein extraction from peanut flours although such extracts were shown to reduce lateral flow signals by 20% (Rudolf et al., 2012). Thus, although a high salt concentration may give a better protein yield, the salt may have an inhibitory effect on immunoassay formats. This is in agreement with our results where the addition of 1 M NaCl resulted in significantly lower recovery of peanut from raw pastry dough with most buffers (Fig. 1A and B). Nevertheless, this effect of NaCl was lower or even non-existent with sodium carbonate buffers, an observation also reported in a recent study that establishes carbonate extracts are less affected by the different ionic strengths compared to more neutral buffers (L'Hocine & Pitre, 2016b).

Strong denaturants (e.g. urea, SDS) as well as reducing agents (e.g. β-ME) are also effective in enhancing protein extraction (Khuda et al., 2015; Poms et al., 2004; Rudolf et al., 2012). However, because of their highly denaturing effect, even following dilution of the sample extracts prior to being used in ELISA, we found that such additives were compatible with only the Morinaga kit and not with the Veratox kit. The effect of strong denaturants can be overcome to a certain extent by using antibodies that has been raised against antigens denatured and reduced with these agents. This allows the detection of proteins that have been denatured by different processing and manufacturing conditions. Additionally, incorporating such agents in the extraction buffer

increases the amount of proteins that can be extracted from both processed and non-processed samples (Watanabe et al., 2005). A similar approach has been used by the Morinaga kit, which accounts for the compatibility of this kit with samples extracted in buffers containing SDS and β-ME. Indeed, the extraction buffer of the kit itself contains β-ME at a concentration of 2%. Such an ELISA is especially beneficial in detecting allergens in samples where processing can result in denaturation and aggregation of proteins. The addition of these agents helps to re-solubilize such aggregated proteins (Morishita et al., 2008). This is in agreement with our results where we see better recovery of peanut with the Morinaga kit than with the Veratox kit for highly processed samples (HPP for 30 min, baking for 20 and 30 min). In a study carried out by Fu, Maks, and Banaszewski (2010), the effect of heat treatment on the detection of egg proteins was assessed using three commercial ELISA kits and, while all kits underestimated the amount of egg proteins in heat-treated samples, the Morinaga kit gave the highest recoveries. The authors report that this underestimation is due to heat-induced changes in the immunoreactivity of the proteins targeted by the ELISA and not due to a decrease in extraction efficiency.

Other than the different extraction additives, several other factors including roasting, buffer type, extraction temperature and sample to buffer ratio have a significant effect on the percentage recovery of proteins from peanuts in which carbonate (77.6%) and borate (68.7%) buffers were shown to be more effective than phosphate (58.6%) buffer in recovering peanut (L'Hocine & Pitre, 2016a, 2016b). Nonetheless, different buffer types and buffer additives will affect only the protein yield of peanut extracts but not the composition of the extracted proteins (Zeleny & Schimmel, 2010) as shown in Fig. 3. A study comparing the protein profiles of peanut flour extracts reported lower band intensities for phosphate buffer compared to carbonate and Tris buffers (Westphal et al., 2004), which is in agreement with our observations for light roast peanut flour extracts in different buffers (Fig. 3). However, as is evident with the protein profile of the Morinaga extract, the addition of a reducing agent (β-ME) to the extraction buffer does result in a significantly higher yield of several peanut proteins (Fig. 3). With the exception of the Morinaga extract, we observed a decrease in the band intensity of Ara h 1 compared to the other peanut allergens which is in agreement with previously published studies on roasted peanuts and peanut flours (Fu & Maks, 2013; Koppelman et al., 1999; Schmitt et al., 2010). The presence of a reducing agent in the Morinaga extraction buffer most likely helps to re-solubilize peanut proteins aggregated during roasting of the peanut flour.

Although our results indicate that processing causes a decrease in the recovery of peanut, the allergenicity of these proteins may remain unchanged or even enhanced. Several studies have established that IgE from peanut allergic patients binds more strongly with roasted peanut extracts compared to raw peanuts and also to Ara h 1 and Ara h 2 purified from roasted peanuts compared to these same allergens purified from raw and boiled peanuts (Mondoulet et al., 2005; Nesbit et al., 2012; Blanc et al., 2011). Moreover, the IgE recognition sites of roasted peanut proteins differ from those of raw peanuts, suggesting that heat treatment may expose previously unexposed IgE binding epitopes or may even create new IgE binding sites (Maleki, Chung, Champagne, & Raufman, 2000). Thus, even if the ELISA kits show decreased recovery from processed matrices, with human sera based assays, we see higher IgE binding affinities, indicating that processing does not make peanuts less allergenic.

5. Conclusions

Replacing the kit supplied extraction buffer of the Veratox kit with a high pH buffer or PBS containing 1 M GuHCl significantly improved the recovery of peanut from both unprocessed and processed pastry samples. On the other hand, with the Morinaga kit, the kit supplied buffer performed better than the selected buffer under most conditions of processing, indicating that with this particular ELISA kit, the performance of this kit supplied buffer is adequate to detect peanut with the processing conditions assessed in the current study. Moreover, although the Veratox kit outperformed the Morinaga kit in recovering peanut from unprocessed and less severely processed pastry samples, with more severe processing conditions, better recovery was observed with the Morinaga kit. These observations suggest that by using a kit that targets heat stable allergens, a more accurate representation of peanuts present in highly processed samples can be achieved. Additionally, while the processing results appear to suggest that the sensitivity of ELISA kits is adversely affected by heat processing, these results may also be matrix dependent.

Conflict of interests

The authors declare no competing financial interest.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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