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## Analytical Methods

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**Quantitative and qualitative optimization of allergen extraction from peanut and selected tree nuts. Part 1. Screening of optimal extraction conditions using a D-optimal experimental design**

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**Abstract**

A D-optimal design was constructed to optimize allergen extraction efficiency simultaneously from roasted, non-roasted, defatted, and non-defatted almond, hazelnut, peanut, and pistachio flours using three non-denaturing aqueous (phosphate, borate, and carbonate) buffers at various conditions of ionic strength, buffer-to-protein ratio, extraction temperature, and extraction duration. Statistical analysis showed that roasting and non-defatting significantly lowered protein recovery for all nuts. Increasing the temperature and the buffer-to-protein ratio during extraction significantly increased protein recovery, whereas increasing the extraction time had no significant impact. The impact of the three buffers on protein recovery varied significantly among the nuts. Depending on the extraction conditions, protein recovery varied from 19% to 95% for peanut, 31% to 73% for almond, 17% to 64% for pistachio, and 27% to 88% for hazelnut.

A modulation by the buffer type and ionic strength of protein and immunoglobuline E binding profiles of extracts was evidenced, where high protein recovery levels did not always correlate with high immunoreactivity.

**Keywords:** Allergen; Extraction; Peanut; Tree nut; Almond; Hazelnut; Pistachio; Experimental design; D-optimal; Optimization; Roasting; Defatting.

## 1. Introduction

Peanut and tree nuts are among the most potent food allergens. Together, peanut and tree nuts are responsible for approximately 80% of anaphylactic reactions and account for over 50% of child fatalities related to food allergies in North America (Bock, Muñoz-Furlong, & Sampson, 2001). These allergies are generally lifelong and seem to becoming more prevalent, particularly among children. Recent studies estimated that peanut and tree nut allergies affect over 1% of the Canadian and U.S. populations (Ben-Shoshan et al., 2010; Sicherer & Sampson, 2010).

Despite our increasing knowledge about the molecular and immunological properties of peanut and tree nut allergens, we are still a long way from fully understanding the processes and factors that govern the severity of allergic reactions to peanut and tree nuts. Precise knowledge about the physical and biochemical characteristics of allergenic molecules is best acquired with allergens from natural sources (i.e., foods), to avoid the possible erroneous chain folding that may occur in heterologous expression systems (Lorenz, Scheurer, Haustein, & Vieths, 2001; Verdino & Keller, 2004). The limitation of utilization of recombinant allergens as standardized allergen materials has been demonstrated due to incorrect folding, aggregation, poor solubility, and insufficient stability (Chapman et al., 2008). Proteins need to be extracted efficiently and without degradation to ensure, as much as possible, that an accurate

representation of allergenic proteins from the source material is obtained. The preparation of high-quality allergen extracts is required for not only scientific research but also a variety of other applications, including the development of robust allergen detection tests for the food industry as well as reference materials for diagnostic purposes (skin testing and food challenges) and immunotherapy (Akkerdaas et al., 2003; Westphal, Pereira, Raybourne, & Williams, 2004).

Several studies aimed to optimize the extraction of allergens from peanut (Chassaigne, Brohée, Nørgaard, & van Hengel, 2007; Ma, Wang, & Wu, 2010; Poms, Capelletti, & Anklam, 2004) and tree nuts (Platteau, Bridts, Daeseleire, De Loose, Ebo, & Taverniers, 2010; Sathe, Venkatachalam, Sharma, Kshirsagar, Teuber, & Roux, 2009;; Albillos, Al-Taher, & Maks, 2011) by testing different factors that are known to influence protein extractability, such as the buffer type, its pH and ionic strength, the extraction temperature, the duration of extraction, etc. Optimization was, however, often done by testing a large number of conditions, with no underlying model, and then selecting the best ones. In other instances, optimization was done on one or two factors only or by varying one factor at a time while keeping the others constant. This classical one-variable approach generally fails when interactions between more than one factor might be involved. Therefore, an extensive and systematic study of the concerted application of extraction conditions is still lacking. Moreover, little attention was given to how these extraction conditions affected the protein profile and immunoglobulin E (IgE) reactivity of the extract. The quality of the allergen extract is also influenced by pre-extraction procedures (ex. milling, defatting, processing, etc.) (Mills, Sancho, Rigby, Jenkins, & Mackie, 2009). The thermal processing of food is known to cause protein denaturation or alteration, which often results in decreased solubility and either decreased or increased immunoreactivity (Mondoulet et al., 2005; Poms et al., 2004; Schmitt, Nesbit, Hurlburt, Cheng, & Maleki, 2010). Defatting prior to extraction, also expected to affect the allergen

composition of the extract, because they may lead to low recovery of the more hydrophobic protein/allergen species, such as lipid-transfer proteins (LTP) and oleosins (Platteau et al., 2010; Zuidmeer-Jongejan et al., 2014). This effect has not been verified for all selected nuts, however.

The objective of this work was therefore to set up optimized conditions for the non-denaturing, efficient, and representative extraction of allergens from peanut and selected tree nuts (almond, hazelnut, and pistachio). The design of the experiment provided us with an efficient working strategy to explore both the individual and interactive effects of several factors together, thereby ensuring optimal selection of extraction conditions that provided high protein recovery while maintaining consistent and representative allergen profiles. Because the experimental effort to achieve the best extraction grows exponentially with the number of factors, the optimization procedure included two steps: the screening, and the fine-tuning of the most relevant factors. This first part of the study concerns the screening step, where an algorithmic D-optimal experimental design was constructed to assess the individual and interactive effects of eight relevant factors on the protein recovery, profile, and IgE binding of peanut and three selected tree nuts (almond, hazelnut, and pistachio).

## 2. Materials and methods

### 2.1. Preparation of peanut and tree nut samples

Raw seeds of peanut (*Arachis hypogaea*) and selected tree nuts (almond (*Prunus dulcis*), hazelnut (*Corylus avellana*), and pistachio (*Pistacia vera*)) were obtained from local nut distributors and were stored under vacuum at 4 °C until use. The peanut and tree nut seeds were roasted in a convection oven (Fisher & Paykel Appliances Inc., Huntington Beach, CA, USA). Peanut, almond, and pistachio were roasted at 176 °C for 20, 15, and 12 min, respectively. Hazelnut seeds were roasted at 135 °C for 25 min. The

raw and roasted seeds were frozen in liquid nitrogen and ground to a fine flour in an IKA A11 analytical mill (IKA Works, Wilmington, NC, USA). Where applicable, the peanut and tree nut flours were defatted with hexane at a ratio of 1:5 (w/v). The defatted samples were dried overnight in a chemical hood to remove all traces of residual solvent. The defatted flours were then homogenized for 30 s in a coffee grinder (Custom Grind Deluxe; Hamilton Beach, Washington, NC, USA) and stored at  $-80^{\circ}\text{C}$  until further use.

## 2.2. Proximate analysis of peanut and tree nut flour samples

Fat content was determined using a Soxtec apparatus (Foss Tecator Soxtec System HT-6, 1043 extraction unit; Foss, Brampton, ON, Canada) according to AACC Method 30-25 (AACC, 2003). Protein content was calculated from the nitrogen content that was determined by the Dumas combustion method (AOAC, 1995) using an FP-428 protein/nitrogen analyzer (LECO Corp., St. Joseph, MI, USA). The nitrogen conversion factors used were 5.18 for almond, 5.46 for peanut, and 5.3 for hazelnut and pistachio, as recommended by Venkatachalam and Sathe (2006). Moisture was determined by drying 0.3 g of the sample in a Fisher Isotemp Vacuum Oven (Fisher Scientific, Montreal, QC, Canada) for 5 h at  $100^{\circ}\text{C}$  (AACC, 1983). All determinations were done in triplicate, and average values were computed.

## 2.3. Protein extraction and analysis

Phosphate buffer (0.03 mol/L, pH 7.2), borate buffer (0.2 mol/L, pH 8.5), and carbonate buffer (0.05 mol/L, pH 9.6) solutions were prepared in-house at three different ionic strengths ( $I = 0.075, 0.15$ , and  $0.5$ ) using indications from the Buffer Maker software package, version 1.0.1.55. The various ionic strengths were achieved by adding the appropriate amount of NaCl. Each extraction buffer was used to extract raw, roasted, defatted, and non-defatted peanut and tree nut flour samples (0.5 g) at different combinations of buffer-to-protein ratio (100:1, 150:1, or 250:1 [v/w]), temperature (4, 25,

or 50 °C), and duration (1, 4, or 24 h). All extractions were conducted in 50-ml centrifuge tubes under constant shaking at 45 rpm using a LabRoller Rotator (Labnet International, Woodbridge, NJ, USA)) inclined at a 45° angle. The crude extracts were then centrifuged (Beckman Model J2-21 centrifuge (Beckman Coulter, Brea, CA, USA).) at 16 000×*g* for 30 min at 4 °C. The supernatant was vacuum-filtered (Whatman filter paper No. 4; Whatman International Ltd., Maidstone, UK). The pH of the extracts was measured at the beginning and the end of the extraction time to verify their stability. The clarified extracts were aliquoted in 2-ml cryogenic vials and stored at −80 °C. Protein concentration for the various extracts was determined using the Dumas combustion method (LECO Corp.) as described earlier and was used to calculate the percentage of recovered protein. Deionized filtered water was used throughout.

## 2.4. Experimental design

The D-optimal experimental design was constructed using the OPTEX procedure in the Statistical Analysis System (SAS software package (version 9.1; SAS Institute Inc., Cary, NC, USA). The single and interactive effects of eight different factors on the percentage of protein recovery, the electrophoretic protein and IgE-binding profiles of the peanut and tree -nut extracts were investigated. The tested factors were type of nut (peanut, almond, hazelnut, or pistachio), roasting and defatting of the nuts, type of extraction buffer (phosphate buffer [pH 7.2], borate buffer [pH 8.5], or carbonate buffer [pH 9.6]), ionic strength of the buffer ( $I = 0.075, 0.15, \text{ or } 0.5$ ), buffer-to-protein ratio (100:1, 150:1, or 250:1 [v/w]), temperature (4, 25, or 50 °C), and duration of extraction (1, 4, or 24 h). The D-optimal design generated 123 extraction trials consisting of various combinations of these factors in random order. The selection of levels for independent variables was based on a review of the relevant literature.

## 2.5. Statistical analysis

The effects of the different factors as well as their two-way interactions on protein recovery were statistically analyzed. All measured protein recovery values were subjected to analyses of variance (ANOVA) and subsequent multiple pairwise comparisons. The qualitative factors (type of nut, type of buffer, roasting, and defatting) were tested using the General Linear Model (GLM) procedure of SAS (version 9.1; SAS Institute Inc.), and the quantitative variables (ionic strength, temperature, duration of extraction, and buffer-to-protein ratio) were assessed by modeling with the SAS response surface regression model PROC RSREG. Comparisons of means were performed using Duncan's multiple range tests. The significances of all terms were judged statistically according to their  $p$ -values. Differences were considered significant at  $p < 0.05$ . Graphics (box plots, main effects plots, and interaction plots) were created with the free trial version of the Minitab 16.0 software program (Minitab Inc., State College, PA, USA).

## 2.6. Protein profiling by gel electrophoresis

Protein profiling of the various peanut and tree nut extracts was carried out by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Before these analyses, aliquots from the peanut and tree nut protein extracts were analyzed for soluble proteins by the Bradford method (Bio-Rad protein assay; Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin (BSA) as the standard protein in appropriate buffers. The aliquots were then standardized to the same protein concentration and mixed with an equal volume of Laemmli sample buffer (62.5 mmol/L Tris-HCl [pH 9.6], 25% glycerol, 2% SDS, 5%  $\beta$ -mercaptoethanol, and 0.01% bromophenol blue) and boiled for 5 min. For protein detection, 9  $\mu$ g of protein for peanut, almond, hazelnut, and pistachio was loaded per well onto 10–20% Criterion Tris-HCl

gels (Bio-Rad Laboratories). For Western blots, 2 µg of protein was loaded per well. The gels were run in TGS buffer (25 mmol/L Tris, 192 mmol/L glycine, and 0.1% SDS) in a Criterion cell (Bio-Rad Laboratories) at 150 V for 90 min. A molecular weight ladder (Precision Plus Protein standard; Bio-Rad Laboratories) was included on each gel. For Western blots, the standard was diluted 1:40 in Laemmli sample buffer, and a pre-stained protein standard marker (Kaleidoscope standard; Bio-Rad Laboratories) was used to monitor transfer efficiency. When the run was finished, the gels were stained with Coomassie Brilliant Blue (Bio-Rad Laboratories) or used for Western blotting. The stained gels were scanned and calibrated using an Image Scanner III (GE Healthcare, Amersham Biosciences, Piscataway, NJ, USA) operated by LabScan 6.0 software (GE Healthcare), and the images were analyzed by ImageQuant TL 7.0 software (GE Healthcare).

## 2.7. IgE immunoblotting

The pattern of specific IgE binding of the various extracts was determined by immunoblotting using human sera or plasma allergic to peanut and the selected tree nuts. Sera and plasma samples were purchased from PlasmaLab International (Everett, WA, USA). Levels of specific IgE were established by ImmunoCAP testing (Phadia, Uppsala, Sweden). For almond extracts, a pool of two sera with almond-specific IgE levels of 5.27 and 7.15 kU/L, respectively, was used. For hazelnut, a serum with a hazelnut-specific IgE level of 53.8 kU/L was chosen. For peanut, a pool was made from three sera with peanut-specific IgE levels of 5.12, 52.8, and 50.7 kU/L, respectively. For pistachio, a plasma with a pistachio-specific IgE level of 57.7 kU/L was used. An atopic control serum from a patient with a history of asthma and dust mite allergy but no food allergies (PlasmaLab International) was used throughout the study. After SDS-PAGE electrophoresis, the proteins were transferred onto polyvinylidene difluoride (PVDF)

membranes (Immun-Blot; Bio-Rad Laboratories) in a Criterion Blotter (Bio-Rad Laboratories) using Tris-glycine transfer buffer (20% methanol [v/v], 25 mmol/L Tris, and 192 mmol/L glycine, pH 8.3) at 100 V for 40 min, after which the membranes were recovered and air-dried. Staining of the gels after transfer showed that these conditions transferred all of the detectable protein from the gel to the membranes (results not shown). The membranes were pre-wet in methanol, washed three times in phosphate-buffered saline with 0.1% Tween-20 (PBST), and then blocked in 5% (w/v) skim milk powder dissolved in PBST for 1 h at room temperature. The membranes were then washed three times in PBST and incubated for 1 h at room temperature with an appropriate dilution of blood serum (1:50 for almond, hazelnut, and pistachio and 1:100 for peanut) with 1% BSA in PBST. After being washed three times with PBST, the membranes were incubated for 1 h at room temperature with horseradish peroxidase conjugated to goat anti-human IgE antibody (Sigma A-9667; Sigma, Saint Louis, MO, USA) at a dilution of 1:3000 as well as with Precision Plus Protein StrepTactin labeled with horseradish peroxidase conjugate (1:7500; Bio-Rad Laboratories). The membranes were washed extensively after incubation and then developed with amplified optimized 4-chloro-1-naphthol (Opti-4CN) reagent (Bio-Rad Laboratories) following the recommendations of the manufacturer. The immunoblots were scanned and analyzed in the same manner as the SDS-PAGE gels.

### 3. Results and discussion

#### 3.1. Effects on protein extractability and recovery

Statistics from the multi-factorial analysis of variance (ANOVA) of global data are listed in Table 1. The model's *p*-value, *R*-squared value, and root mean square error (Root MSE) indicated that there were significant differences in protein recovery and there was a good fit between the model and the experimental data (*R* = 97.88%). The multi-

factorial ANOVA results showed significant differences in protein recovery among the studied nuts. Recovery was significantly affected by roasting ( $p \leq 0.0001$ ), defatting ( $p = 0.0250$ ), type of extraction buffer ( $p = 0.0189$ ), and temperature and buffer-to-protein ratio ( $p = 0.0003$ ). The  $F$ -values indicated that the highest variance was due to roasting ( $F = 94.69$ ), followed by the buffer-to-protein ratio ( $F = 20.15$ ). The effects of the ionic strength of the extraction buffer and the duration of extraction were found to be non-significant overall. The prevailing influence of roasting, extraction temperature, and buffer-to-protein ratio on the level of protein recovery became further evident when illustrated by main effects plots (Fig. 1A). Collectively, roasting had a significant negative impact on the protein recovery levels for all nuts. This result was expected, because it is well known that roasting reduces protein extractability owing to protein denaturation and aggregation (Mills et al., 2009; Scaravelli, Brohée, Marchelli, & van Hengel, 2009; Schmitt et al., 2010). The results also showed that not defatting nut flours prior to extraction lowered protein recovery levels, in comparison with hexane defatting. This difference could be due to the higher loss of lipid-associated protein fractions when the extracted fat layer is skimmed out manually.

Increasing the buffer-to-protein ratio to 250:1 (v/w) and the temperature of extraction to 50 °C resulted in considerably higher protein recovery levels. Although some previous studies reported a non-significant effect of increasing the solvent-to-flour ratio on protein solubility (Sathe, 1992), our result was expected, because a higher solvent content leads to lower viscosity of the solution, thereby promoting molecular diffusion and facilitating protein extraction. Similarly, the mild heating of protein solutions reduces viscosity and could increase mass transfer and solubility (Martínez-Maqueda, Hernández-Ledesma, Amigo, Miralles, & Gómez-Ruiz, 2013). As shown in the response surface graph in Fig. 1A, combining these extraction conditions therefore seemed advantageous in terms of protein recovery but would have required further optimization,

because true maxima were not obtained. However, increasing extraction temperatures above 50 °C is not advisable, because it could lead to irreversible protein denaturation and changes in immunoreactivity, and further increasing the buffer-to-protein ratio was not practical, because it made handling the extract more problematic. Increasing the extraction time also did not improve protein recovery levels (Fig. 1).

The analysis of two-way interactions between the different factors (Table 1) revealed significant interaction between the type of nut and the type of extraction buffer ( $p = 0.0136$ ), meaning that the three studied buffers extracted different proteins from the four nuts under study (Table 1). Pistachio proteins were the least soluble proteins in the aqueous buffers tested (Fig. 1A). In contrast, the lack of significant interaction between other studied factors, such as between roasting and buffer type, between roasting and defatting, or between defatting and buffer type, means that these effects are independent of one another. These observations suggest that optimal extraction conditions might be different among nuts but that for each nut, similar conditions would theoretically apply to both roasted and raw seeds and to both defatted and non-defatted nut flours.

Statistical analysis of the data by type of nut confirmed the negative impact of roasting on protein extractability for all the nuts under study (Table 2). The impact of non-defatting, however, was found to be particularly significant for almond and hazelnut, which could be related to their higher fat content (47.02% and 56.31%, respectively), in comparison with peanut (45.93%). Variations in the effects of the extraction buffer on protein recovery among the nuts were also corroborated (Table 2). Depending on the extraction conditions, the protein yield varied from 19% to 95% for peanut, 31% to 73% for almond, 27% to 88% for hazelnut, and 17% to 64% for pistachio.

The ANOVA results and multiple comparison tests showed that the carbonate and borate buffers were significantly more efficient than the phosphate buffer in solubilizing peanut and almond proteins, whereas for hazelnut and pistachio, the effect of

buffer type was found to be non-significant, despite a tendency for the borate and phosphate buffers to enhance protein recovery levels in comparison with the carbonate buffer (Fig. 2). Analysis of the data by nut revealed that ionic strength had a significant influence on protein recovery for pistachio ( $I = 0.5$  resulting in higher levels) but not for almond, hazelnut, or peanut, despite some noticeable differences, particularly for peanut. These results agree only partially with those in the study by Sathe et al. (2009), who reported a non-significant effect of increasing salt concentration and ionic strength for both almond and pistachio nuts. Splitting the analysis of the data by nut has the disadvantage of reducing the power of statistical tests (the likelihood of detecting differences) because of the smaller numbers of observations for each nut. Because of their importance, the effects of buffer and ionic strength on protein recovery merit further examination.

### 3.2. Effect of extraction conditions on protein and IgE-binding profiles

The determination of protein recovery yields provides a quantitative measurement of total protein extracted under the studied conditions, but additional information about the qualitative and IgE-binding aspects of the extracts was needed in order to better characterize the ability of the different sets of conditions to extract fully representative and immunoreactive protein/allergen profiles. This task is usually quite challenging, because allergenic foods contain a mixture of allergenic and non-allergenic proteins with diverse structural and chemical properties; ideally, extraction conditions should be optimized so that the maximum number of specific proteins and the largest possible amount of proteins are extracted from the food of interest (Westphal et al., 2004).

As shown in Fig. 3, clear differences were observed in the resolved protein banding patterns and IgE-binding profiles among the extracts of all nuts. For peanut

(Fig. 3A), roasting clearly had a negative effect on the extractability of Ara h 1 (64 kDa) as well as an unidentified 29-kDa polypeptide; however, other peanut proteins did not seem to be affected by roasting. Surprisingly, defatting favored the extraction of low-molecular-weight polypeptides generally associated with Ara h 2 (doublet at 17 and 19 kDa), Ara h 6 (15 kDa), and Ara h 7 (15.8 kDa) ([www.allergome.org](http://www.allergome.org); Allergome Platform, 2014). The results also showed that carbonate extracted a high-molecular-weight (MW) polypeptide (338 kDa) from all roasted, non-roasted, defatted, and non-defatted samples. The appearance of this polypeptide was associated with the disappearance of the 64-kDa polypeptide of Ara h 1, suggesting that the 338 kDa polypeptide could be an aggregated form of the 11S globulin Ara h 1. Some protein degradation due to proteolytic activity *in situ* was clearly observed, particularly for the phosphate buffer extracts (pH 7.2) and the long extraction period (24 h), given the appearance of new hydrolyzed protein fragments.

For almond (Fig. 3B), roasting and defatting strongly affected protein SDS-PAGE and IgE-binding profiles. The acidic  $\alpha$ -chains (40 and 42 kDa) and basic  $\beta$ -chains (20 and 22 kDa) of Pru du 6 (amandin 11S globulin) were less affected, however, and seemed to retain IgE-binding potency even after harsh heat-processing conditions. The results also showed that phosphate and borate favored the extraction of IgE-reactive bands with MW less than 10 kDa in non-roasted almond.

Hazelnut protein and IgE-binding profiles were visually unaffected by roasting but appeared to be affected negatively by defatting (Fig. 3C). In particular, the extractability of polypeptides with of 50, 17, 11, and 13 kDa was better in non-defatted hazelnut samples. These polypeptides may correspond to the allergenic proteins vicilin Cor a 11, fat-soluble/associated protein oleosin Cor a 12, lipid-transfer protein ()Cor a 8, and oleosin Cor a 13, respectively (Allergome Platform, 2014). These results are different from those obtained by Platteau et al. (2010), who reported that defatting had no impact

on hazelnut SDS and IgE profiles. Our findings are, however, supported by the fact that oleosins are localized in oil bodies and could therefore be lost during the defatting process.

For pistachio (Fig. 3D), roasting seemed to increase the IgE reactivity of two protein bands of 55 and 53 kDa that potentially correspond to the allergens Pis v 3 and Pis v 2/Pis v 5, respectively. As with hazelnut, non-defatting favored the recovery of 17- and 11-kDa polypeptide bands for pistachio. In addition, SDS-PAGE and IgE immunoblotting revealed important differences in protein and IgE-binding profiles among the various buffer systems, where the phosphate buffer in particular increased the extraction of polypeptide bands with of 36, 10, and 9 kDa.

For all the nuts studied, the SDS-PAGE analyses revealed important protein degradation for the 24-h extraction period. The optimal extraction time is a compromise between yield and the degradation/denaturation of the allergens (Larsen & Dreborg, 2008). In general, extraction time should be minimized, and extraction should be performed at low temperatures; protease inhibitors are also often added during allergen extraction to prevent proteolytic degradation and preserve the allergenic activity. The addition of such inhibitors, however, is generally not recommended in extracts intended for *in vivo* applications (Rudeschko, Fahlbusch, Henzgen, Schlenvoigt, Herrmann & Jager, 1995). To minimize protein degradation, an extraction duration of 1 h would be recommended, particularly because increasing the extraction time did not improve protein recovery levels.

#### 4. Conclusions

In this study, the use of a D-optimal design allowed us to explore both the individual and interaction effects of several extraction factors simultaneously, in order to identify the conditions that resulted in improved protein extractability and recovery levels.

The investigation of protein and IgE-binding profiles added crucial information about the qualitative aspects of the extraction. Our results demonstrated that high protein recovery levels did not always correlate with high immunoreactivity.

In summary, it can be concluded that roasting had a significant negative impact on protein recovery levels for all nuts. Roasting also strongly affected almond protein IgE-binding. Not defatting lowered protein recovery levels, in comparison with hexane defatting, particularly for almond and hazelnut, but analysis of protein electrophoretic profiles revealed a favorable impact on the recovery of some hydrophobic immunoreactive polypeptides. The lack of interaction between the effects of roasting and defatting and of other factors suggests that the same extraction conditions would theoretically apply to roasted or raw, and to defatted or non-defatted nut flours.

Furthermore, the present study showed that protein recovery increased significantly with increased temperature and increased ratio of extraction buffer to protein, with no major impact on the protein and immunoreactivity profiles. Increasing the extraction time, however, resulted in increased proteolytic degradation with no improvement in protein recovery.

The impact of various ionic strengths on protein recovery was statistically inconclusive. However, the modulation of protein and IgE-binding profiles by ionic strength was clearly demonstrated by the SDS-PAGE and immunoblotting analyses. The results of D-optimal plans are generally viewed as exploratory so that initial information on significant effects can be collected when several factors (eight in this study) are examined and where it is impossible to use a plan with only repetitions.

Part 2 of this study, which will appear in a separate article, will be devoted to assessing the impact of ionic strength along with buffer type on protein extractability and immunoreactivity using a decisive full factorial design.

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482 **Figures captions**483 Fig. 1. A) Main effect plots for protein recovery (%). B) Surface plot of protein  
484 recovery (%) vs buffer-to-protein ratio and temperature (°C).

485 Fig. 2. Interaction plots for protein recovery (%).

486 Fig. 3. Impact of various combinations of extraction conditions on protein profile,  
487 IgE-binding and recovery. A) Peanut; B) Almond; C) Hazelnut; D) Pistachio; R:  
488 Roasted; N: Non-Roasted; B: Borate; C: Carbonate; P: Phosphate; I: Ionic  
489 Strength; T: Temperature; R: Ratio.

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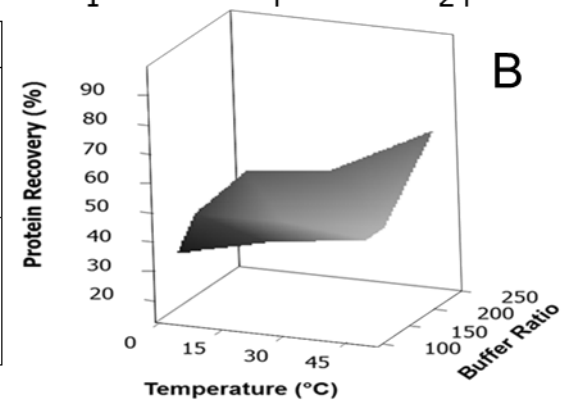
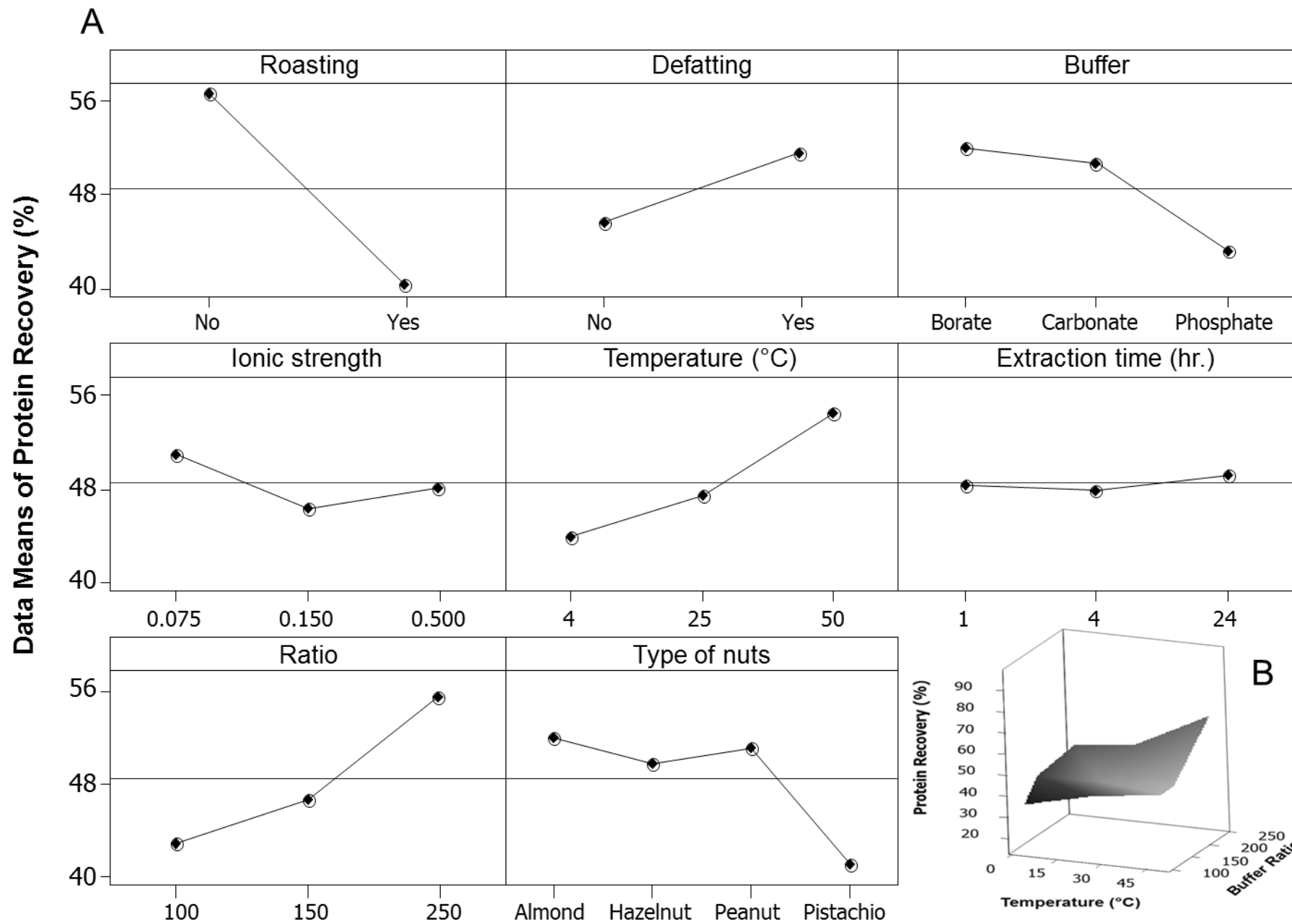
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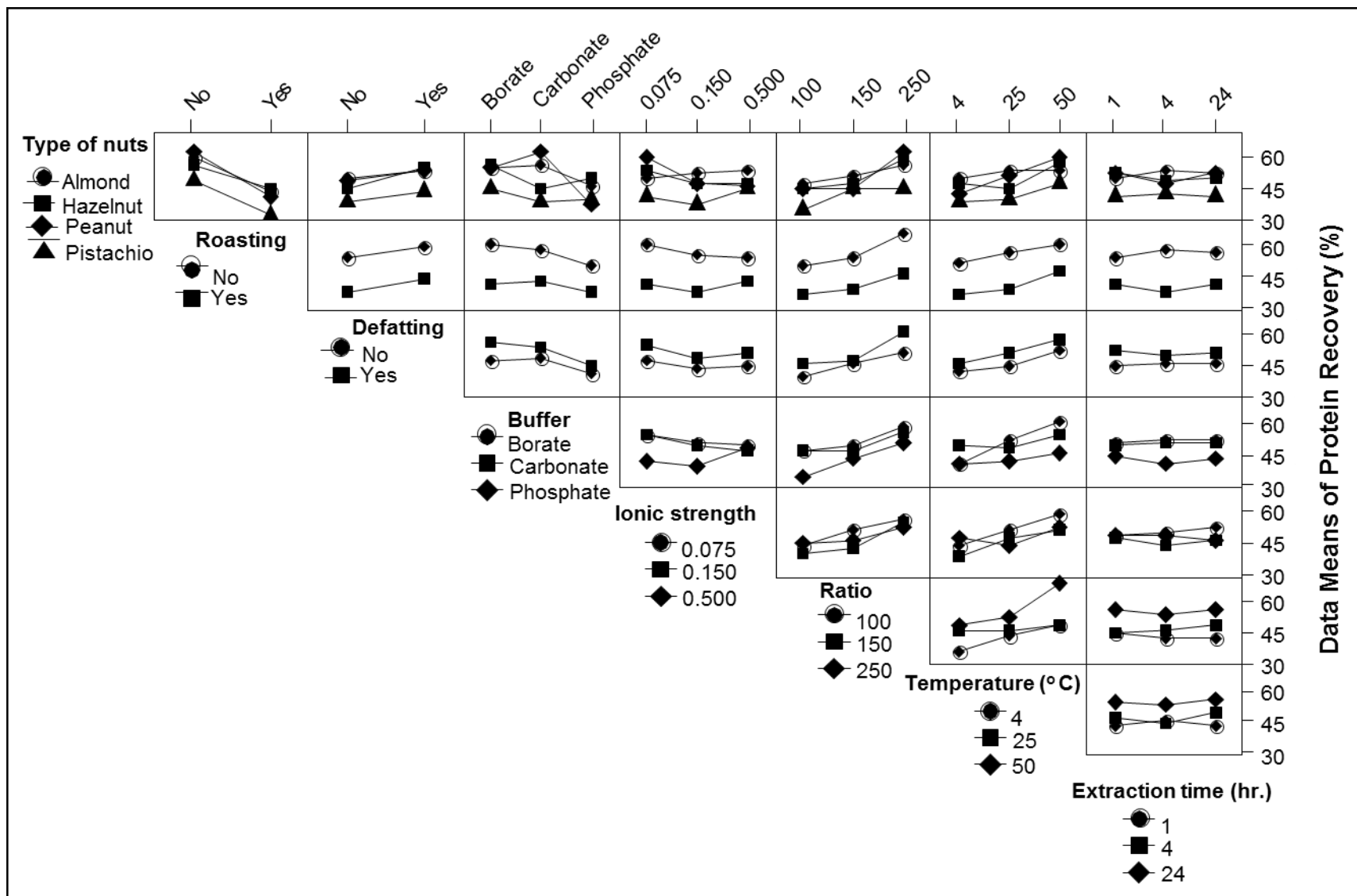
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Surface Plot of Protein Recovery (%) vs Buffer Ratio, Temperature (°C)



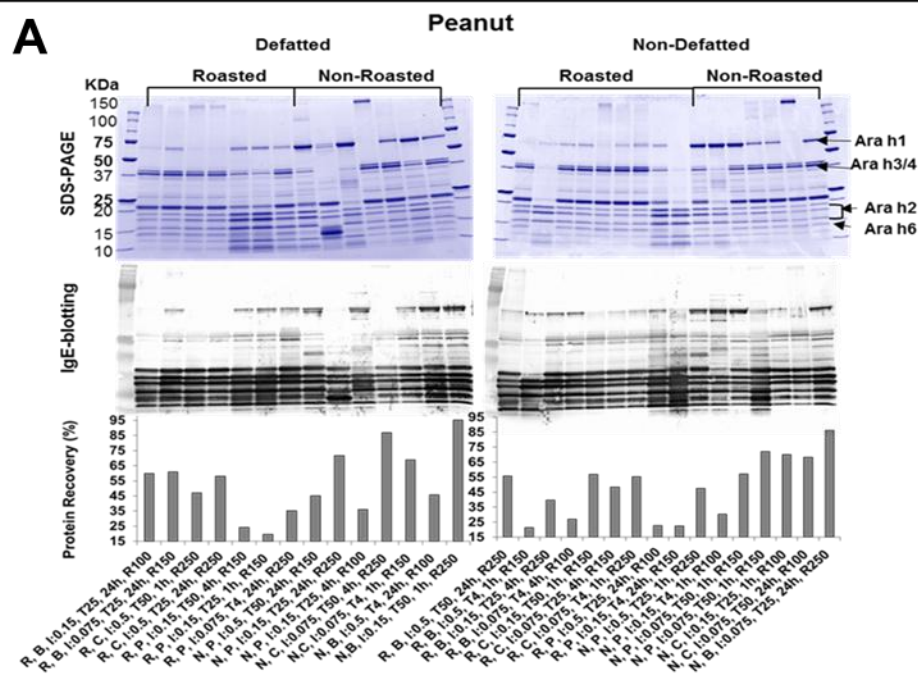
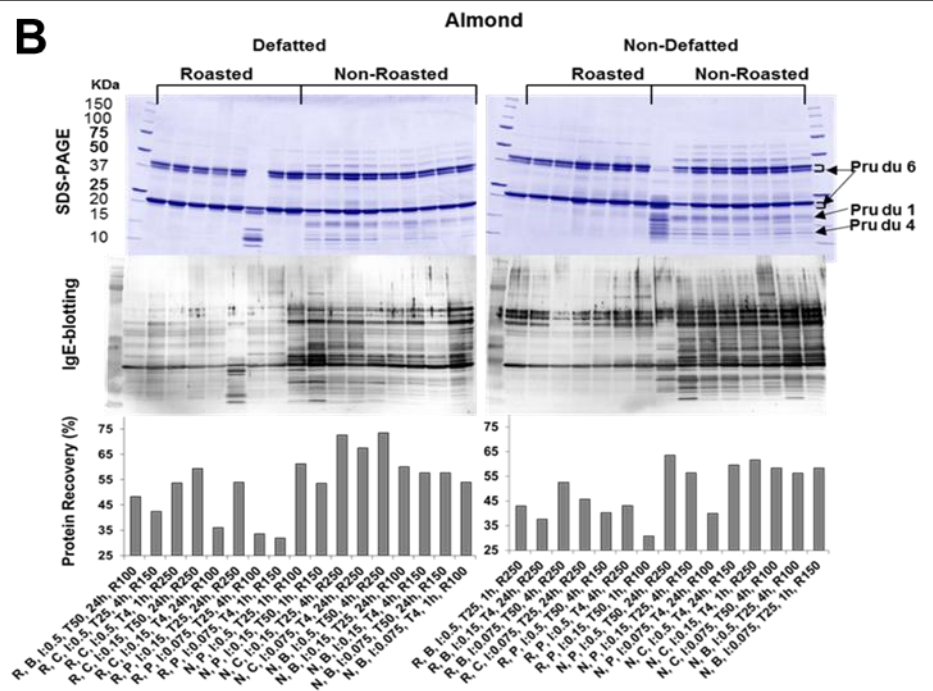
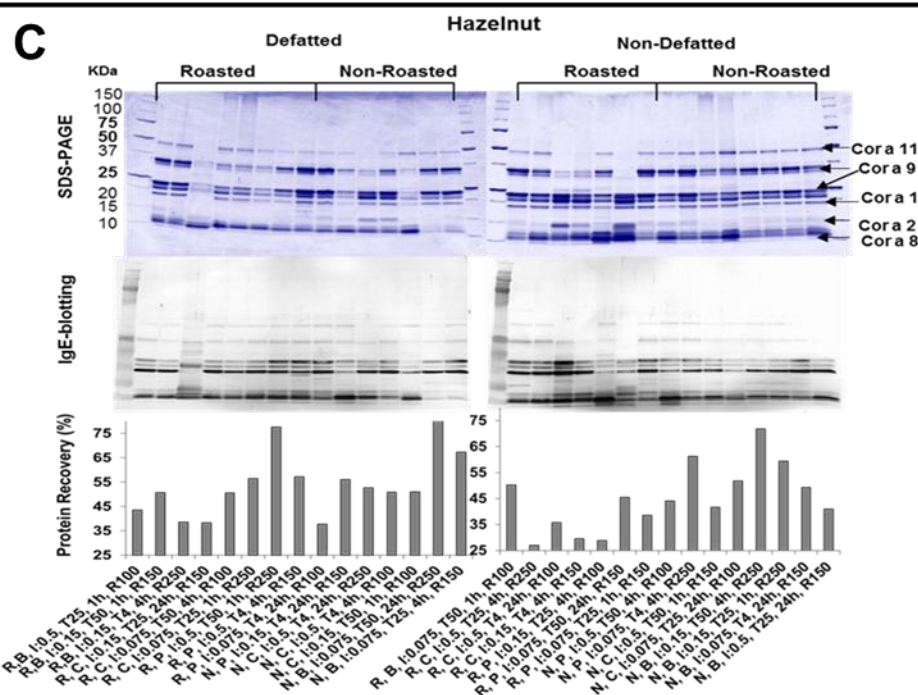
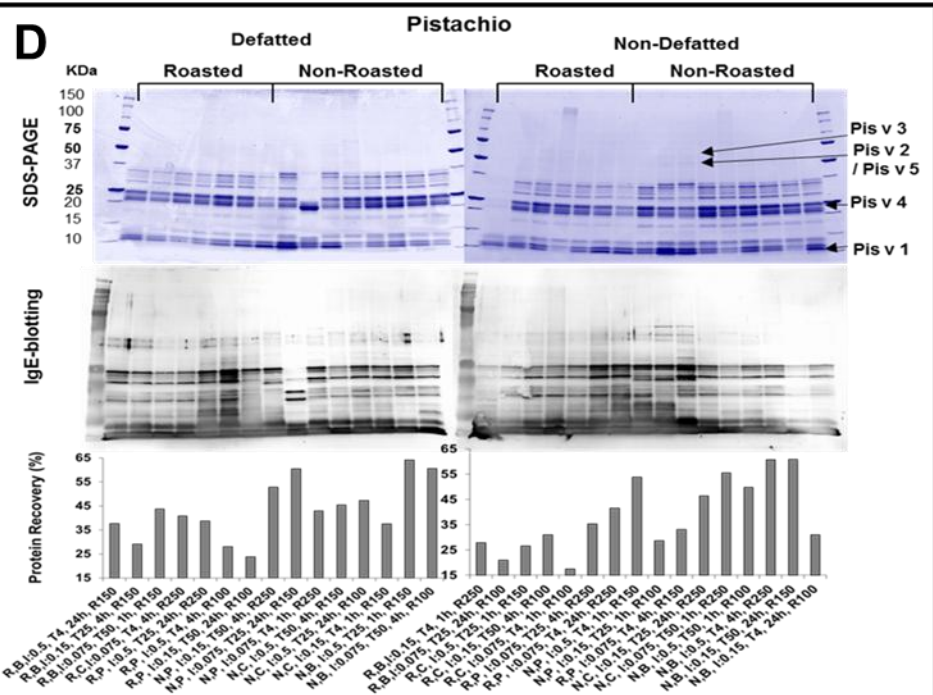
**A****B****C****D**

**Table 1.** Multi-factorial analysis of variance (ANOVA) of global data.<sup>a</sup> The *F*-value for a term is the test for comparing the variance associated with that term with the

| Source                        | DF       | Sum of Squares | Mean Square | <i>F</i> Value <sup>a</sup> | <i>p</i> -Value <sup>b</sup> |
|-------------------------------|----------|----------------|-------------|-----------------------------|------------------------------|
| Model                         | 112      | 29288.93       | 261.51      | 4.13                        | 0.0088                       |
| Error                         | 10       | 632.61         | 63.26       |                             |                              |
| Corrected Total               | 122      | 29921.54       |             |                             |                              |
| R-Square                      | Root MSE | Mean           | CV          |                             |                              |
| 0.98                          | 7.95     | 48.51          | 16.40       |                             |                              |
| Source                        | DF       | Type III SS    | Mean Square | <i>F</i> Value <sup>a</sup> | <i>p</i> -Value <sup>b</sup> |
| A: Type of nuts               | 3        | 1633.67        | 544.56      | 8.61                        | <b>0.0040</b>                |
| B: Roasting                   | 1        | 5990.22        | 5990.22     | 94.69                       | <b>&lt;.0001</b>             |
| C: Defatting                  | 1        | 438.76         | 438.76      | 6.94                        | <b>0.0250</b>                |
| D: Type of buffer             | 2        | 766.56         | 383.28      | 6.06                        | <b>0.0189</b>                |
| E: Ionic strength             | 2        | 219.45         | 109.73      | 1.73                        | 0.2256                       |
| F: Temperature                | 2        | 926.84         | 463.42      | 7.33                        | <b>0.0110</b>                |
| G: Extraction duration        | 2        | 70.09          | 35.04       | 0.55                        | 0.5913                       |
| H: Ratio of buffer to protein | 2        | 2549.04        | 1274.52     | 20.15                       | <b>0.0003</b>                |
| <b>Interactions</b>           |          |                |             |                             |                              |
| A*B                           | 3        | 209.08         | 69.69       | 1.10                        | 0.3933                       |
| A*C                           | 3        | 68.62          | 22.87       | 0.36                        | 0.7822                       |
| B*C                           | 1        | 30.09          | 30.09       | 0.48                        | 0.5061                       |
| A*D                           | 6        | 1866.47        | 311.08      | 4.92                        | <b>0.0136</b>                |
| B*D                           | 2        | 95.45          | 47.73       | 0.75                        | 0.4953                       |
| C*D                           | 2        | 155.65         | 77.83       | 1.23                        | 0.3329                       |
| A*E                           | 6        | 547.74         | 91.29       | 1.44                        | 0.2897                       |
| B*E                           | 2        | 171.05         | 85.52       | 1.35                        | 0.3022                       |
| C*E                           | 2        | 28.82          | 14.41       | 0.23                        | 0.8003                       |
| D*E                           | 4        | 90.78          | 22.68       | 0.36                        | 0.8325                       |
| A*F                           | 6        | 220.24         | 36.78       | 0.58                        | 0.7391                       |
| B*F                           | 2        | 305.58         | 152.79      | 2.42                        | 0.1394                       |
| C*F                           | 2        | 154.32         | 77.16       | 1.22                        | 0.3357                       |
| A*F                           | 4        | 314.64         | 78.66       | 1.24                        | 0.3535                       |
| E*F                           | 4        | 278.97         | 69.74       | 1.10                        | 0.4072                       |
| A*G                           | 6        | 210.73         | 35.12       | 0.56                        | 0.7567                       |
| B*G                           | 2        | 34.09          | 17.05       | 0.27                        | 0.7692                       |
| C*G                           | 2        | 45.71          | 22.85       | 0.36                        | 0.7055                       |
| D*G                           | 4        | 132.01         | 33.00       | 0.52                        | 0.7223                       |
| E*G                           | 4        | 107.29         | 26.82       | 0.42                        | 0.7882                       |
| F*G                           | 4        | 67.71          | 16.93       | 0.27                        | 0.8922                       |
| A*H                           | 6        | 86.07          | 14.35       | 0.23                        | 0.9586                       |
| B*H                           | 2        | 122.34         | 61.17       | 0.97                        | 0.4131                       |
| C*H                           | 2        | 12.75          | 6.37        | 0.10                        | 0.9050                       |
| D*H                           | 4        | 247.97         | 61.99       | 0.98                        | 0.4608                       |
| E*H                           | 4        | 296.54         | 74.13       | 1.17                        | 0.3798                       |
| F*H                           | 4        | 231.93         | 57.98       | 0.92                        | 0.4912                       |
| G*H                           | 4        | 118.26         | 29.56       | 0.47                        | 0.7588                       |

residual variance. It is the mean square for the term divided by the mean square for the residual.

<sup>b</sup> This is the probability value that is associated with the *F*-value for this term. It is the probability of getting an *F*-value of this size if the term did not have an effect on the response. In general, a term that has a probability value less than 0.05 would be considered a significant effect; a probability value greater than 0.10 is generally regarded as not significant. Values significant at the 5% level are printed in bold-faced type.

**Table 2.** Analysis of variance (ANOVA) of independent variables for protein recovery by type of nut.

| Statistical Significance on % Protein Recovery ( <i>p</i> -value)* |                   |                   |               |                   |
|--------------------------------------------------------------------|-------------------|-------------------|---------------|-------------------|
| Source                                                             | Peanut            | Almond            | Hazelnut      | Pistachio         |
| Roasting                                                           | <b>&lt;0.0001</b> | <b>&lt;0.0001</b> | <b>0.0040</b> | <b>&lt;0.0001</b> |
| Defatting                                                          | 0.4598            | <b>0.0024</b>     | <b>0.0046</b> | 0.3548            |
| Buffer type                                                        | <b>&lt;0.0001</b> | <b>0.0256</b>     | 0.2053        | 0.1578            |
| Ionic strength                                                     | 0.0562            | 0.1332            | 0.1477        | <b>0.0376</b>     |
| Temperature                                                        | <b>0.0186</b>     | <b>0.0082</b>     | 0.0742        | <b>0.0444</b>     |
| Extraction time                                                    | 0.0610            | 0.7188            | 0.9595        | 0.5736            |
| Ratio of buffer                                                    | <b>0.0016</b>     | <b>&lt;0.0001</b> | <b>0.0258</b> | <b>0.0054</b>     |
| Protein Recovery (%)                                               |                   |                   |               |                   |
| Number of assays                                                   | 30                | 32                | 30            | 31                |
| Minimum recovery                                                   | 19.5              | 30.8              | 27.0          | 17.4              |
| Maximum                                                            | 95.2              | 73.4              | 87.7          | 64.2              |
| recovery                                                           | 20.9              | 11.4              | 14.0          | 13.2              |
| STD                                                                |                   |                   |               |                   |

\* Values significant at the 5% level are printed in bold-faced type.

**Full title:**

Quantitative and qualitative optimization of allergen extraction from peanut and selected tree nuts. Part 1. Screening of optimal extraction conditions using a D-optimal experimental design.

Lamia L'Hocine and Mélanie Pitre

**Short Running title:**

Quantitative and qualitative optimization of allergen extraction-Part1

**Highlights**

- Optimization of allergen extraction from selected nuts was investigated using a D-optimal design.
- Extraction with high protein recovery level did not always correlate with high Immunoglobuline E (IgE)-binding.
- Roasting decreased protein recovery from all nuts, and particularly affected almond IgE-binding.
- Defatting improved almond and hazelnut protein recovery, but affected IgE-binding.
- Protein recovery was significantly improved by increasing the buffer-to-protein ratio during extraction.