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Effect of roasting history and buffer composition on peanut protein extraction efficiency

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Peanut is a major allergenic food. Undeclared peanut (allergens) from mis-formulation or contamination during food processing pose a potential risk for sensitized individuals and must be avoided. Reliable detection and quantification methods for food allergens are necessary in order to ensure compliance with food labelling and to improve consumer protection. The extraction of proteins from allergenic foods and complex food products is an important step in any allergen detection method. In this study, the protein extraction efficiency of various buffers prepared in-house and some extraction buffers included in some commercial allergen enzyme-linked immunosorbent assay (ELISA) test kits for peanut determination in food products were tested. In addition, the effect of roasting history on the extractability of peanut protein was investigated by the biuret and the bicinchoninic acid (BCA) assays. Elevated roasting temperatures in food processing were found to have a major impact on protein extraction efficiency by reducing protein yields of oil and dry roasted peanuts by 50 – 75% and 75 – 80%, respectively, compared with the raw material. Extraction buffers operating in the higher pH range (pH 8 – 11) showed best yields.

Keywords: Allergen / Peanut / Protein extraction

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1 Introduction

Peanut ranks among the most important allergenic foods [1]. Epidemiological studies estimate that peanut allergy affects approximately 0.6% of the general population in the USA [2] and 0.5% in the UK [3]. Peanut allergy accounts for 10 – 47% of food-induced anaphylactic reactions and for more than 50% of food allergy fatalities [4]. In a highly sensitized individual, minute amounts of peanut (some mg or even less) are capable of inducing allergic reactions [5 – 7] ranging from mild to severe gastrointestinal (nausea, diarrhoea), respiratory (rhinitis, asthma), and skin reactions (acute urticaria) [8]. For some allergic individuals, the contact with a certain food allergen can even lead to severe systemic reactions (anaphylaxis) that are potentially fatal [9].

For an allergic individual total avoidance of the allergen-containing food is currently the only effective treatment. Sensitive individuals may also be inadvertently exposed to allergenic protein by consumption of supposedly peanut-free foods contaminated with hazelnut or peanut proteins. This can occur during shipping and storage, during processing, from carry-over due to inadequate cleaning of shared processing equipment, or in reuse (rework) of allergen-containing products [10, 11]. Peanut may be present in breakfast cereals, ice cream, and in highly processed, refined products, such as syrups, sauces, soups, *etc.* [12], and it has been detected in supposedly peanut-free chocolate and biscuits [13], however, contamination of other food matrices are likely to occur.

It is important that products not declared to contain peanut are truly free of the respective protein. Reliable detection and quantification methods for food allergens are necessary in order to ensure compliance with food labelling and to improve consumer protection. Various immunochemical and DNA-based methods for the determination of peanut in food products have been published (summarized in [14]), including several ELISAs. Recently, several ELISA test kits for peanut determination in food products have become commercially available [15]. It was shown that the analytical results obtained with different peanut allergen detection kits may vary substantially [16, 17]. In some cases, highly processed peanuts may not be detected by commercial ELISA kits, but the same samples showed high binding capacity to human sera immunoglobulin E (IgE) from pea-

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Abbreviations: BCA, bicinchoninic acid; **Ig**, immunoglobulin

nut allergic individuals ([18]; Poms unpublished). This may be due to low affinity of the employed animal IgG antibodies of the ELISA assay to the antigen (*e. g.*, antibodies raised against raw material can have low affinity to heat-treated analyte) or inefficient extraction procedures (matrix effects, impaired solubility of denatured proteins, hydrophobic proteins remain bound in the food matrix). Moreover, animal IgG and patient IgE might react to different epitopes having different stabilities (*e. g.*, after heat processing) and neo-epitopes might be generated by food processing, which are variably recognized by IgG and IgE antibodies.

In this study, we investigated the influence of various commonly used processing procedures (*i. e.*, different heat treatments) on the extraction efficiency for peanut protein. In addition, we tested the performance of several commercially available extraction buffers, which are included in ELISA kits for peanut detection in food. We also tested various extraction buffers produced in-house for the extraction of peanut protein, which might have the potential to improve protein extraction efficiency from allergenic foods and maybe also from complex food matrices.

2 Materials and methods

2.1 Test samples

Raw American and Chinese peanuts were obtained from the University of Vienna (Vienna, Austria). Raw peanuts were either used in the original state (raw, unshelled) or after further heat-processing in-house by employing conventional industrial temperature/time profiles. Dry roasted and oil-roasted peanuts were produced to yield light and dark products, respectively. Prior to roasting the peanuts where dry-blanched at 120°C for 20 min in a General Electric oven, Model JRP 36 GIV 3 SB, maximum power 3770 W, and shelled manually. For the production of dry-roasted peanuts the blanched, shelled peanuts were heated under circulating hot air in the General Electric oven at 160°C for 40 min and 60 min, and at 190°C for 20 min and 25 min, respectively. To produce oil-roasted peanuts, the blanched, shelled peanuts were fried in refined peanut oil (obtained from a local supermarket) in a household deep fryer (Mulinex Olea plus, France) at 140°C for 5 min and 10 min, and at 160°C for 3 and 5 min, respectively. After frying excess oil was removed by agitating the fried peanuts between two sheets of absorbent paper for about 30 s. Dry roasted and oil roasted peanuts, respectively, were cooled rapidly after heat-processing and kept at −20°C until analysis. Samples from industry were dry-roasted peanuts (light color) received from Nestlé Česko S.R.O. (Czech Republic) and oil-roasted peanuts (light color) were obtained from Migros (Zürich, Switzerland). The peanut products are listed in Table 1.

Table 1. List of various peanut products prepared in-house and obtained from commercial sources

Origin of peanuts	Processng	Roasting temperature (°C)	Roasting time (min)
American and Chinese	Raw, unshelled	–	–
	Blanched, shelled	120	20
	Oil-roasted	140	5
	Oil-roasted	140	10
	Oil-roasted	160	3
	Oil-roasted	160	5
	Dry-roasted	160	40
	Dry-roasted	160	60
	Dry-roasted	190	20
	Dry-roasted	190	25
Nestle	Dry-roasted	?	?
Migros	Oil-roasted	?	?

2.2 Extraction buffers

Various buffers were used to extract peanut proteins (Table 2). All salts necessary for preparing the buffers were obtained from Sigma (St. Louis, MO, USA) and purified water (Millipore, Bedford, MA, USA) was used to prepare the solutions. Concentrated and 1 M HCl (hydrochloric acid), and 1 M sodium hydroxide (NaOH) were used to adjust pH to the desired levels. Two citrate buffers (each with 1.6 M citric acid 1-hydrate and 1.6 M sodium citrate 2-hydrate) at pH 3 and 4, respectively; two TBS buffers (each with 20 mM Tris and 150 mM sodium chloride) at pH 7.4 and 8.2, respectively; a PBS buffer (with 2 mM potassium chloride, 1 mM potassium phosphate, 136 mM sodium chloride, and 8 mM sodium phosphate), a 6 M urea buffer, and two borate buffers at concentrations of 7 mM (0.3%) and 15 mM (0.6%), respectively, and a sodium carbonate buffer (50 mM) were prepared in-house. In addition to the buffers prepared in-house, several commercial buffers, which are included in commercial ELISA test kits for peanut allergen detection in food products, were tested. The commercial buffers were taken from the BioSystems Peanut kit from (Tepnel Deeside, Flintshire, UK; TBS: 6 g/L Tris, 11.6 g/L sodium chloride, pH 8.2), the Peanut Residue kit from ElisaSystems (Windsor, Queensland, Australia; no information of composition), Prolisa Peanut PAK from Pro-Lab (Richmond Hill, Ontario, Canada, no information of composition), the Ridascreen Peanut from R-Biopharm (Darmstadt, Germany; no information of composition), and the buffer from the Veratox Peanut Kit from Neogen (Lansing, MI, USA; PBS: 0.01 sodium phosphate, 0.138 M sodium chloride, 0.0027 M potassium chloride, pH 7.4). The commercial buffers were prepared according to the manufacturers' instructions. Extraction additives, or skim milk powder and fish gelatine, recommended for the extraction procedure for complex food matrices (*e. g.*, dark chocolate) were not included in the extraction protocol, in order to make a protein determination feasible.

Table 2. List of tested extraction buffers (prepared in-house and commercially available)

Source	No.	Buffer	pH
Exraction buffer prepared in-house	1	Citrate 3	3
	2	Citrate 4	4
	3	TBS pH 7.4	7.4
	4	PBS	7.4
	5	Water bidest.	7.5
	6	TBS pH 8.2	8.2
	8	Urea	8.7
	9	Sodium borate (0.3%)	9.2
	10	Sodium borate (0.6%)	9.2
	11	Sodium carbonate (50 mM)	11
Commercial extraction buffer	12	BioKits Tepnel	8.2
	13	ElisaSystems	7.6
	14	Prolisa-Prolab	6.7
	15	Ridascreen-R-Biopharm	8.4
	17	Veratox-Neogen	7.4

2.3 Sample preparation

Peanuts were ground in liquid nitrogen in a Steril Mixer II (PBI International) for 30 s and subsequently dried in an oven at 30 ± 3°C and over silica gel overnight, to evaporate the residual nitrogen and the condensed moisture (due to the temperature difference of the liquid nitrogen and the surrounding environment).

Each extraction was done at a sample to buffer ratio of 1 : 25 by weighing 1 g fine ground peanut flour into 50 mL plastic tubes and adding 25 mL of the respective extraction buffer. Extraction was done either at 60°C for 20 min, or at ambient temperature or 4°C over night. All extractions were done under constant shaking at approx. 300 rpm. The crude extract was cleared by centrifugation at 5000 rpm (approx. 3000 × *g*). Twice the supernatant was extracted with *n*-hexane. The defatted extract was aliquoted and kept at 4°C until analysis within 48 h or frozen for future use.

2.4 Protein quantitation

Soluble protein in the peanut extracts was determined by the biuret and the BCA assays. The biuret assay was performed to get a rough estimate of the protein content and to determine the dilution factor for the more accurate BCA protein assay. The biuret reagent (Fluka, Buchs, Switzerland; ready-to-use) standards (0, 1, 2.5, 5, 7.5, and 10 mg/mL) were prepared with BSA (bovine serum albumin; Sigma). The BCA kit including calibration standards (BSA at 1 mg/mL to be diluted to 0, 0.2, 0.4, 0.6, 0.8, 1 mg/mL) was received from Sigma. All dilutions were made in 0.15 M sodium chloride solution. In short, for the biuret assay 500 µL standard or sample extract were incubated with 500 µL water water and 2000 µL biuret reagent at 37°C for 20 min and the absorbance was measured at 540 nm wavelength on a UV 500 UV-visible spectrometer from UNICAM. For the BCA assay a working reagent consisting of 1 : 50 copper II sulfate : BCA was prepared. Hundred µL standard and sample were each incubated with 2000 µL BCA working reagent at 37°C for 30 min. Absorbance was measured at 562 nm wavelength. For quantitative analysis the absorbances obtained with the sample extracts were plotted against readings of the standards. Blank values were determined by analyzing extraction buffers (no sample protein) and these values were substracted from the calculated yield.

3 Results and discussion

Information about processing parameters commonly employed by the food industry was collected from the literature [19] and various peanut manufacturers in Europe and North America (personal communications). Typically, oil-roasted peanuts are used in snacks (such as nut-mixes) and biscuits whereas dry-roasted peanuts are more often used in confectionary due to their strong aromatic properties. Compared to dry-roasting the temperatures and times for oil-roasting are typically lower and respectively shorter, due to the faster and more efficient heat transfer (to the core of the kernels) in hot oil than by hot air. Temperature/time profiles were selected to produce light and dark products of oil and dry roasted American and Chinese peanuts in-house (see Table 1), in order to determine the effect of the roasting history on the extraction efficiency by employing various extraction buffers. In this study, two different temperatures and two different roasting times at each temperature were investigated for each procedure (oil- and dry-roasting), thus the lower and upper limits (according to the information by the manufactures) for each procedure and product (light and dark) were covered.

Extraction with Tris-buffered saline (TBS, pH 8.2) at 4°C overnight yielded approximately 127 mg and 104 mg of peanut protein from American and Chinese peanuts, respectively (Fig. 1). These quantities correspond to about 40 – 50% of the average total protein content in peanuts [20]. Generally, approximately 20% less protein was extracted from Chinese peanuts compared to American. In relation to raw peanuts the extraction efficiency decreased with the degree of processing, *i.e.*, with increasing temperatures (Fig. 1). Dry-blanching at 120°C for 20 min did not seem to significantly affect the protein solubility. However, oil-roasting decreased the yield of soluble peanut protein by approx. 50% and dry-roasting by 75 – 80% after TBS (pH 8.2) extraction. The roasting temperature appeared to affect the solubility of peanut proteins to a greater extent than the time of exposure to the respective roasting temperatures

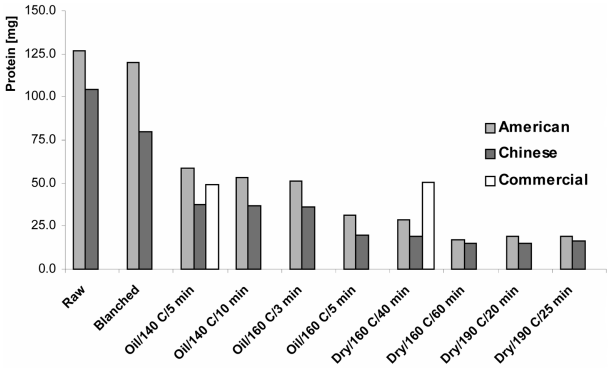

Figure 1. Effect of heat-processing on the extraction efficiency of protein peanut. Yield of peanut protein per gram peanut material, which had undergone various roasting procedures, after extraction with TBS (pH 8.2) at 4°C overnight.

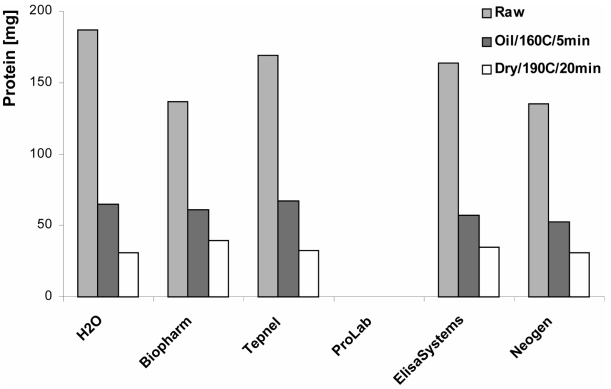

Figure 2. Protein extraction efficiency of various commercial allergen extraction buffers. Yield of peanut protein per gram peanut material, which had undergone various processing procedures, after extraction with purified water, and extraction buffers included in commercial peanut allergen detection kits at 60°C for 20 min.

(140 – 190°C), when time and temperature profiles commonly employed in industry were investigated. However, in previous studies the time of exposure at a constant roasting temperature showed a significant effect on the detectability of peanut protein by specific peanut ELISA tests [17, 18], when extended roasting times were tested (in part beyond the lower and higher limits of commonly employed protocols in peanut manufacturing). This indicates that the time of exposure to a certain roasting temperature might show a stronger effect on the antigen recognition by the IgG antibodies of ELISA test systems (due to alteration of specific epitopes) than the effect on the change of the general solubility of total peanut protein.

The quantities of extracted protein of the commercial peanut samples were at a comparable level with yields obtained with the peanuts processed in-house employing the lower temperature/time profile for oil- and dry-roasted peanuts, respectively. This confirmed the relevance of the processing parameters selected for the in-house production. When commercial protein extraction buffers, usually included in commercial allergen detection test kits, and water were tested at 60°C the difference in yield for raw, oil-roasted (160°C, 5 min) and dry-roasted peanuts (190°C, 20 min) followed a similar pattern (see Fig. 2). Extracted protein quantities ranged between 187 and 135 mg soluble protein/g whole peanut and the yield was decreased for oil-roasted peanuts by approximately 75% and for dry-roasted peanuts by 80%. All of the commercial buffers rendered similar results and they did not differ significantly from the yield of extracted protein obtained with water at 60°C. The quantity of the extracted protein employing the extraction buffer of the Prolisa-Prolab Peanut PAK could not be determined due to a strong background from the extraction buffer (contained about 23 mg/mL protein). Providers of the commercial extraction buffers claim that their buffers had been optimized for the extraction of peanut protein from a variety of food matrices and they also take into account the compatibility of the extraction buffer with the ELISA system. Therefore, they are operating in a neutral pH range and may not include denaturing reagents. However, they seem to perform poorly for the extraction of (highly) processed protein. A sufficient quantity of extracted protein is the prerequisite for a successful detection of low, but relevant amounts of allergenic foods/proteins from potentially "contaminated" food products. Apparently, an inefficient extraction procedure may be the reason – at least in part – for false-negative results in some analyses of spiked/contaminated food products (Poms, unpublished).

In search for a superior extraction protocol we investigated the performance of 11 different protein extraction buffers for hazelnut and peanut proteins (Table 2). Ten buffers were prepared in-house, and purified water was also included (Table 2). Each buffer system was incubated at two different temperatures (4°C overnight, and 60°C for 20 min), respectively. Performance characteristics were assessed by determining the yield of extractable protein by the biuret and BCA assays and the obtained resolution after protein separation with SDS-PAGE. The effects of pH and salt concentration of the various buffers and the extraction temperature/time were evaluated. Results are illustrated in Fig. 3.

For peanut protein extraction efficiency the most significant factor appeared to be the pH of the employed extraction buffer (Fig. 3). Best yields were obtained with buffers in the range of pH 8 – 11. Most protein was extracted with the sodium borate buffers at pH 9, different salt concentrations (0.3% and 0.6%) did not majorly affect the extraction efficiency for peanut protein. The impact of the pH was also clearly notable by comparing the yields of the two TBS buffers at pH 7.4 and 8.2. The TBS buffer at the higher

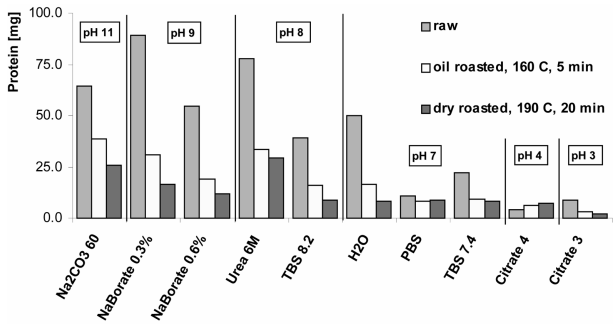

Figure 3. Protein extraction efficiency of various extraction buffers for the extraction of protein from differently processed peanuts. Yield of peanut protein per g peanut material after extraction with nine buffers prepared in-house and purified water at 60°C for 20 min.

pH (8.2) extracted approximately 35% more protein from peanut than the TBS buffer at pH 7.4. The citrate buffers at pH 3 and 4, purified water, the PBS buffer, and the commercial extraction buffer from one commercial ELISA test kit extracted similar quantities of peanut protein. Yields for these buffers ranged between 6 and 12 mg/mL. Surprisingly, elevated temperatures (60°C for 20 min) did not increase the yield of extractable protein compared to an extraction at 4°C overnight. Unfortunately, all of the tested buffers showed significantly decreased protein yields, when protein from processed peanuts was extracted (Fig. 3). Buffers at higher pH, such as sodium carbonate buffer (pH 11) and sodium borate buffer (pH 9) achieved generally higher protein yields. Thus, these buffers are preferable, when extraction efficiency is an important issue. Depending on the robustness of the protein assay and the sensitivity of the assay to high pH, these buffers may also be applicable in the extraction procedures of commercial test kits. Also the urea buffer (pH 8.7) showed relatively high yields for processed peanut protein, however, this buffer is highly denaturing and its employment in allergen detection assays may thus be limited.

However, due to the decreased extraction efficiency of any of the tested extraction procedures for conventionally heat-processed peanut products, it is important that allergen detection assays and test kits are standardized and calibrated against protein extracts from relevant sources with a relevant processing history. Apparently, impaired extraction efficiency for peanut protein from conventionally processed peanuts is responsible – at least in part – for impaired detection of peanut in the surveillance of the presence of allergenic foods in food products. Additionally, the choice of antigen for antibody production for any immunochemical allergen detection method must correlate with the standards. Only by optimizing the extraction procedure and a thorough selection of antigen(s) for antibody production and assay calibration the necessary determination of relevant allergen contents in food products is likely to be achieved.

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4 References

[1] Hefle, S. L., Nordlee, J. A., Taylor, S. L., Allergenic foods. *Crit. Rev. Food Sci. Nutr.* 1996, *36*, 69 – 89.

[2] Sicherer, S. H., Munoz-Furlong, A., Burks, A. W., Sampson, H. A., Prevalence of peanut and tree nut allergy in the US determined by a random digit dial telephone survey. *J. Allergy Clin. Immunol.* 1999, *103*, 559 – 562.

[3] Emmett, S. E., Angus, F. J., Fry, J. S., Lee, P. N., Perceived prevalence of peanut allergy in Great Britain and its association with other atopic conditions and with peanut allergy in other household members. *Allergy* 1999, *54*, 380 – 385.

[4] Bock, S. A., Munoz-Furlong, A., Sampson, H. A., Fatalities due to anaphylactic reactions to foods. *J. Allergy Clin. Immunol.* 2001, *107*, 191 – 193.

[5] Hourihane, J. O'b., Kilburn, S. A., Nordlee, J. A., Hefle, S. L., *et al.*, An evaluation of the sensitivity of subjects with peanut allergy to very low doses of peanut protein: A randomised, double-blind, placebo-controlled food challenge study. *J. Allergy Clin. Immunol.* 1997, *100*, 596 – 600.

[6] Wensing, M., Penninks, A. H., Hefle, S. L., Akkerdaas, J. H., *et al.*, The range of minimum provoking doses in hazelnut-allergic patients as determined by double-blind, placebo-controlled food challenges. *Clin. Exp. Allergy* 2002a, *32*, 1757 – 1762.

[7] Wensing, M., Penninks, A. H., Hefle, S. L., Koppelman, S. J., *et al.*, The distribution of individual threshold doses eliciting allergic reactions in a population with peanut allergy. *J. Allergy Clin. Immunol.* 2002b, *110*, 915 – 920.

[8] Ortolani, C., Ispano, M., Scibilia, J., Pastorello, E. A., Introducing chemists to food allergy. *Allergy* 2001, *56 (Suppl. 67)*, 5 – 8.

[9] Wüthrich, B., Lethal or life-threatening allergic reactions to food. *J. Invest. Allergol. Clin. Immunol.* 2000, *10*, 59 – 65.

[10] Yunginger, J. W., Gauerke, M. B., Jones, R. T., Dahlberg, M. J. E., Ackerman, S. J., Use of radioimmunoassay to determine the nature, quantity and source of allergenic contamination of sunflower butter. *J. Food Protect.* 1983, *46*, 25 – 628.

[11] Huggett, A. C., Hischenhuber, C., Food manufacturing initiatives to protect the allergic consumer. *Allergy* 1998, *53 (Suppl. 46)*, 89 – 92.

[12] Singh, B., Singh, U., Peanut as a source of protein for human foods. *Plant Foods Hum. Nutr.* 1991, *41*, 165 – 177.

[13] Koppelman, S. J., Bleeker-Marcelis, H., Duijn, G., Hessing, M., Detectiong peanut allergens. The development of an immunochemical assay for peanut proteins. *World Ingred.* 1996, *12*, 35 – 38.

[14] Poms, R. E., Klein, C., Anklam, E., Methods for allergen analysis in food – A review. *Food Add. Contam.* 2004, *21*, 1 – 31.

[15] Poms, R. E., Anklam, E., Current trends in detecting food allergens. *GIT Lab. J. Eur.* 2004, *1*, 43 – 46.

[16] Hurst, W. J., Krout, E. R., Burks, W. R., A comparison of commercially available peanut ELISA test kits on the analysis of samples of dark and milk chocolate. *J. Immunoassay Immunochem.* 2002, *23*, 451 – 459.

[17] Poms, R. E., Lisi, C., Summa, C., Stroka, J., Anklam, E., In-house validation of commercially available ELISA test kits for peanut allergens in food. Report from the Institute for Reference Materials and Measurements, Joint Research Centre, European Commission, EUR 20767 EN, 2003.

[18] Koch, P., Schäppi, G. F., Poms, R. E., Wüthrich, B., *et al.*, Comparison of commercially available ELISA kits with human sera-based detection methods for peanut allergens in foods. *Food Add. Contam.* 2003, *20*, 797 – 803.

[19] Woodroof, J. G., Peanuts: Production, Processing, Products, 3rd Edition, AviPublishing Company, Westport, CT 1983.

[20] Souci, S. W., Food Composition and Nutrition Tables. Medpharm Scientific Publications, Stuttgart 1994.

