



Analytic investigations on protein content in refined seed oils: Implications in food allergy

Matteo Ramazzotti^a, Nadia Mulinacci^b, Luigia Pazzagli^a, Maria Moriondo^c, Giampaolo Manao^a, Franco Francesco Vincieri^b, Donatella Degl'Innocenti^{a,*}

^a Dipartimento di Scienze Biochimiche, Università degli Studi di Firenze, Viale Morgagni 50, 50134 Firenze, Italy

^b Dipartimento di Scienze Farmaceutiche, Università degli Studi di Firenze, Via Ugo Schiff 6, 50019 Sesto Fiorentino (Firenze), Italy

^c Dipartimento di Pediatria, Università degli Studi di Firenze, Ospedale Pediatrico Meyer, viale Gaetano Pieraccini 24, 50134 Firenze, Italy

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ABSTRACT

Background: A number of scientific reports have investigated the possible implications of refined seed oils in allergic reactions, resulting in conflicting points of view. Also the total amount of residual proteins after refinement is still a matter of debate. Nevertheless, seed oils are now blamed as possible cause of allergic reactions.

Objective: To determine the true amount of proteins after oil refinement and to shed new lights on allergenic properties of refined seed oils.

Methods: We optimized a protein extraction procedure on several commercial refined seed oils. Both colorimetric and amino acid analysis were used to measure residual protein content. SDS–PAGE was also used for characterizations of protein band patterns. Sensitized child patients sera were tested by Western blot on PAGE-resolved proteins.

Results: Our extraction method proved to be effective and reproducible. Amino acid analysis resulted more accurate in determining the protein content with respect to colorimetric methods, indicating a higher protein content than that previously reported. IgE responsive residual proteins were found in peanut oil extracts.

Conclusions: Our preliminary data suggest that fully refined seed oils should be taken into account in the context of allergic reactions and would benefit of further toxicological studies.

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1. Introduction

The refining of edible oils is usually performed in two major routes, indicated as chemical and physical, respectively. These steps involve several treatments among which acid pre-treatment, degumming, neutralization, bleaching and deodorization by heating the oil up to 230 °C. Such processes usually alter the proteins present in these oils, affecting their solubility and resulting in a dramatic decrease of total protein content (Venkatesh and Prakash, 1993). Despite no adequate data exist on the effect of different refining methods, it has been reported that the refining process lowers the total protein content up to more than 100 folds (Crevel, 2005). This is usually sufficient to grant an almost complete removal of proteins which can act as allergens. Nevertheless, it is uncertain whether these residual traces may provoke allergic reaction in highly susceptible individuals (Hefle and Taylor, 2002; Keating et al., 1990; Crevel et al., 2000; Zitouni et al., 2000).

Due to susceptibility to inactivation during the extraction procedures, the preparation of purified food allergen is generally considered a difficult task to accomplish.

Food allergies have become a major health concern over the past two decades and are associated with a significant negative impact on the quality of life (Marklund et al., 2006). In effect, their real impact in terms of human population is still unclear since different research groups often use different inclusion criteria, diagnostic definitions and methods (Sampson, 2005; Asero et al., 2007). In order to unify such criteria, recently the EuroPreval Project was founded, constituting an unified framework for measuring the social impact of food allergy across Europe (de Blok et al., 2007).

The prevalence of food allergies is certainly overestimated in the public perception. Nonetheless, it is known that, in adults, food allergies are less relevant than in children; nearly 1–4% of the population in the USA suffers from some kind of food allergy (Asero et al., 2007) and 6–8% of children experience food-allergic disorders during the first 3 years of life (Bock, 1987). European reports suggest that 0.3–7.5% of children and about 2–4% of adults have food allergy (Kanny, 2002; Mills et al., 2007). Despite such a small percentage, it is important to bring into perspective that allergic

* Corresponding author. Fax: +39 055 4598905.

E-mail address: donatella.deglinnocenti@unifi.it (D. Degl'Innocenti).

reactions may arise also from cosmetic and therapeutic formulations in addition to normal food consumption (*International Journal of Toxicology*, 2001).

The EFSA (European Food Safety Authority) counts the allergy problem among its major topics, including the potential allergenicity of refined food product, especially for peanut and soybean oils. Since the 25th November 2004 every European Union Member State was obliged, according to the Directive 2003/89/EC, to list in the food label the major potential allergens. Among them, soybean and peanut are included.

European Medicines Agency (EMA) in its document n EMA/HMPWP/37/04 blamed edible oils for causing allergic reactions in people, even if there are conflicting views for the inadequate scientific reports regarding protein contents and their allergenicity. Moreover, there is a lack of solid data regarding the levels of proteins needed to cause an allergic reaction and, more generally, of allergen tolerance in human. Furthermore the sensitivities for allergens in food in allergic individuals may vary widely, and not all allergenic foods present the same tolerance level (*Hafleand Taylor*, 1999).

In our study we optimized a single step PBS based protein extraction method and we applied it to six different commercial refined oils from soybean, peanut, sunflower and maize. Extracted proteins were quantified by colorimetric assays and amino acid analysis. Moreover, preliminary tests were conducted to investigate the allergy involvement of the extracted proteins by using sera of susceptible young patients.

2. Materials and methods

2.1. Refined seed oils

Protein extractions were performed on commercial edible oils purchased from Italian market. We used refined oils obtained from seed of soy (*Glycine max* L., two different products, here labelled as A and B), peanut (*Arachis hypogaea* L.), sunflower (*Helianthus annuus* L.) and maize (*Zea mays* L., two different products, here labelled as A and B).

2.2. Protein extraction from refined oils

Due to the extremely reduced protein content, much attention was paid to the use of appropriately cleaned glassware or disposable plastics in order to avoid dust and heterologous protein contamination.

Protein extraction was based on the method reported by Zitouni et al. (2000), modified and optimized to be reproducible and applicable to all the different oils in exam. Extraction mixture was composed by 20 ml of 50 mM phosphate buffered saline (PBS) pH 6.5 added to 60 ml of refined oil. This 80 ml mixture was maintained at 55 °C for 24 h in a 200 ml glass flask under rapid stirring and, after cooling at room temperature for 20 min, was centrifuged at 1500×g for 10 min at RT. Only in the case of soybean oil, for which PBS/oil separation was poorly efficient, 2% (v/v) isobutanol was added prior to centrifugation. The lower phase (PBS) was carefully recovered by slow aspiration with a peristaltic pump. To avoid oil contamination, only 90% of the PBS phase was taken. The samples were then exhaustively dialyzed using Spectra/Por 3.5 kDa cut-off membranes (Spectrum) for 48 h at room temperature against two changes of 5 l of distilled water. The salt-free protein solution was finally dried with a vacuum-concentrator centrifuge (Univapo® 150 H) and stored at –20 °C until use.

2.3. Quantification of total protein content

The residual protein content was firstly determined by standard protein assays such as bicinchoninic acid (BCA, Pierce) or Bradford reagent (Bio-Rad). Usually, the dried protein samples from 60 ml of oil were dissolved in 50 µl bidistilled water. The insoluble material after dissolution was removed by ultracentrifugation. Protein titration was made with bovine serum albumin (BSA) ranging from 2 to 20 µg.

For the determination of total amino acid content, the dried samples were reconstituted and subjected to acid hydrolysis according to the modified procedure of Spackman (*Piez and Morris*, 1961). With this procedure, no insoluble material was observed. Amino acid separation and quantification was made with an amino acid analyzer (model 3A29, Carlo Erba) equipped with a Na⁺-high-performance column (4 × 120 mm, Beckman) employing salt/pH based gradients (*Carresi et al.*, 2006). Detection system was based on post-column derivatization of primary amino groups of amino acids with *o*-phthalaldehyde in presence of 2-mercap-

toethanol (*Benson and Hare*, 1975). Titrated amino acid standards were used to calculate the amino acid concentration in extracts and finally to estimate average total protein content.

2.4. SDS-PAGE analysis

Electrophoresis of the protein extracts was performed with 12% polyacrylamide gel using Mini-PROTEAN 3 cell (Bio-Rad), according to *Laemmli* (1970). Dried samples extracted from 60–120 ml of oil were dissolved in 50 µl of 10 mM Tris-HCl buffer pH 6.8 containing 1% (w/v) SDS, 2% (v/v) glycerol, 5% (v/v) β-mercaptoethanol and 0.1% (w/v) bromophenol blue and heated for 4 min at 95 °C. Molecular weights were estimated using Precision Plus Protein Unstained™ markers (Bio-Rad), ranging from 10–250 kDa.

After electrophoresis, gels were stained both with coomassie brilliant blue R-250, sensitive in the micrograms range, and silver nitrate staining (*Morrissey*, 1982), sensitive in the nanogram range.

2.5. Western-blot analysis

Then extracted proteins were electro-transferred onto nitrocellulose membrane (Millipore) by Western blot. Membranes were blocked for 1 h at room temperature with TBST (50 mM Tris-HCl pH 7.5, 0.15 mM NaCl, 1% w/v BSA and 0.1% v/v Tween-20) and then were incubated over night at 4 °C with sera from allergic children patients diluted 1:5 in TBST. After washing, membranes were treated with HRP-conjugated polyclonal anti-human IgE antibodies (DakoCytomation) diluted 1:3000 in TBST for 3 h at room temperature and, after washing, peroxidase activity on the membrane was detected with an enhanced chemiluminescence detection system (Bio-Rad) on hyperfilms (GE Healthcare).

The sera of children sensible to soybean, maize or peanut allergens were collected at the paediatric Meyer Hospital of the University of Florence. Specific IgE levels (kU/l) were determined with ImmunoCAP RAST (Pharmacia Diagnostics) and rated in six classes according to the manufacturer's instructions, namely Class 0, negative: <0.35; Class 1: 0.35–3.5; Class 2: 3.5–17.5; Class 3: 17.5–35; Class 4: 35–50; Class 5: 50–100; Class 6: >100. Negative control sera were drawn from non allergic children (checked for both total and specific IgE). The study was reviewed and approved by local ethical committees. Children's parents provided written informed consensus before enrolment in the study.

2.6. Data collection and analysis

Colorimetric assay results were the average of at least three different extractions. For the amino acid analysis, at least two extractions were taken into account. In all cases, two/three different amounts of extracts were quantified to check for linearity and accuracy of the methods. The data reported in text and tables are expressed in terms of average ± standard deviation.

3. Results

3.1. Protein extraction

Although several approaches have been suggested to recover the protein fractions from vegetable oils (*Crevel et al.*, 2000), no official and standardized method exist up to now. In this study we found that a correct combination of extractive solution, agitation, incubation temperature and time was determinant for maximizing protein extraction efficiency and reproducibility. Several buffer/aqueous and organic solvent-based systems were tested (*Zitouni et al.*, 2000; *Olszewski et al.*, 1998; *Awazuhara et al.*, 1999; *Hidalgo et al.*, 2001), searching for a method that could give good results for all the oils in analysis. Finally, we optimized a single step heat/PBS method that allowed obtaining the highest amount of proteins in the most easy, reproducible and consistent way possible. To increase protein concentration to levels suitable for assays, a drying procedure was chosen, preceded by an extensive dialysis to remove salts. Due to the dialysis membrane cut-off, peptides smaller than 3.5 kDa were lost and consequently excluded from protein analysis.

3.2. Protein content assay by colorimetric methods

Protein content from dried extracts was measured with different colorimetric assays, calibrating and setting out a wide range of values (1–20 µg). We experienced scarce reproducibility with

Table 1

Protein content in refined oils as determined with standard colorimetric methods and with amino acid analysis

Oil type	Protein quantification method		
	Colorimetric	Amino acid analysis	
	µg/100 ml	µg/100 ml	nmol/100 ml
Soybean A	16.4 ± 3.0	96 ± 1	810 ± 9
Soybean B	19.0 ± 3.2	166 ± 8	1448 ± 144
Maize A	6.5 ± 1.3	64 ± 8	572 ± 76
Maize B	16.1 ± 2.9	33 ± 1	284 ± 27
Peanut	9.3 ± 1.7	35 ± 2	352 ± 43
Sunflower	18.0 ± 3.1	95 ± 0	823 ± 3

Values are expressed as average ± standard deviation deriving from at least three different extractions per oil.

standard protein assays such as BCA and Bradford. This poor reliability was possibly due to the low solubility of extracted material or to residual lipid traces. The assays measured a total residual protein content varying from 6.5 to 19 µg/100 ml of oil in all the tested samples (see Table 1), in agreement with previous data reported for sunflower (Zitouni et al., 2000), peanut and soybean (Crevel et al., 2000).

3.3. Amino acid analysis

Since standard protein colorimetric assays proved to be scarcely reproducible and prone to under or overestimations, total protein quantification was also assessed by amino acid analysis. Protein extracts was considered not to contain free amino acids or small peptides because of the exhaustive dialysis.

The amino acid peak patterns obtained were integrated for quantification and their elution times were compared to peaks from standard calibrated mixture of amino acids. Hydrolyzed protein extracts resulted free of unknown peaks, allowing a correct and unambiguous detection and quantification of every amino acid apart from cysteine and tryptophan and proline, lost during the acid hydrolysis or derivatization (Piez and Morris, 1961). Table 2 details the contribution of each single amino acid on the total amino acid content.

Total protein content was calculated from amino acid values and are reported in Table 1. According to amino acid analysis, the highest protein content was found in both the soybean oils

and sunflower oil. According to our data, protein content estimated with amino acid analysis resulted up to 9 times higher when compared to that obtained by the colorimetric assays (Table 1).

3.4. Protein profiling by SDS-PAGE

We evaluated the protein pattern of each oil extract by SDS-PAGE, mainly for its ability to resolve the proteins according to their molecular weight. In all cases, distinct protein band patterns were evidenced (Fig. 1), clearly differentiating each seed oil.

Soybean oil A and B (Fig. 1a and b, respectively) appeared very different in terms of total protein content, being the former much richer than the latter. This appeared in agreement with the results shown in amino acid analysis. In addition, also band patterns were roughly different, possibly evidencing different efficiencies in the refinement process in the two commercial products. Whatever, no major protein bands were evidenced by this technique. It should be taken into account that SDS-PAGE cannot be considered as a quantitative technique since its results are influenced by a number of factors, especially if stained with silver nitrate.

Very similar results were instead underlined for the two maize oils (Fig. 1c and d, respectively). Coomassie staining was not able to differentiate the two oils at all and silver staining showed only minor differences. In addition, both staining methods agreed in detecting three major protein bands at 12, 60 and >200 kDa.

Peanut edible oils SDS-PAGE is shown in Fig. 1e. It showed three evident major bands around the 30–40 kDa region with coomassie staining, whilst silver staining exalted two other major bands at about 14 (and lower) and 66 kDa. This heterogeneity in bands detection should not surprise since the two techniques do not differ only for the sensitivity but also in the specificity. Interestingly, the band at about 66 kDa possessed a molecular weight similar to that of known allergens in peanut, e.g. Ara h 1, a well known 64.5 kDa allergenic glycoprotein. The silver stain showed proteins banded at about 10–12, 14, 18, 35–40, 60–66 and above 200 kDa, that resembled those previously reported for peanut oils (Olszewski et al., 1998).

Sunflower oil SDS-PAGE (Fig. 1f) showed some major bands with coomassie staining. This number was greatly increased with silver staining that was able to resolve additional bands at 14 and 23–28 kDa.

Table 2

Amino acid composition in different refined oils

Amino acid		Soybean A		Soybean B		Maize A		Maize B		Peanut		Sunflower	
		nmoles	%	nmoles	%	nmoles	%	nmoles	%	nmoles	%	nmoles	%
Ala	A	79.4 ± 0.3	9.8	122.1 ± 21	8.4	49.7 ± 2.6	8.7	8.7 ± 0.7	8.7	22.4 ± 3.1	6.4	78 ± 1.9	9.5
Arg	R	20.7 ± 1.0	2.6	15.8 ± 0.2	1.1	5.8 ± 0.9	1	1.4 ± 1.4	1.4	2.8 ± 0.1	0.8	4.6 ± 0	0.6
Asx	Z	55.9 ± 3.2	6.9	96.2 ± 5.7	6.6	34.5 ± 7.9	6	6.2 ± 0.1	6.2	13.9 ± 2	3.9	51.9 ± 3.1	6.3
Glx	B	152.7 ± 19.7	18.9	250.8 ± 3.7	17.3	112.9 ± 11.6	19.7	18.2 ± 3.5	18.2	46.6 ± 4.8	13.2	162.5 ± 11.6	19.7
Gly	G	143 ± 8.4	17.6	293.3 ± 17.6	20.2	123.3 ± 23.8	21.5	19.6 ± 3.2	19.6	153.3 ± 13	43.4	146.8 ± 1.8	17.8
His	H	23 ± 2.4	2.8	57.2 ± 1	4	22.9 ± 0.8	4	4.1 ± 0.6	4.1	6.7 ± 0.1	1.9	36.7 ± 0.3	4.5
Ile	I	24.8 ± 0.6	3.1	31.5 ± 0.2	2.2	13.3 ± 3.9	2.3	2.1 ± 0.2	2.1	6.3 ± 0.7	1.8	16 ± 0.2	1.9
Leu	L	43.3 ± 7.3	5.3	54.1 ± 0.1	3.7	15.2 ± 5.4	2.7	3.5 ± 0.3	3.5	7.1 ± 2.5	2	24 ± 0.6	2.9
Lys	K	31.8 ± 2.1	3.9	35.7 ± 0.4	2.5	10.2 ± 2.6	1.8	2.4 ± 0.1	2.4	5.6 ± 1.2	1.6	19.2 ± 0.6	2.3
Met	M	6.6 ± 0.7	0.8	7.1 ± 0.2	0.5	1.7 ± 1.7	0.3	0.2 ± 1	0.2	1.4 ± 0.1	0.4	3.1 ± 0.1	0.4
Ser	S	25.2 ± 9.5	3.1	33.5 ± 1.4	2.3	9.3 ± 2.1	1.6	4.2 ± 2.2	4.2	6.3 ± 1.5	1.8	16.3 ± 2.6	2
Phe	F	102.4 ± 1	12.6	285 ± 4.6	19.7	121.4 ± 16.9	21.2	18.8 ± 2.2	18.8	54 ± 7.1	15.3	176.9 ± 9.8	21.5
Thr	T	40.6 ± 0.8	5	80.8 ± 6	5.6	26 ± 2.2	4.5	4.7 ± 0.5	4.7	11.1 ± 0	3.2	45.6 ± 2.4	5.5
Tyr	Y	20.4 ± 1.6	2.5	33.5 ± 1	2.3	9.7 ± 2	1.7	2.3 ± 0.1	2.3	6.6 ± 1.8	1.9	11.9 ± 0.3	1.4
Val	V	40.4 ± 0.2	5	52 ± 0.5	3.6	16.7 ± 3.6	2.9	3.5 ± 0.6	3.5	8.8 ± 0.8	2.5	30.2 ± 1.6	3.7
Total		810 ± 9	–	1448 ± 9	–	572 ± 76	–	284 ± 27	–	352 ± 43	–	823 ± 3	–

Values are expressed as nmoles/100 ml of refined oil ± standard deviation deriving from at least three different extractions per oil. Percentage values refer to the contribution of each single amino acid to the total amino acid content in each oil.

Asx: asparagine + aspartic acid; Glx: glutamine + glutamic acid.

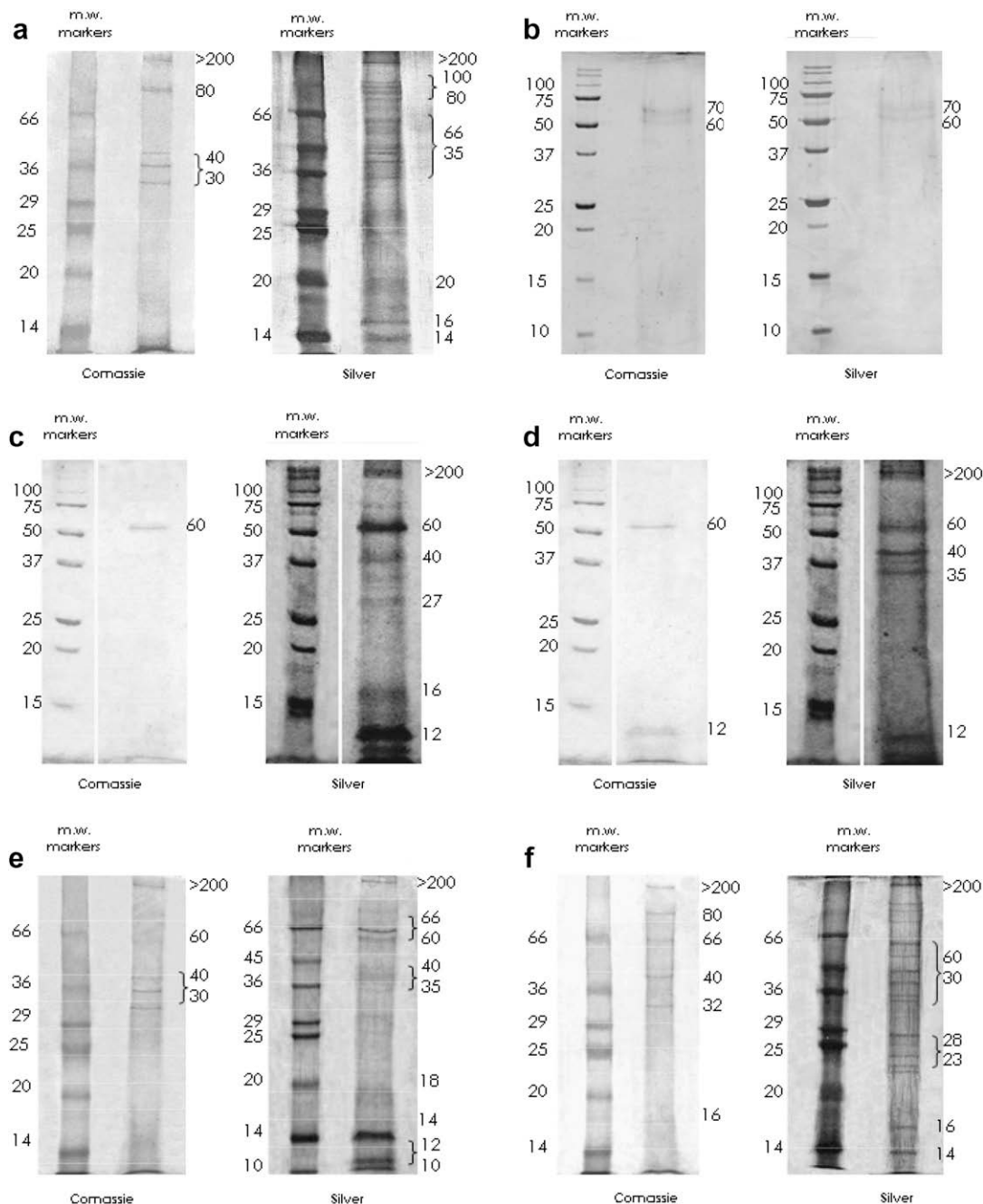


Fig. 1. SDS-PAGE of various oil extracts stained in parallel with coomassie and with silver methods: (a) soybean oil A, (b) soybean oil B, (c) maize oil A, (d) maize oil B, (e) edible peanut oil and (f) edible sunflower oil. The labels flanking gels report in kDa the molecular weight of the observed protein bands.

As a final note, we might observe that the enhanced capability of the silver staining procedure in protein detection, with respect to the coomassie staining, is rarely associated to an increased ability in protein pattern characterization.

The amount of protein estimated from the SDS-PAGE resulted more similar to that obtained from the standard protein assays than that calculated from the amino acid values (Table 1). This led us to suppose that peptides, ranging 10–3.5 kDa, hardly detectable on SDS-PAGE, could be present in the residual protein extraction pools. Despite silver staining is more sensitive for detection of SDS-PAGE separated proteins than the coomassie one, it showed some difficulties in evidencing some peculiar proteins, making it difficult to be certain that all proteins have been detected (Goldberg and Warner, 1997).

3.5. Immuno-recognition of the extracted proteins

The possibility for oil protein extracts to be recognized by IgE of allergic children's sera was tested by Western blot. We used different sera of patients CAP-RAST positive to peanut (three samples: 43.3, 36.2 and <0.35 kU/l), soybean (four samples: 13, 5.78, 0.83 and 1.27 kU/l) and maize (four samples: 0.79, 0.78, 4.45 and 12.5 kU/l). The blotted proteins were incubated with serum from child patients and visualized with HRP-conjugated anti-human IgE. No immuno-recognition was obtained using negative sera (i.e. class 0 or near). Since a study of Foucard et al. (1999) reported strong cross reactions of peanut specific IgE for soybean proteins, we tested our samples by crossing sera and extracted samples (e.g. sera from patients with peanut specific

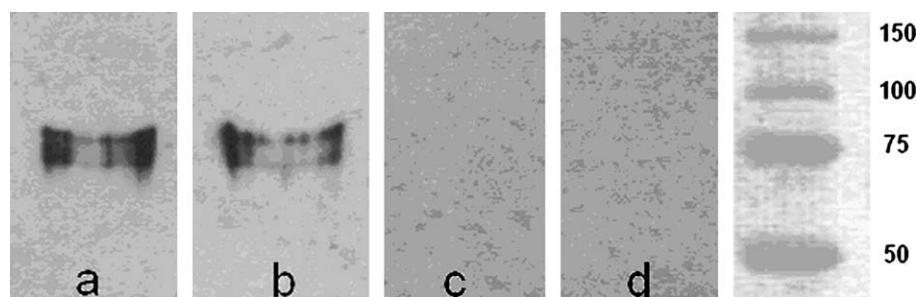


Fig. 2. Immuno-recognition of protein extraction from refined peanut oil. The different lanes represent the same extract incubated with different sera from allergic patients with variable levels of IgE: (a) IgE = 43.3 kU/l, (b) IgE = 36.2 kU/l, (c) IgE = 28.3 kU/l and (d) negative control (IgE < 0.35 kU/l). Last lane report pre-stained molecular weight standards, with labels expressed in kDa.

IgE incubated with non-peanut oil extracts), obtaining no cross reactions.

Among the several combinations of serum samples and seed oil extractions, we obtained positive signals only by incubating extracts of refined peanut oils with sera from peanut allergic patients with 43.3 and 36.2 kU/l specific IgE (class 4), as shown in Fig. 2. The last class 4 serum available, with 28.3 kU/l IgE, gave no signals. Taken together, our results indicated that there could be a visualization limit of Western-blot technique with levels of specific IgE i.e. <35 kU/l. This may also explain the lack of response for other oil extract tested. In fact, IgE levels for soybean and maize were all classifiable in RAST CAP classes 1 and 2, therefore with levels below 17.5 kU/l.

We could not exclude, consequently, the absence of possible immuno-response in patients given a negative result. For both positive cases, the molecular weight of the two recognized proteins was estimated around 70–80 kDa. Both coomassie and silver staining did not display any major bands at that molecular weight, possibly indicating a strong IgE specificity for such proteins. To our knowledge, at this moment no peanut allergen is known associated with such molecular weights, opening up interesting perspectives for further studies.

4. Discussion

Several studies are available on protein content in seeds, particularly for soybean and peanut. It must be underlined that the seeds are a completely different matter with respect to the corresponding derived oils. In fact, the refining process that the seed oils undergo before commercialization and human consumption strongly affects the protein fraction. Such rectifying procedures are usually thought to drastically reduce the allergenicity of refined oils. Nevertheless, a number of works cast doubts on this safety (Yunginger and Calobrisi, 2001; Paschke et al., 2001; Crevel et al., 2000; Hourihane et al., 1997), forcing international efforts to adopt specific food labels for such products.

Since the method we applied for extracting the residual proteins was based on aqueous solution, it is likely to suffer from phospholipids contamination problems (Frémont et al., 2004). In addition, it cannot be reasonably validated by comparison with other standard procedures. In fact, there is no *a priori* knowledge about the identity of the extracted proteins and, consequently, it is not reasonable to perform spotting tests or to prepare reference samples. The major problem with lipid based contaminants is the possibility of severe interferences with standard colorimetric assays, resulting in an inaccurate estimation of the protein content (Morton and Evans, 1992). Other factors that can alter the results of colorimetric methods could be ascribed to glycosylation degree (Fountoulakis et al., 1992) and amino acid composition (Sapan et al., 1999).

In contrast, the amino acid analysis we used is *per se* not affected by the abovementioned interference, due to pre-treatments of samples and to chromatographic separation. Our data let us to propose amino acid analysis as a candidate technique for protein content measurement after the refining process, to possibly be taken into account for product labelling and safety assessment.

We found two proteins in peanut oil extracts that were able to raise significant signals in Western blot with peanut sensitized patient's sera. It is remarkable that neither coomassie nor silver stains were able to detect at significant levels protein bands in that 70–80 kDa region. This result in peanut oils points out that the sensitivity and the specificity of the immuno-recognition is much higher than that of standard protein staining techniques. In fact, it should be observed that the different reactivity of proteins to staining procedures with respect to antibody recognition could be related to the post-translational modifications (e.g., glycosylation) that can hide binding sites to dye molecules (Osset et al., 1989; Lauber et al., 2001) but that can constitute the major effectors of antibody binding. It is well known, in fact, that the glycosylation degree is an important feature of proteins that increases their allergenicity (Huby et al., 2000).

Given the protein bands that were found as positive in Western blot of peanut oil extracts, it cannot be excluded that allergic patients may present specific IgE for a number of proteins larger than that known, some of them still present in the refined oils. According to our results we suggest that immuno-based methods should be applied to a larger extent before confidently invoking the safety of refined seed oils, especially for sensitized patients.

In conclusion, we would like to stress the importance of having reliable methods to diagnose food allergy in order to exclude only specific foods from the diet of allergic people, avoiding excessive and unnecessary dietary restrictions.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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