

Influence of refining steps on trace allergenic protein content in sunflower oil

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Background: Although allergy to sunflower seed and oil is a relatively rare occurrence, several cases of sunflower seed allergy have been observed, and we have already described one case of anaphylaxis after eating sunflower oil and margarine. **Objective:** The aim of our study was to determine and characterize the allergens from sunflower oil at the different steps of the refining process: crude pressed oil (step A), acidification and neutralization (step B), pregumming by centrifugation (step C), washing (step D), bleaching (step E), gumming by filtration (step F), and deodorization (step G).

Methods: A sample of oil from each step of the process (steps A to G) was heat extracted with PBS. The protein concentration of each extract was evaluated by using the micro-Bradford assay. Samples were run on SDS-PAGE. The immunoblot was performed with the serum of a patient sensitized to sunflower seed and oil.

Results: The extracts obtained after each step reveal a decrease in total protein concentration from 13.6 µg/mL to 0.22 µg/mL. The result of SDS-PAGE shows 5 bands, from 67 kd to 145 kd, with the most abundant being the 67-kd protein. The amount of this protein decreases after each step of the process. It is, however, still present in trace amounts in the refined oil. The 67-kd protein, which is mainly present in the crude oil and slightly in the refined oil, has been shown to be allergenic.

Conclusion: Because of the presence of allergenic proteins, refined sunflower oil may pose a threat to people highly sensitized to sunflower seeds. (*J Allergy Clin Immunol* 2000;106:962-7.)

Key words: Food allergy, sunflower oil, crude oil, refined oil, food processing, Western blot

Food allergy is particularly frequent in infants with atopic dermatitis.¹ Attention has been drawn to the eventual risk of food allergy to peanut oil.² After recent publications on clinical reaction to sesame³ or sunflower

Abbreviation used

TBS: TRIS-buffered saline

oil,⁴ we wondered about the current risk of a widely used vegetable fat like sunflower oil. In France, its consumption represents 40% of the human vegetable oil consumption. It represents 20% of consumption in Europe and 12% in the world (Proléa–Documentation; ONIDOL, Paris, France).

The aim of this study was to assess the allergenicity of sunflower oils at the different steps of the refining process.

METHODS

Serum

The serum used in this study was from a 36-year-old woman. She experienced 3 episodes of anaphylaxis within a 2-month period, which started 1 hour after eating a meal containing sunflower oil and margarine. Skin prick test results were positive for sunflower pollen (4 mm), seeds (4 mm), and refined oil (9% codeine phosphate control, 3 mm).

A double-blind, placebo-controlled, food challenge was performed with 5 mL of sunflower oil. Abdominal pain occurred 2 and a half hours later. After a further 8 hours, the patient had a cough, impaired respiration, and facial angioedema.⁴

Sunflower oil

Sunflower oil from each step of the refining process was rigorously sampled by a French oil producer (Table I).

Protein extraction from sunflower oil

Oil protein extractions were performed on 7 samples. A sample of oil from each step of the process was heat extracted with PBS (pH 6.5).

A volume of 80 mL of 50 mmol of PBS was added to 240 mL of each oil and rocked for 24 hours at 60°C. The samples were centrifuged at 1500g for 15 minutes, and the 80-mL clear aqueous layer was collected and dialyzed (Spectrum Laboratories, Breda, The Netherlands) against distilled water for 48 hours. The dialysate was then lyophilized.

Protein extraction from sunflower seeds and shells

The sunflower seeds were shelled. The proteins of the sunflower seeds and shells were extracted with water after stirring for 15 minutes at room temperature.

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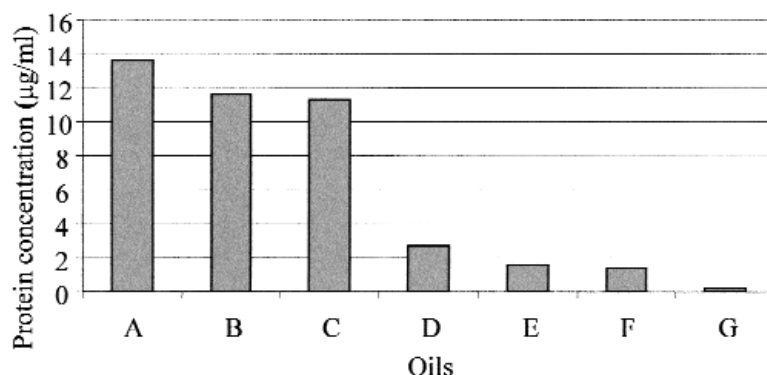


FIG 1. Protein concentration of oil extracts from the different steps of the refining process. *Lane A*, Crude sunflower oil; *lane B*, neutralized oil; *lane C*, pregummed oil; *lane D*, washed oil; *lane E*, bleached oil; *lane F*, gummed oil; *lane G*, refined oil.

Protein assay

The protein concentration of each extract was evaluated by using the Bradford assay (Bio-Rad, Hercules, Calif).

Protein concentrations were calculated in micrograms per milliliter of oil by extrapolation from the concentration in extracts, assuming complete extraction.

The relative amount of protein removed (Y) after one step (step x) of the refining process was calculated as follows: $Y = 100(1 - B/A)$, where A is the protein concentration of the oil sample before step x, and B is the protein concentration of the oil sample after step x.

Rabbit immunization

A rabbit was immunized against protein extract of sunflower seeds. Three intradermic injections of protein extract (1 mg/mL) were administered on days 0, 21, and 42. The sera were collected on day 0 (control serum) and day 49.

Polyacrylamide gel electrophoresis

SDS-PAGE was carried out according to the method of Laemmli⁵ in a vertical slab gel (Bio-Rad). An acrylamide gradient concentration from 4% to 20% was used. Protein samples were boiled for 5 minutes in sample buffer (0.125 mmol/L TRIS-HCl, 4% SDS, 20% glycerol, 10% mercaptoethanol, and 0.004% bromophenol blue [pH 6.8]). The same volumes of each extract were loaded and run at a constant voltage of 150 V.

Protein transfer and immunoblotting

The proteins were transferred onto polyvinylidene difluoride paper (Millipore, Bedford, Mass) for 1 hour at 300 mA on a semi-dry apparatus (Bio-Rad), and the immunoblot was obtained by using the catalyzed reporter deposition method.⁶

The blots were probed in triplicate with the serum from the patients with sunflower seed allergy, sera from nonsensitized patients, and sera in TRIS-buffered saline (TBS) containing 0.1% Tween-20. The sera were diluted (1:5) in TBS (pH 7.4) containing 1% BSA for 24 hours on a rocker at 4°C. After washing two times for 15 minutes with TBS and 0.1% Tween-20, the blot was incubated for 2 hours at room temperature with biotinylated anti-human IgE (Vector, Burlingame, Calif). The blots were washed twice and blocked with BSA incubated with streptavidin-peroxidase (Boehringer, Mannheim, Germany) and then washed again. Incubation with diluted biotinylated tyramide in TBS-imidazole containing H₂O₂ was followed by two washings. Finally, the blots were incubated with streptavidin-alkaline phosphatase (Boehringer),

washed twice, and then revealed with 5-bromo 4-chloro-3-indoyl phosphate/nitro blue tetrazolium (Sigma, St Louis, Mo).

The Western blots using rabbit sera were performed as follows. The blots were probed with diluted rabbit sera (1:10) in 1% BSA in TBS (pH 7.4) for 24 hours on a rocker at 4°C. The blots were again washed twice and incubated for 2 hours at room temperature with anti-rabbit IgG labeled with alkaline phosphatase (Sigma), washed twice for 15 minutes with TBS and 0.1% Tween-20, and revealed with 5-bromo 4-chloro-3-indoyl phosphate/nitro blue tetrazolium for 5 minutes.

RESULTS

Protein concentrations

The protein concentrations determined in each oil of the different steps of the refining process are 13.6 µg/mL in crude oil (step A), 11.56 µg/mL in neutralized oil (step B), 11.30 µg/mL in pregummed oil (step C), 2.75 µg/mL washed oil (step D), 1.63 µg/mL in bleached oil (step E), 1.41 µg/mL in gummed oil (step F), and 0.22 µg/mL in refined oil (step G, Fig 1).

Molecular weight

The SDS-PAGE of sunflower oil extracts shows 5 bands from 67 to 145 kD, with the most abundant being the 67-kD protein (Fig 2, A). The amount of this protein decreases after each step of the process. Trace amounts are still present in the refined oil.

The SDS-PAGE of sunflower shell extract shows only one band of 67 kD (Fig 2, B). A protein of the same size is present among the proteins extracted from sunflower seed (Fig 2, C).

Allergenic proteins

The immunoblot of sunflower oil extracts shows one major band at 67 kD in each oil from crude oil to refined oil but reveals a lower intensity in the bleached oil, gummed oil, and refined oil than in the others. Four faint bands are also present at molecular weights of 145, 132, 131, and 114 kD in crude oil (step A), neutralized oil (step B), pregummed oil (step C), and washed oil (step D). In bleached oil (step E), gummed oil (step F), and refined oil (step G), the single 67-kD band can be observed (Fig 3).

MW (kDa)

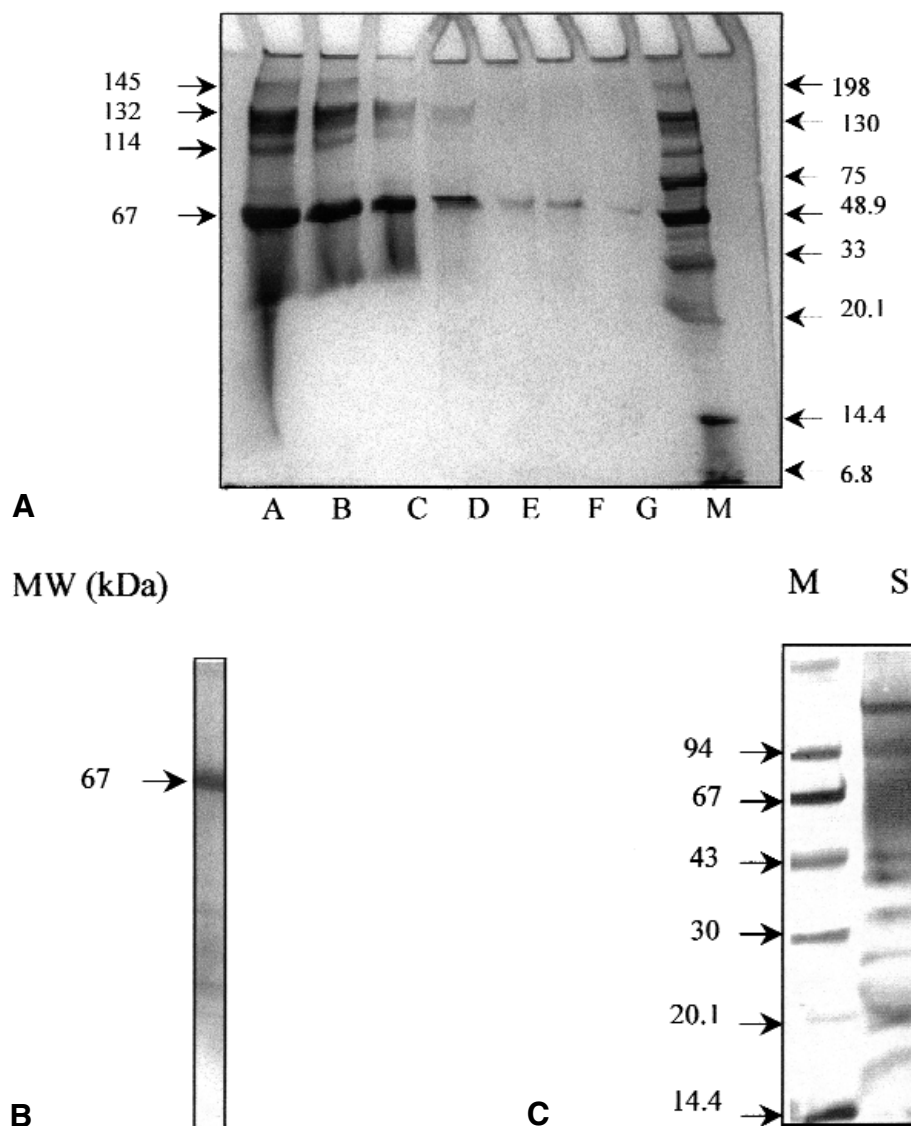


FIG 2. Silver-stained SDS-PAGE. *M*, Molecular weight markers. **A**, Sunflower oil extracts from the different steps of the refining process. *Lane A*, Crude sunflower oil; *lane B*, neutralized oil; *lane C*, pregummed oil; *lane D*, washed oil; *lane E*, bleached oil; *lane F*, gummed oil; *lane G*, refined oil. **B**, Sunflower shell extract. **C**, Sunflower seed extract (*S*).

In the most hydrophobic fraction eluted from hydrophobic interaction chromatography of the sunflower seed protein extract, a protein of 67 kD was recognized by a pool of 5 sera from patients sensitized to sunflower pollen and sunflower seed (data not shown). One strip incubated with the secondary antibody without any serum to control for nonspecific binding and another strip incubated with the serum of an individual not sensitized to sunflower seed protein did not demonstrate any binding (data not shown).

Antigenic protein

The protein extract obtained from refined sunflower oil showed that the 67-kD protein is recognized by rabbit sera (Fig 4).

DISCUSSION

Sunflower (*Helianthus annuus*) belongs to the Compositae family and is the third biggest source of vegetable

MW (kDa)

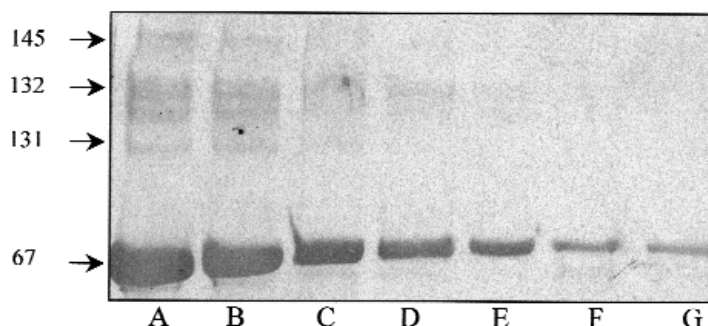


FIG 3. Western blotting of sunflower oil extracts with the serum of a patient allergic to sunflower oil. *Lane A*, Crude sunflower oil; *lane B*, neutralized oil; *lane C*, pregummed oil; *lane D*, washed oil; *lane E*, bleached oil; *lane F*, gummed oil; *lane G*, refined oil.



FIG 4. Western blotting (IgG pattern) of refined sunflower oil extract with sera of rabbit immunized against extract of sunflower seed protein.

oil in the world. In France, the consumption of sunflower oil was up 63% between 1981 and 1997, at the expense of the consumption of peanut oil (Proléa-Documentation, ONIDOL).

Hypersensitivity to seeds has been described,^{7,8} as well as one single case of allergy to sunflower oil.⁴ However, some authors have stated that sunflower oil does not contain any allergenic protein.⁹

Different studies on peanut oil described conflicting results.^{10,11} Teuber et al¹² found that the extracts from oils that had undergone less processing at lower temperatures tended to demonstrate greater IgE binding.

In our work the results obtained indicate the presence of a global protein concentration of 220 ng/mL oil in the refined sunflower seed oil. This concentration is greater than the concentration of 54 ng/mL oil obtained by Halsey et al⁹ and lower than the 849 ng/mL concentration obtained by Klurfeld et al.¹³ It should, however, be noted that the sunflower seed oils analyzed were not of the same origin and were probably not subject to the same type of refining. Noteworthy also is that the protein determination methods were different. Halsey et al⁹ used the Lowry method, which has a detection limit of 10 µg/mL and that led these authors to consider that 54 ng/mL is well below the lower recognition level.^{3,14} In contrast, both Klurfeld et al¹³ and ourselves carried out protein analysis by using the Bradford method,¹⁵ a method 10 times more sensitive than that of Lowry. In a more recent study encompassing the allergenicity of refined food oils using the bicinchoninic acid micro-method, Teuber et al¹² estimated the protein concentrations of almond, peanut, and walnut oils taken to maximum temperatures of 230°C to 260°C during the refining process at between 2.2 ± 0.7 and 16.7 ± 0.8 µg/mL. The thermal treatments can exercise a denaturing influence on the structure of the proteins and consequently on their solubility. In this respect Venkatesh and Prakash¹⁶ observed that application of a temperature of 100°C to 150°C to sunflower seed proteins led to a reduction in their hydrosolubility. They noted an increase in the binding of lipid to the proteins through the hydrophobic nonpolar amino acid side chains and the exposure of buried hydrophobic amino acids after thermal denaturation. This property could explain the very hydrophobic character of the 67-kD protein, which is present in considerable quantity in refined sunflower oil after hexane extraction of seeds. Furthermore, Lapanje¹⁷ demonstrated that the thermal treatment of sunflower proteins can lead to the substitution of amine groups of lysine residues by reaction either with the reducing sugars or the free chlorogenic acid present in the microenvironment of the protein.

TABLE I. Description of the steps of refining process of sunflower oil

Step	Oil obtained	Treatment used	Temperature applied	Time	Removed components
0: Expeller and chemical extraction	Crude oil (A)				
1: Acidic and neutral treatment	Neutralized oil (B)	H ₃ PO ₄ , NaOH	85°C	15 min	Phospholipids, free fatty acids, metals, pigments, products from oxidation, some contaminants
2: Pregumming by centrifugation	Pregummed oil (C)	Soap and water	10°C	4 h	Plant wax
3: Washings	Washed oil (D)	Water	85°C	Few minutes	Soap, phospholipids
4: Bleaching	Bleached oil (E)	Filters	85°C	20 min	Pigments, residues of soap, phospholipids, metals, products from oxidation
5: Gumming by filtration	Gummed oil (F)	Filters	10°C	5 h	Plant wax
6: Deodorization	Refined oil (G)	Water steam	220°C-4 min under vacuum	2 h, 30 min	Peroxides, odors, some contaminants

The 67-kd protein is the most abundant protein of the oil. It may come from the albumen of the sunflower seed. Indeed, a protein of 67 kd, the hydrophobic characteristics of which have been determined by hydrophobic interaction chromatography, is recognized by the polyclonal serum of rabbit immunized against the protein extract of sunflower seed. Moreover, the SDS-PAGE of sunflower shell shows a unique bande at 67 kd, and the slot blot reveals its allergenicity (data not shown).

The crude sunflower oil contains a main 67-kd protein in addition to the less-abundant proteins of higher molecular weights eliminated during refining. In contrast, the 67-kd protein is present in the sunflower seeds in relatively lower quantities than in the oil and is accompanied by other smaller size proteins. The extraction conditions, which have a determining influence on the protein concentration, may explain these observations.¹⁸ Indeed, in both cases extraction is carried out with PBS solutions of a different NaCl molarity, pH, and temperature. A PBS solution containing 1 mol/L NaCl (pH 7.0) at 4°C was used to extract the sunflower seeds as opposed to a PBS solution made up of 50 mmol/L NaCl (pH 6.5) at 60°C for the oil. The incubation time with the extraction buffer is also a factor involved in the selective extraction of proteins.¹⁹ This could explain the absence of small-size protein bands in our oil extracts because the incubation time in PBS at 60°C is 24 hours.

Another hypothesis is that the phospholipids present in the oil extracts could be able to bind the low-molecular-weight proteins. They could be responsible for hiding these proteins, making them undetectable on SDS-PAGE by silver-staining coloration.

The allergenic properties of the 67-kd protein in the oils tested, from the crude oil to the refined oil, were highlighted by Western blotting.

Allergy to sunflower oil has been the subject of a limited number of studies. Halsey et al,⁹ after sunflower oil-negative skin and negative RAST test results in patients having undergone anaphylaxis to seeds, concluded to the nonallergenicity of sunflower oil. Our laboratory related one case documented by double-blind,

placebo-controlled, food challenge.⁴ At the present time, the data on the allergenicity of crude and refined oils remain conflicting. Taylor et al²⁰ concluded that there was an absence of evidence to indicate any potential danger of refined peanut oil in peanut-sensitized patients, whereas Hoffmann and Williams,¹⁰ using slot blots, highlighted the presence of allergens in crude cold-pressed peanut oils but not in crude hot-pressed oils. More recently, Olszowski et al¹¹ isolated and identified an 18-kd allergen in refined peanut oil. In addition, Teuber et al¹² demonstrated the presence of allergens ranging between 14 and 80 kd in peanut oil having undergone low-temperature refining.

This study has provided additional information on the gradual modification of the allergenicity of the proteins present in the oil according to the refining stage. The 67-kd allergenic protein resists all the processes, including application of a temperature of 220°C in a vacuum for 4 minutes. Thermal denaturation of the proteins may lead to modifications of the allergenicity, as was recently shown for β -lactoglobulin.²¹ Heating pecan nuts has also revealed a neoallergen.²² As regards peanuts, it has been confirmed that a major allergen (65 kd) is recognized by specific IgEs before and after cooking at 140°C for 45 minutes.²³ Therefore the allergenicity of certain food proteins can be maintained after cooking. With respect to the 67-kd protein, the allergenicity could be recognized both before and after oil extraction and refining. It cannot be confirmed that this protein characterizes a native allergen because inhibition of the immunoblot by the sunflower flour was not performed. Furthermore, it cannot be excluded that the allergenicity appeared on account of the chemical processes of extraction and heating.

Some other patients with sunflower seed allergy may not be reactive to the 67-kd allergen. Kelly et al²⁴ show that other proteins of the seed (10.5-48.9 kd) are recognized by 5 sensitized patients. This can be explained by differences in the protein extraction methods and by the interindividual variability. More research is needed with more patients to determine which sunflower seed proteins are the major allergens.

The 67-kD protein is present in the refined oil at a concentration of 0.22 µg/mL. Assuming a mean consumption of 15 to 30 mL of oil per person per day, the daily consumption of this protein would be the equivalent of 6.6 µg.

The hydrophobic proteins of the seeds present a potential nonnegligible risk of allergy highlighted by the occurrence of allergic reaction to peanut oil or, as demonstrated in this work, to sunflower oil, although the quantity consumed is in the order of a microgram.^{10,25,26} Although no study has determined the minimum reactive quantity of sunflower seeds, the minimum reactive quantity of peanut seed is very much higher than 1 µg, having been evaluated at 100 µg.^{26,27} Concerning peanut oil, it was shown that as little as 1 to 10 mL of crude oil are reactive.²⁸ In our case the patient reacted to 5 mL of refined sunflower oil (ie, 10-40 µg of proteins).⁴

Among the possible explanations, we favor the hypothesis according to which the hydrophobic proteins able to interact with the phospholipids could be capable of permeating the intestinal barrier, as demonstrated for mustard allergen.²⁹

REFERENCES

1. Sicherer H, Sampson HA. Food hypersensitivity and atopic dermatitis: pathophysiology, epidemiology, diagnosis and management. *J Allergy Clin Immunol* 1999;104:114-22.
2. Moneret-Vautrin DA, Hatahet R, Kanny G, Ait-Djafer Z. Allergenic peanut oil in milk formulas. *Lancet* 1991;338:1149.
3. Kanny G, De Hauteclouque C, Moneret-Vautrin DA. Sesame seed and sesame seed oil contain masked allergens of growing importance. *Allergy* 1996;51:952-7.
4. Kanny G, Frémont S, Nicolas JP, Moneret-Vautrin DA. Food allergy to sunflower oil in a patient sensitized to mugwort pollen. *Allergy* 1994;49:561-4.
5. Laemmli UK. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* 1970;227:680-5.
6. Bobrow MN, Shaughnessy KJ, Litt GJ. Catalysed reporter deposition, a novel method of signal amplification. *J Immunol Methods* 1991;137:103-12.
7. Axelsson IKG, Ihre E, Zetterström O. Anaphylactic reactions to sunflower seed. *Allergy* 1994;49:517-20.
8. Garcia Ortiz JC, Cosmes Martin P, Lopez-Asundo A. Allergy to foods in patients monosensitized to artemisia pollen. *Allergy* 1996;51:927-31.
9. Halsey AB, Martin ME, Ruff ME, Jacobs FO, Jacobs RL. Sunflower oil is not allergenic to sunflower seed-sensitive patients. *J Allergy Clin Immunol* 1986;78:408-10.
10. Hoffman DR, Williams CC. Cold processed peanut oils may contain peanut allergen. *J Allergy Clin Immunol* 1994;93:801-2.
11. Olszowski A, Pons L, Mouté F, Aimone-Gastin I, Kanny G, Moneret-Vautrin DA, et al. Isolation and characterization of proteic allergens in refined peanut oil. *Clin Exp Allergy* 1998;28:850-9.
12. Teuber SS, Brown RL, Haapanen LA. Allergenicity of gourmet nut oils processed by different methods. *J Allergy Clin Immunol* 1997;99:502-7.
13. Klurfeld DM, Kritchevsky D. Isolation and quantification of lectins from vegetable oils. *Lipids* 1987;22:667-8.
14. Lowry OH, Rosenbrough NJ, Farr AL, Randall J. Protein measurement with the folin phenol reagent. *J Biol Chem* 1951;193:265-75.
15. Bradford MM. A rapid sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248-54.
16. Venkatesh A, Prakash V. Functional properties of the total proteins of sunflower (*Helianthus annuus*). *J Agric Food Chem* 1993;41:18-23.
17. Lapanje S. Protein denaturation. New York: Wiley (Interscience); 1978.
18. Gheysaiddin S, Cater CM, Mattil F. Effect of several variables on the extractability of sunflower seed proteins. *J Food Sci* 1970;35:453-6.
19. Voitenko V, Poulsen LK, Nielsen L, Norgaard A, Bindlev-Jensen C, Stahl Skov P. Allergenic properties of kiwi-fruit extract: cross-reactivity between kiwi-fruit and birch-pollen allergens. *Allergy* 1997;52:136-43.
20. Taylor SL, Busse WW, Sachs MI, Parker JL, Yunginger JW. Peanut oil is not allergenic to peanut-sensitive individual. *J Allergy Clin Immunol* 1981;68:372-5.
21. Davis PJ, Williams SC. Protein modification by thermal processing. *Allergy* 1998;53:102-5.
22. Malanin K, Lundberg M, Johansson SGO. Anaphylactic reaction caused by neoallergens in heated pecan nut. *Allergy* 1995;50:988-91.
23. Vieths S, Hoffmann A, Holzhauser T, Muller U, Reindl J, Hausteiner D. Factors influencing the quality of food extracts for in vitro and in vivo diagnosis. *Allergy* 1998;53(Suppl 46):65-71.
24. Kelly JD, Hlywka JJ, Hefle SL. Identification of sunflower seed IgE-binding proteins. *Int Arch Allergy Immunol* 2000;121:19-24.
25. Moneret-Vautrin DA, Kanny G, Thevenin F. L'asthme par allergie alimentaire. *Rev Med Int* 1996;17:551-7.
26. Moneret-Vautrin DA, Rancé F, Kanny G, Olsewski A, Guéant JL, Dutau G, et al. Food allergy to peanuts in France—evaluation of 142 observations. *Clin Exp Allergy* 1998;28:1113-9.
27. Hourihane JO'B, Bedwani SJ, Dean TP, Warner JO. Randomised, double blind, crossover challenge study of allergenicity of peanut oils in subjects allergic to peanuts. *BMJ* 1997;314:1084-8.
28. Hourihane JO'B, Kilburn SA, Nordlee JA, Hefle SL, Taylor SL, Warner JO. An evaluation of the sensitivity of subjects with peanut allergy to very low doses of peanut protein: a randomized, double-blind, placebo-controlled food challenge study. *J Allergy Clin Immunol* 1997;100:596-600.
29. Onaderra M, Monsalve RI, Mancheno JM, Villalba M, Martinez Del pozo A, Gavilan JG, et al. Food mustard allergen interaction with phospholipid vesicles. *Eur J Biochem* 1994;225:609-15.