

Allergenicity of gourmet nut oils processed by different methods

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Background: No information is available on allergenicity of tree nut oils, and information on peanut oils has been conflicting. Many of the nut oils now on the market undergo minimal processing and may contain residual antigen.

Objective: This study was carried out to determine whether several of the new "gourmet" tree nut oils, as well as peanut oils, contain residual proteins that could bind IgE from sera of patients with allergy.

Methods: Several brands of walnut, almond, hazelnut, pistachio, and macadamia nut oils were examined. Peanut oils, both unrefined oils (which have been shown to contain allergenic proteins) and refined oils (without previously demonstrable allergens), were also examined to confirm reproducibility of immunoreactivity as reported by other investigators. Oils were extracted with 0.2 mol/L ammonium bicarbonate, and protein concentrations in the aqueous extracts were measured. IgE binding was assayed by slot-blot and Western immunoblotting. Pooled sera from patients with a history of systemic reactions to various tree nuts or peanuts were used as appropriate.

Results: The oil extracts known to be from oils that had undergone less processing at lower temperatures tended to demonstrate qualitatively greater IgE binding and higher protein concentrations.

Conclusion: Tree nut and peanut oils may pose a threat to patients with allergy, depending on the method of manufacture and processing. (*J Allergy Clin Immunol* 1997;99:502-7.)

Key words: Food allergy, oils, tree nuts, peanut

Peanuts and tree nuts are among the most common foods responsible for IgE-mediated food hypersensitivity in adults and children. Reactions to these nuts are also among the most serious. In a recent survey of patients with food-related fatal or near-fatal anaphylaxis seen over a 14-month period, 10 of the 13 patients described had a reaction to peanuts or tree nuts.¹ Although many childhood food allergies are "outgrown," peanut or tree nut allergy is usually lifelong.²

Previous studies have demonstrated how difficult it is for patients with known peanut allergy to avoid accidental ingestion. In one study 24 of 32 patients with known

Abbreviations used

PBS:	Phosphate-buffered saline
PDH:	Pyruvate dehydrogenase
SDS-PAGE:	Sodium dodecylsulfate-polyacrylamide gel electrophoresis

peanut allergy had subsequent unintentional exposure.² It is currently unclear whether nut or peanut oils pose a risk to patients with known hypersensitivity to the actual nuts or peanuts. It has been demonstrated that peanut oils that are "hot processed" do not contain antigen and thus do not pose a threat to patients with peanut allergy. Indeed, 10 patients with histories of peanut allergy and positive results for in vitro specific IgE assays to peanut were able to ingest up to 8 ml of hot-processed peanut oil with no untoward reaction.³ More recently, a study was conducted to determine whether there were antigenic differences between various peanut oils that had different methods and temperatures of manufacture.⁴ The study used extracts of peanut oil from seven manufacturers including four "cold-pressed" and three "hot-pressed" oils. These were each incubated with pooled sera from patients with peanut allergy. Immunoassays with iodine 125-labeled anti-human IgE demonstrated significant IgE binding to two of the four cold-pressed oil extracts but none of the hot-pressed oils. These two oils were also the only peanut extracts with significant concentrations of protein. This suggested that the lower temperature or reduced processing used in the cold-pressed oils allowed significant protein to remain behind, which theoretically could pose a threat to patients allergic to peanuts. Our study was initiated after a patient with walnut allergy reported anaphylaxis after eating a few bites of a salad that supposedly did not contain nuts; however, the dressing did contain gourmet walnut oil. We wished to determine whether IgE binding to the extracts of various tree nut and peanut oils processed in different ways could be demonstrated.

METHODS

Sera

Sera used in this study were chosen from a food allergy sera bank collected at the University of California at Davis. Informed consent was obtained from all subjects, and approval was obtained from the institution's human subjects review committee. Sera from 17 patients with nut or peanut allergy were chosen on the basis of a history of anaphylaxis and a specific IgE score greater than class 3 (Pharmacia Immuno-

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TABLE I. Oil characteristics and relative immunoreactivity of extracts

Oil type	Protein concentration (μg/ml)	Approximate protein loaded (μg/l)	Maximum processing temp. (C)	Immunoassay relative reactivity*
Almond				
Flora	62.2 ± 2.2	2.0	57	+/-
Hain	2.2 ± 0.7	2.0	230-260	-/+
Spectrum	16.7 ± 0.8	2.0	230-260	-/+
Gourmet Int.	12.7 ± 2.8	2.0	90; 190†	++
Nut extract	5300	2.0	4	+++
Peanut				
Flora	10.5 ± 0.4	1.0	54	+++
Hain	3.0 ± 0.3	1.0	230-260	+/-
Spectrum	10.7 ± 0.8	1.0	65-93	++
Hollywood	5.7 ± 1.2	1.0	230-260	-
Nut extract	9001	1.0	4	++++
Walnut				
Flora	16.5 ± 2.4	2.0	71	+
Hain	9.2 ± 3.1	2.0	230-260	-
Spectrum	7.0 ± 2.5	2.0	230-260	+/-
Gourmet Int.	20.4 ± 1.8	2.0	90; 190†	++
Diamond	7.0 ± 0.8	2.0	230-260	+
Nut extract	4671	2.0	4	+
Hazelnut				
Gourmet Inc.	62.0 ± 1.1	2.0	90; 190†	++
Nut extract	3900	2.0	4	+++
Macadamia				
Oil of Aloha	21.3 ± 0.9	2.0	65-93	-/+
Loriva	81.1 ± 0.8	2.0	65-93	+
Nut extract	3000	2.0	4	++
Pistachio				
Gourmet Int.	36.0 ± 1.5	1.4	90; 190†	+
Nut extract	5000	1.4	4	+++
Corn				
Mazola	8.7 ± 2.8	1.0-2.0	NA	Varied‡
PDH	9800	1.0-2.0	4	-

Values for protein concentration are expressed as means ± SD. NA, Not applicable.

*Relative reactivity was based on a subjective visual scale with "++++" assigned to the peanut extract as the most reactive protein extract tested; "-/+" meant that a band was barely visible, and "+/-" meant that a very light band was present.

†Gourmet International Oils are a blend of refined and unrefined oils.

‡Varied from trace positive with pooled sera for almond, walnut, peanut, hazelnut, and pistachio assays to strongly positive with the patient sera pooled for the macadamia assay. No patient had a history of corn allergy. Many were highly allergic to grass pollen.

CAP; Pharmacia Diagnostics Inc., Columbus, Ohio). Sera were pooled; sera from four to six patients with allergy to the various nuts were included in each group. Sera from atopic patients without food allergy were pooled and used as negative controls.

Oils

A total of 17 nut and peanut oils from eight different manufacturers were obtained. Nut oils from six different manufacturers were purchased from health food stores or distributors in California and Washington. These manufacturers included Flora Manufacturing (Burnaby, British Columbia), Hain Pure Food Co. (Los Angeles, Calif.), Spectrum Marketing (Petaluma, Calif.), Oils of Aloha (Hawaiian Macadamia Nut Oil Co., Waialua, Hawaii), Diamond Walnut Growers, Inc. (Stockton, Calif.), and Hollywood Peanut (distributed by Hain Pure Food Co., Inc.). Two manufacturers, Gourmet International (Anglia Oils, Ltd., Hull, United Kingdom) and Loriva Supreme Foods, Inc. (Hauppauge, N.Y.), donated nut oils. All test oils were mechanically extracted. Mazola corn oil (Best

Foods Division, CPC International Inc., Englewood Cliffs, N.J.) is solvent (chemically) extracted and was used as an oil "negative" control, although patient sera had shown moderately significant IgE binding to the protein extract of this oil in a previous study.⁴ Information on the processing methods for each oil was obtained directly from each of the companies.

All Flora oils are unrefined with no high temperatures, alkali, or ultrafiltration used. Spectrum peanut oil is similarly unrefined. Spectrum almond and walnut oils undergo refining, bleaching, and deodorization. All Hain and Hollywood oils are refined, bleached, and deodorized. Gourmet International uses a blend of refined and unrefined oils in all its products. The proportions vary according to the type and quality of the individual oils used. The proportions for the oils used in this study are unknown. Table I provides a summary of the temperatures reached during manufacture for each oil.

Oil protein extractions were performed on all nut and peanut oils, as well as on corn oil. In a 50 ml conical tube, 35 ml of each oil was added to 7 ml of 0.2 mol/L ammonium bicarbonate, pH

7.8, and rocked for 48 hours at 4° C. The samples were spun at 2200 g for 10 minutes (Sorvall RC-3B centrifuge with the H2000 rotor), and the 7 ml clear aqueous layer was collected. Additional ultracentrifugation was performed for 1 hour at 67,100 g (Beckman L5-75B ultracentrifuge with rotor 50.4 ti) at 4° C to enhance aqueous and lipid separation. After ultracentrifugation, the clear aqueous phase was collected, stored in 500 µl aliquots, and frozen at -70° C.

Nut and peanut protein extractions

Nuts obtained for the study were unprocessed and unsalted. Spanish peanuts, almonds, and hazelnuts were previously shelled and were purchased from a local health food store. Walnuts and pistachios were received in their shells from the Pomology Department at University of California, Davis. Shelled macadamia nuts were received as a gift from Mauna Loa.

Ten to twelve grams of almond, walnut, peanut, pistachio, hazelnut, and macadamia were blended individually in a Waring blender (Waring Products Div., New Hartford, Conn.) for 20 to 30 seconds. Five to six grams of the blended nuts was added to 15 ml of 0.05 mol/L ammonium bicarbonate, pH 7.8, in a 50 ml conical tube. The tubes were placed on ice, blended (Polytron PT-MR 3000; Brinkmann, Kinematica Ag, Littau, Switzerland) for 10 minutes per tube, and rocked at 4° C for 48 hours. The mixture was spun at 45,447 g for 20 hours at 4° C, and the top layer of fat plugs was removed and discarded. The sample was again spun at 45,447 g for 1 hour; the fat plugs were removed; and the aqueous layer was retrieved, stored in 500 µl aliquots, and frozen at -70° C.

Protein assays

Protein concentration of the nut extracts was measured by use of the Bradford protein assay (Bio-Rad Laboratories Inc., Hercules, Calif.). The nut oil protein extract concentrations were measured by the bicinchoninic acid assay (Pierce Chemical Co., Rockford, Ill.); protein concentrations were calculated in micrograms per milliliter of oil by extrapolation from the concentration in extracts, assuming complete extraction.

Slot-blot immunoassays

Immunoblots were prepared by filtering 1.0 to 2.0 µg of each nut oil protein, Mazola corn oil protein, and nut protein onto Nitropure 0.45 µm nitrocellulose paper (Micron Separations Inc., Westboro, Mass.) by using a Hybri-Slot Manifold (Bethesda Research Laboratories, Gaithersburg, Md.). Additionally, pyruvate dehydrogenase (PDH) (Sigma Chemical Co., St. Louis, Mo.) was applied to each test strip as an irrelevant protein control because a previous study had shown that Mazola corn oil extract could bind IgE from atopic sera⁴ and thus was not an ideal control. Extracts were applied to the Hybri-Slot Manifold, and a house vacuum was run for 5 minutes or until all slots were clear of liquid. The amount of protein applied, from 1.0 to 2.0 µg, depended on the viscosity of the samples: if one sample in a group reached maximum penetration through the nitrocellulose at 1 µg, the entire group of extracts was held at that level to allow comparison of relative IgE binding. The nitrocellulose paper was cut into 2 mm by 10 cm strips and placed in an incubation tray for blocking in 4% milk with phosphate-buffered saline (PBS), pH 7.4, at room temperature for 1 hour. The strips were probed in triplicate with pooled sera from patients with food allergy, pooled sera from atopic patients without food allergy, and an additional control of the secondary antibody alone. The positive and

negative sera pools were diluted 1:10 and prepared with 4% milk in PBS, pH 7.4, and incubated for 24 hours on a rocker at 4° C. Strips were washed five times for 10 minutes with PBS-0.05% Tween-20 and incubated for an additional 24 hours on a rocker at 4° C with ¹²⁵I-labeled anti-human IgE (Sanofi Diagnostics; Pasteur, Chaska, Minn.). Strips were then washed five times with PBS-0.05% Tween-20, dried, and exposed to film (Fuji Medical X-Ray film-RX; Fuji Photo Film Co., Ltd., Tokyo, Japan) for 1 to 3 days at -70° C.

Western immunoblotting

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on concentrated peanut oil protein extracts. Two milliliters of each peanut oil sample was spun for 3 to 6 hours, depending on the viscosity of the sample, in a Centricon 10 filter (Amicon, Beverly, Mass.) in an SS34 rotor in the Sorvall RC35 centrifuge at 5000 g. Samples were boiled for 5 minutes in sample buffer (50 mmol/L Tris [pH 6.8], 10% glycerol, 2% SDS, 100 mmol/L dithiothreitol, and 0.0025% bromophenol blue). Approximately 2 µg of protein was loaded in each lane of a 15% polyacrylamide mini-gel (Mighty Small electrophoresis Unit; Hoefer Pharmacia Biotech, San Francisco, Calif.) and run at a constant current of 20 mA. Transfer of the proteins onto Nitropure 0.2 µm nitrocellulose paper (Micron Separations Inc.) was performed on the Transphor TE 42 Unit (Hoefer Pharmacia Biotech) overnight at 30 V. The blots were then cut into strips, and immunoblotting was carried out as described previously.

Immunoblot inhibition with grass extract

To explore the previously reported reactivity of Mazola corn oil extract with atopic patient sera, immunoblot inhibition was performed. Samples of 0.7 ml of the pooled control and patient sera (1:10 dilution of each), used in the macadamia nut oil assay, were subjected to extensive incubation with 4 mg of perennial ryegrass pollen (*Lolium perenne*; Bayer Inc., Spokane, Wash.) for 6 hours at room temperature, followed by overnight incubation at 4° C on a rocking platform; samples were then spun, and the supernatants were collected. Sera, both preabsorption and postabsorption samples, were then incubated with nitrocellulose strips onto which 2 µg each Mazola corn oil extract, PDH, and ryegrass pollen extract had been slotted; and immunoblotting was carried out as described above.

RESULTS

Protein concentrations determined in the various oils differed significantly within each oil type. Table I shows the mean protein concentrations and standard deviations. Flora (unrefined oils) and Gourmet International oils (blend of unrefined and refined) tended to yield relatively high concentrations of protein across all oil types. Spectrum yielded the highest concentration of protein for refined almond oil and lower concentrations for unrefined peanut and refined walnut oils. Diamond walnut, Hain peanut and almond, and Hollywood peanut oils, which are all deodorized, yielded relatively low protein concentrations. Within nut oil groups, a generalization could be made: those that underwent the least processing (reflected by lower maximum temperature in Table I) had the higher protein concentrations. The only exception to this was Spectrum almond oil (refined), which had a higher protein concentration than Gourmet International almond oil (blend of refined and unre-

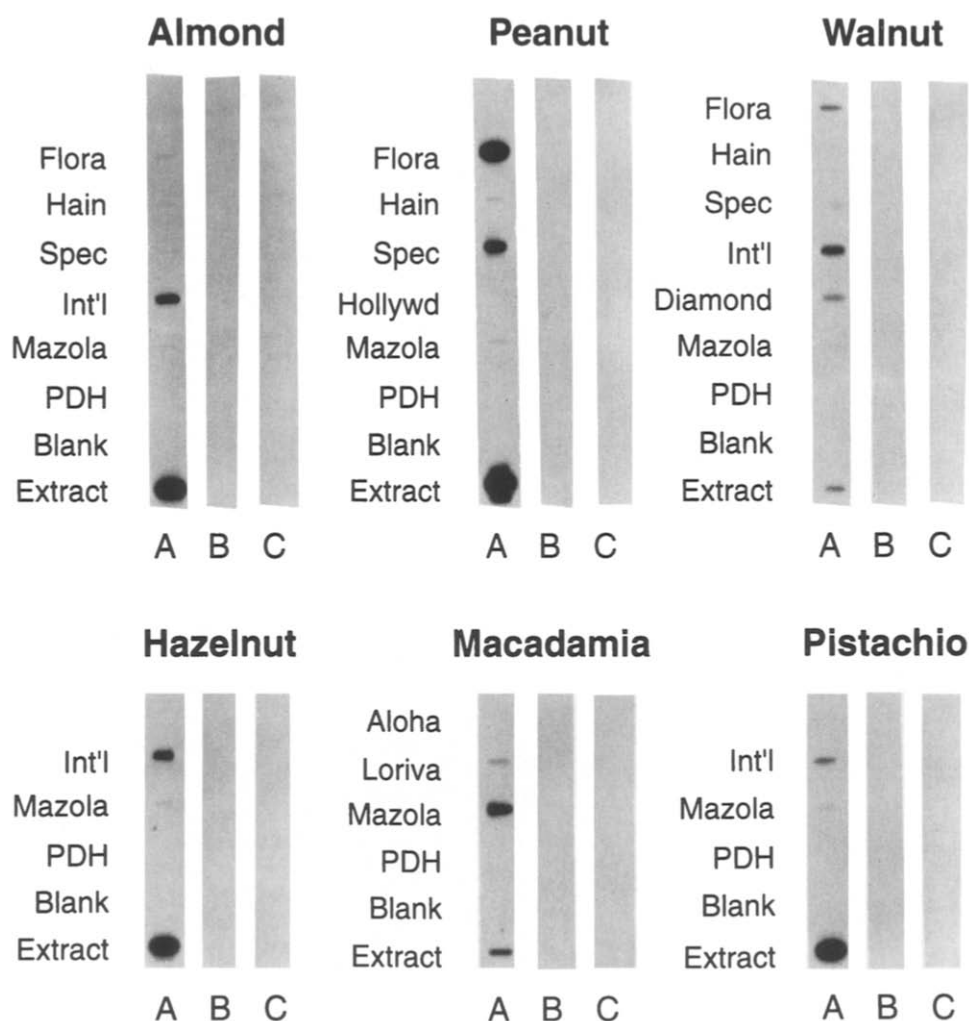


FIG. 1. Slot-blot immunoblots of extracts and controls. Lane A, Oil extracts versus sera from patients with food allergy. Lane B, Oil extracts versus sera from subjects without food allergy. Lane C, Oil extracts versus secondary antibody only. Spec, Spectrum; Int'l, Gourmet International; Hollywd, Hollywood.

fined, ratio unknown). The two macadamia oils, Loriva and Oil of Aloha, showed high protein concentrations, with the concentration of Loriva oil being very high. Positive control extracts made from unprocessed nuts all had many fold higher protein concentrations than any of the oils. Mazola corn oil yielded a low concentration of protein in the extract.

Autoradiographs of the slot-blot immunoassays are shown in Fig. 1. The test oil extracts demonstrated widely variable reactivity. The nut and peanut extracts all bound IgE strongly, as expected. Controls included two duplicate strips for each nut or peanut assay. One strip was incubated with the secondary antibody alone without sera to control for nonspecific binding. The other was incubated with sera from patients with allergic rhinitis in response to various aeroallergens including grass, ragweed, dust mites, and molds but no allergy to any nuts or peanuts. The controls in both instances, compared with all peanut oil or nut oil extracts, did not

demonstrate any binding. Additionally, Mazola corn oil extract and PDH, an irrelevant protein, were also slotted onto each strip. PDH did not show any IgE binding. Mazola corn oil extract showed slight reactivity to most of the pooled sera and moderately strong IgE binding to the sera used for the macadamia nut oil assay. This was also seen in a previous study in which this oil extract was used as a control.⁴ Most of the patients in this study were allergic to grass, but no patient or control subject had any history of corn food allergy. We hypothesize that one or more of the serum samples in each pool was from a patient with grass or grain IgE because this IgE has been demonstrated to cross-react *in vitro* with corn.⁵

Indeed, Fig. 2 shows the results of immunoblot inhibition of one group of patient pooled sera (macadamia) and the control pooled sera with ryegrass pollen extract. Absorption of the sera with ryegrass pollen completely removed reactivity to the Mazola corn oil extract.

When the slot-blot immunoassay results were exam-

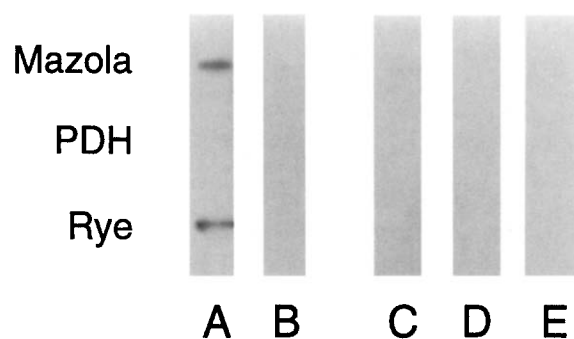


FIG. 2. Slot-blot immunoblot inhibition of reactivity to Mazola corn oil extract by preincubation of sera with perennial ryegrass pollen extract. *Lane A*, Pooled sera used for the macadamia assay. *Lane B*, Pooled sera used for the macadamia assay after incubation with perennial ryegrass pollen. *Lane C*, Pooled sera from patients not allergic to food. *Lane D*, Pooled sera from patients not allergic to food after incubation with perennial ryegrass pollen. *Lane E*, Secondary antibody alone.

ined individually (Fig. 1), the least refined almond oil extracts, Gourmet International (blend of unrefined and refined) and Flora (unrefined), showed the most IgE binding, as judged visually by the intensity of the banding pattern. Hain and Spectrum almond oil extracts, which are both refined, showed only a barely visible banding pattern. All bands, however, were less intense than that produced by the almond kernel extract itself.

Within the peanut group, Flora oil extract, which claims the lowest temperature and minimal processing, showed the greatest amount of IgE binding, duplicating the results of the previously reported study.⁴ Spectrum oil extract also demonstrated a dark band (unrefined oil). Hain, which was refined, showed a light banding pattern. Hollywood peanut oil extract, also refined, did not demonstrate any IgE binding.

Results for walnut oil extracts were somewhat surprising. Gourmet International revealed the most intense banding pattern (blend of unrefined and refined), but Diamond, a refined oil, followed and was equal to Flora (unrefined) in band intensity. Spectrum (refined) showed a very light band, whereas refined Hain showed no band.

There was one sample oil each for hazelnut and pistachio, this being Gourmet International. These two oil extracts were reactive against their respective sera but less so than the positive control extracts. One macadamia oil extract, Oil of Aloha, had a barely visible band; and the other, Loriva, was more reactive. Both are unrefined oils.

SDS-PAGE IgE immunoblotting results for peanut oil extract are shown in Fig. 3. Flora oil extract showed lighter band intensity at 24 hours than the peanut kernel extract, with approximately the same banding pattern on this mini-gel immunoblot. However, a band faintly present at 17 kd on the peanut kernel extract lane does not appear to be present in the Flora lane; rather, there is a faint band at approximately 14 kd. IgE binding

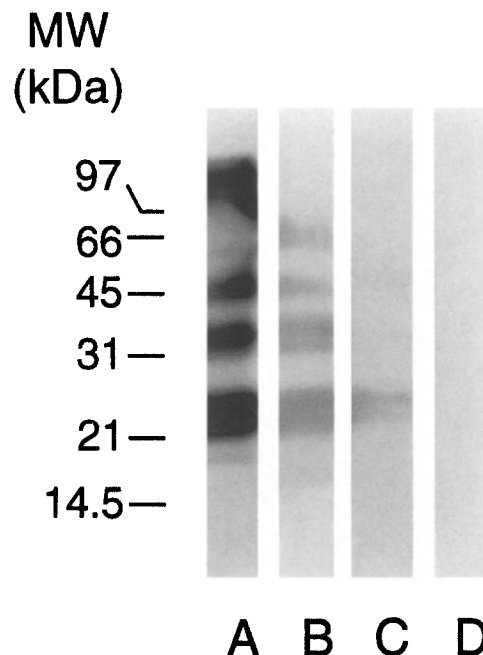


FIG. 3. Western immunoblotting of peanut extract and peanut oil extracts. Approximately 2 μ g of protein loaded per lane. *Lane A*, Peanut extract, exposed to film 24 hours. *Lane B*, Flora peanut oil extract exposed to film 24 hours. *Lane C*, Spectrum peanut oil extract, exposed to film 72 hours. *Lane D*, Pooled sera from patients not allergic to food, exposed to film 72 hours. There is a bubble artifact at 66 kd in lane A. Note that the band normally seen (faint) at 17 kd in the peanut extract is not seen in lane B, but a band at a lower molecular weight is present. In addition, higher molecular weight moieties are not present in the oil extracts.

showed up for the Spectrum oil extract at 72 hours. Neither Flora nor Spectrum extracts showed banding at the highest molecular weights present for the peanut kernel extract. Neither of the other peanut oil extracts had IgE binding demonstrated with longer exposure of the film (Hain or Hollywood). Flora had the most intense binding of the oil extracts on the slot-blot immunoassay, followed by Spectrum.

DISCUSSION

The data in this study indicate variability in IgE binding from pooled sera to various brands and types of nut oil extracts in vitro. Until recently, peanut oil was believed to be nonallergenic to peanut-sensitive patients on the basis of a study in which one brand of oil was used.³ The authors detected no protein in this oil and demonstrated negative double-blind placebo-controlled food challenge results, as well as negative skin test responses. However, anecdotal cases have continued to describe hypersensitivity to peanut oils. More recent studies have suggested that some brands or types of oils may indeed pose a threat.^{4,6} In addition, a report describing four cases of infant formula-induced atopic dermatitis suggested that the peanut oil in the formula was responsible.⁷ To our knowledge, peanut oil is not used in infant formulas in the United States.

There is very little information on the protein content of oils, including nut oils. Two earlier studies from the chemist's perspective examined protein in peanut oils. One detected protein,⁸ and the other did not.⁹ The study that detected protein did not state how the oils were processed. We were unable to find any information on protein content or allergenicity of tree nut oils. It is also unknown whether variables such as time after processing, exposure to light, or ambient shelf temperatures affect protein levels and quality. We did not have information, for example, on the time interval between processing and our assays. In addition, the role and nature (including variability) of phytochemicals (e.g., polyphenolics) in nut extracts are unknown and may affect protein extraction and stability.

In recent years there has been an increase in the number of gourmet cooking oils made from a variety of nuts and peanuts. Several of these brands claim minimal processing, including lack of chemicals and low temperatures to maintain the oil's flavor and health properties. Most of these, including all test oils in this study, are expeller pressed to extract the oil from the nut. This is a purely mechanical process without the use of chemicals, such as hexane, which are used to extract some high-volume oils. The temperatures reached in the expeller process, which are created by friction, are 65° to 95° C. The term *cold-pressed* has been used to describe this process because of the lower temperatures achieved as opposed to hot-pressed, in which oil is chemically extracted and distilled at high (above 150° C) temperatures. Instead of this terminology, the food oil industry now uses the more descriptive terms *expeller extraction* (all nut and peanut oils in this study) and *chemical extraction* (Mazola corn oil in this study).

The purification or refining process of the extracted oil involves several steps. These steps include de-gumming, which involves separation of oil and water by centrifugation at 30° to 50° C, refining with alkali and further centrifugation at 60° to 70° C, bleaching with filters at 110° C, deodorizing with steam under vacuum at 230° to 260° C, and winterization in which the oil is cooled to 7° C and filtered. These processes remove free fatty acids, soaps, peroxides, and other impurities that would otherwise impart off-flavors or colors or reduce shelf life. Some of the oils tested in this study underwent this refining process; others did not. One study that attempted to quantify protein in refined and deodorized peanut oil detected no protein.¹⁰

Our data indicate a trend between the amount of residual protein in a given oil and how minimally it was processed. In addition, those oils with the most protein and least processing tended to demonstrate the strongest IgE binding within each group of nut oil extracts. There was generally an inverse relationship between whether

an oil was processed at high temperature and the ability of its extract to bind IgE, although more samples and densitometric grading of the slot-blot immunoassay are needed to subject this to statistical analysis. It has previously been demonstrated that peanut allergens present in vegetable oil used to roast peanuts were reduced by filtration and steam cleaning.⁹

In conclusion, this study suggests that several brands of minimally processed nut and peanut oils may contain enough antigen to pose a threat to hypersensitive individuals. It is not clear which techniques in processing reduce protein or alter or eliminate allergenic epitopes on the proteins, some of which may be conformational. Our data indicate that very high temperatures under vacuum (deodorization) generally correlate negatively with both protein content and in vitro IgE binding; but sample sizes are small, and exceptions are present. A study that uses crude nut extracts and manipulates the various processing steps while controlling for others may demonstrate which steps render the oil extract nonimmunoreactive. If a particular processing step were identified, this could have important implications for product labeling and the advice given to patients with nut or peanut allergy.

Considering the in vitro variability in IgE binding seen here, further investigation to correlate these findings in patients needs to be carried out in vivo. This should include both skin testing and subsequent food challenges with products similar to those used in this study.

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