

## INVESTIGATION OF SOME EXTRACTION METHODS FOR THE RECOVERY OF PEANUT PROTEINS FROM OILS AND FATS

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### Abstract

*Food allergies have a considerable impact on modern society. There is no known cure. As a result, consumers can only avoid offending foods and use pharmacological agents. Some of the most severe allergic reactions occur when peanuts and peanut derivatives as peanut oils are consumed. The food industry will have to comply with requirements set forth by law for all packaged foods sold in the European Union. At times, it can be difficult to measure allergenic proteins in a wide variety of foods. Yet the food matrix can sequester allergens, inhibiting their detection, without significantly affecting allergenicity. The studies about allergenicity of edible oils and related to peanut oils are few and enough controversies. Some studies showed the presence of peanut allergens some not. It has to be emphasised that different studies used different methods for extraction, concentration and detection the peanut traces so the results had difficult been compared. In this context we investigated some extraction and concentration methods for the recovery of proteins from oils and fats derived from, or containing, peanut. The recovery of total protein and peanut allergens are very different for each method. Our result show how much the results depend on the method used to extract or/and concentrate the proteins from different matrices. The influence of solvent plays an important role in that process. Interactions with lipids of protein may alter the possibility to detect and quantify them by a hiding allergen/protein effect.*

**Key words:** peanut allergens, oil, extraction methods, concentration methods.

### INTRODUCTION

Peanut and nut allergens represent nowadays a challenge for health and food manufacturers both. The threat of an adverse reaction can be present for sensitive people everywhere in food.

Peanuts are one of the 8 most common allergenic foods and a large proportion of peanut-allergic individuals have severe reactions, some to minimal exposure. Specific protein constituents in the peanuts are the cause of the allergic reactions in sensitized individuals who ingest the peanuts.

Peanut seeds are rich in oil (40 – 50 %) and as a consequence are used like an excellent source of oil. It seems that peanut oilseeds production is in a slightly increment in the last years.

Refined peanut oil is usually labelled as vegetable oil but according with the European legislation now it is mandatory to specify on label when peanut is used.

The allergenicity of refined peanut oils is not so clear (Moneret-Vautrin, Hatahet et al., 1991; Moneret-Vautrin, Hatahet et al., 1994; Hourihane, Bedwani et al., 1997; Peeters, Knulst et al., 2004).

Some studies showed the allergenicity, some not, of edible peanut oils. However it is obviously that the refining process may affect the allergenicity and the thresholds for adverse reaction vary according to sensitive patient.

The studies about allergenicity of edible oils and related to peanut oils are few and enough controversies.

Edible oils undergo usually extensive processing which removes virtually all the protein from the oil. Scientific studies showed that refined oils don't contain allergic protein in detectable amounts (Hefle, 1999). But some studies showed that vegetable oils/fats, crude or even refined, can contain proteins - in peanut allergens case - even that these were hot-pressed processed (Klurfeld and Kritchevsky,

1987; Hoffman and Collins-Williams, 1994; Teuber, Brown et al., 1997; Koppelman, Bruijnzeel-Koomen, et al., 1999; Zitouni, Errahali et al., 2000; Hidalgo, Alaiz et al., 2001; Hidalgo, 2006). Usually by mechanical or cold press the allergenic proteins are not removed in totality, have been considered impurities. These oils aren't used domestically but are often found in healthy food, with increased nutritional value, or in gourmet food stores. Therefore the restaurants and food service facilities have to specify what kind of oil was used. In the meantime vegetable oils, and obviously the oil from peanut, are used for preparing margarine and spreads, and if oils used contained allergenic protein the product would contain it too. Obviously, if some ingredients contain protein from the source material, they are likely to be allergenic to consumers who are allergic to the source foods (Taylor and Hefle, 2001).

Nowadays are known 17 peanut allergens, Ara h1 - Ara h17.

Major peanut allergens are Ara h1, Ara h2, Ara h3 with Ara h4 as isoallergen of Ara h3 and Ara h6. Minor allergens are Ara h5, and Ara h7 to Ara h17. Belong to these proteins there is Ara h agglutinin whose role is not yet clearly understood.(Olszewski, Pons et al., 1998; Besler, Steinhart et al., 2001; Enrique, Utz et al., 2006; Agrawal et al., 2010; Mueller et al., 2014; Offerman et al., 2015; Schwager et al., 2015).

The published peanut proteins/allergens content of edible peanut oils fluctuate widely being dependent on source of the oil as well as the methodology used for extraction, concentration and analysis. Each manufacturer uses own protein extract method, content determination method and standards. The manufacturing processes are very different and the residual protein content too.

To avoid accidental ingestion of peanut-contaminated food, methods of analysis for the determination of the allergenic proteins in foods are important tools. Such methods could help identify foods inadvertently contaminated with peanuts, thereby reducing the incidence of allergic reactions to peanuts. Commercial immunoassay kits are available but need study for method performance, which requires reference materials for within- and between-

laboratory validations. Alternative methods are necessary too in aim to have a better analysis of allergen proteins.

This study will report a comparison and assessment of some peanut allergens extraction methods.

## MATERIALS AND METHODS

The test samples were obtained spiking pasteurized fresh margarine with peanut reference material 481 (IRMM). Butter was spiked with peanut grinded and peanut extract in Tepnel buffer without gelatine for 10.000 ppm, 1000ppm, 100ppm 10 ppm and 1ppm concentrations.

The test samples were obtained using Unirea Original margarine, 60% fat, produced by SC Orkla Foods Romania SA.



Figure 1. Unirea Original margarine

The reference material containing the peanut allergens, denoted 481, was obtained from the Institute for Reference Materials and Methods (IRMM), Belgium.

The samples for testing were obtained by homogenizing the reference material, which has a content of 1000000 ppm Ara h1, in appropriate amounts to give concentrations of 10 000 ppm, 1000 ppm, 100 ppm, 10 ppm and 1ppm. For homogenization was used a Braun mixer.

For immunochemical analyses of peanut allergens we used BioKit-sandwich Cat ELISA. No.902048Q. The kit was purchased from R-Biopharm, Darmstadt Germania.

Stirrers centrifuge and ultracentrifuge, water baths are necessary to prepare the samples and extracts.

For allergens analysis was used a plaque reading spectrometer Model 3200, serial number 2100, from Awareness Technology Inc. US.

## Extraction methods:

**Method I:** 1.0 g ( $\pm$  0.1 g) of each sample was weighed and extracted with 10 ml Tris-HCl buffer (0.6 % Tris, 1.17 % NaCl and 10 % gelatine; pH 8.2) for 15 min at 60 °C in a water bath with continuous shaking. The extracts were centrifuged at 1730 g for 20 min at 4°C. The supernatant was collected and used in the analyses. This method is Tepnel kit extraction procedure.

**Method II:** 1.0 g ( $\pm$  0.1 g) of each sample was weighed, melted at 40°C and CMC solution was added into the same beaker to a total weight of 8g. The mixtures were homogenized until homogenise emulsions were obtained and the samples were stored in a fridge until using.

**Method III:** 1.0 g ( $\pm$  0.1 g) of each sample was weighed and extracted with 0.8 ml of 0.2mol/L ammonium bicarbonate, pH=7.8, for 48 hours at room temperature (20–22°C) using a rotative stirrer. The extraction tubes were centrifuged, 3800g/30min/4°C and the clear aqueous layers were collected by suction with syringes after a part of fat layer is removed.

## Extraction plus Concentration methods:

**Method IV:** 1 g samples + 5 mL ammonium bicarbonate 0.1 M were shaken overnight at 60°C. 5 ml of hexane was added to each sample; samples were vortexed until they formed an emulsion and then centrifuged at 3800g/30min/4°C. The hexane layer was removed and other 10 ml of hexane were added following the above procedure (2 times). The aqueous layer was removed carefully with a syringe. 2 mL aqueous layer was treated with 20 mL cold 10% TCA in acetone; samples were incubated at 20°C overnight. Samples were centrifuged at 18000g/15min/4°C. Precipitates were dissolved in Tepnel buffer (without gelatine) until 0.75 ml each.

**Method V:** 1 g sample (before add the extraction buffer I melted the samples at 45°C for 10 min) + 20mL 20% ethanol in TBS was vortexed then put in the ultrasonic bath for 20 min/4°C. Centrifugation was made at 9300g/30 min/4°C. Were taken 10 ml aqueous layer without disturbing the lipid layer and put in a clean tube; the rest of solution was discarded. Were added 40 ml 10% TCA in acetone, mixed well with Turax and then incubated on ice 15 min. The samples were centrifuged at

9300/30min/4°C. The supernatant was removed and then added 1 ml mili Q water and vortexed 15 sec. 10 mL cold acetone were added and vortexed until all pellets were dispersed; incubated at -20°C overnight. The samples were centrifuged at 9300/30min/4°C. The supernatant was carefully removed and the pellets dissolved in 1.5 ml Tepnel buffer (without gelatine) and kept the product at freezer.

## RESULTS AND DISCUSSIONS

The extractable proteins consist of both the allergenic proteins and non-allergenic proteins. The proportion of which may vary from product to product and of the matrix composition.

Taking in account the mainly methods used by different laboratories to extract and concentrate the oil/fat proteins we followed the core ideas of some of its. So we extracted by 3 methods and extracted and concentrated the oil/fat proteins by 2 methods.

To evaluate the capacity of extraction and concentration of the methods used we processed and analyzed the same spiked peanut and we reported the results to the same starting quantity 1g margarine spiked with peanut even if the quantities of sample used by various methods were different. So in aim to compare the efficiency of each method to report all results to 1 g original sample it is the best choice.

The general averages of peanut allergens from each extracted sample resulted by extraction methods only are presented in Table 1.

Analyzing the results from the table above we see that in the method II case the allergen proteins detected are less than those detected by the reference method I (Tepnel method). Taking in account the fact that by method II we used more sample (corresponding to 1.25 g margarine spiked processed) following the same procedure as in Tepnel method but without a supplementary buffer extraction, the peanut allergens content obtained showed actually a dilution and the CMC solution even if realizes a better homogeneity and stability in time is not a very good buffer for extraction.

Table 1. Averages of peanut allergen content in samples extracted by different methods

Sample	Method I		Method II		Method III	
	ppm	Recovery, %	ppm	Recovery, %	ppm	Recovery, %
1	0.5	53.0	1.7	177.3	7.7	777.5
10	7.5	75.2	5.23	52.3	93.5	935.2
100	95.2	95.2	37.1	37.2	1242.8	1242.8
1000	1131.3	113.1	561.2	56.1	12465.9	1246.5
10000	13057.5	130.6	11822.3	118.2	127834.3	1278.3

Table 2. Averages of peanut allergen content in samples extracted and then concentrated by different methods

Sample	Method I		Method IV		Method V	
	ppm	Recovery, %	ppm	Recovery, %	ppm	Recovery, %
1	0.5	53.0	0.10	10.0	0	0
10	7.5	75.2	1.7	17.3	0	0
100	95.2	95.2	5.2	5.2	4.6	4.6
1000	1131.4	113.1	34.4	3.4	22.4	2.3
10000	13057.6	130.6	205.2	2.1	240.6	2.5

Method III seems that realizes a concentration in the meantime with the extraction. The explanation is very simple. The quantity of buffer extraction was only 0.8 ml per 1g sample so we obtained a solution 12.5 time concentrated. If we take in account this factor of concentration and calculate the real quantity detected using Tepnel procedure we find that the peanut allergens recovered are less than by method I (1:0.62; 10:7.5; 100:99.4; 1000:997.3; 10000:10226.7). But even so this method has the advantage to concentrate the sample just by extraction in a small quantity of buffer.

The general averages of peanut allergens from each extracted sample resulted by extraction and then concentration methods only are presented in Table 2.

The above results show how much the different preparation method of a sample presumed that contain protein allergens might lead to unreal values, to the loss of allergens. The capacity of recovery it seems that is not depend on starting

concentration being enough similar for all kind of sample in the same method.

Considering the quantity of allergen which was added and the quantity which we found in each sample we calculate the concentration factor.

In Table 3 are presented the concentration factor calculated against the theoretical quantity which was added (CFa) and the concentration factor against the quantity which we found in the original samples (margarine spiked with peanut) (CFb) for each method. As we presumed the methods III have the biggest concentration factor. The large variability between results for the same method can be explained mainly by the strong influence of matrix but by the difficulty of each method too. In the method III case the lower ratio between sample quantity and the extraction buffer (1 g/ 0.8 mL) determined a bigger extraction of allergens/proteins. Nonetheless this method doesn't offer a complete extraction of peanut allergic proteins.

Table 3. Concentration factor of peanut allergens

Sample	Method I	Method II		Method III		Method IV		Method V	
	CFa	CFa	CFb	CFa	CFb	CFa	CFb	CFa	CFb
1	0.50	1.77	3.54	7.78	15.56	0.100	0.200	0.000	0.000
10	0.75	0.52	0.70	9.35	12.47	0.170	0.227	0.000	0.000
100	0.95	0.37	0.39	12.43	13.05	0.052	0.055	0.046	0.048
1000	1.13	0.56	0.50	12.47	11.02	0.034	0.030	0.022	0.020
10000	1.31	1.18	0.91	12.78	9.79	0.021	0.016	0.024	0.018

Taking in account the recovery capacity and concentration factor the best method is III and the weakest VII:  $\text{III} > \text{II} > \text{IV} > \text{V}$ .

Our results show how much the results depend on the method used to extract or/and concentrate the proteins from different matrices, the influence of solvents plays an important role in that process and that some proteins/peptides are soluble in lipid matrixes. In addition fatty acids which are present in dairy products and industrially hydrogenated vegetables (such as margarine) affect the quantity determination of protein content.

Finally, the lowest observed adverse effect levels of allergenic proteins in edible oils should be determined and simple methodologies developed for their analysis. All these advances will contribute to development of naturally nutritionally enhanced and safer edible oils.

## CONCLUSIONS

As a general conclusion to evaluate samples regarding peanut protein included into a mass with a large quantity of an outsider protein it is a difficult task because that protein can hidden the protein what we looking for. There had been some speculations that total extractable proteins were not correlated to their allergenicity or allergen contents.

However our results like other presented in different articles show that between total proteins an allergen proteins exist a significant correlation.

Complex and time consuming protocols cause wastage of proteins, materials and time too.

We proposed simplified method to extract and concentrate the proteins/allergens from oils or vegetable fats.

The use of validated analytical methodologies for extraction/concentration and for establishing proteins/allergens content of oil are required to compare the data obtained by different laboratories. The development of more simple methodologies to extract or/and concentrate oils proteins it is necessary in aim to be applied routinely in research laboratories and industrial plants.

The lack of use of appropriate and validated methodology for protein content determination still pose questions touching the validity of oil proteins data from different published studies.

The lowest observed adverse effect levels of allergenic proteins in edible oils should be determined and simple methodologies developed for their analysis. All these advances will contribute to development of naturally nutritionally enhanced and safer edible oils.

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