JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Quantification and Partial Characterization of the Residual Protein in Fully and Partially Refined Commercial Soybean Oils

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ABSTRACT: A method has been developed to determine residual protein in refined oils, a potential trigger of allergic reactions. High-pH bicarbonate or borate buffers were found to be the most effective extractants, residual oil protein comprising a mixture of proteins of M_r 6000−100000. Extracted protein could be quantified with superior precision using 3-(4-carboxybenzoyl)quinolone-2-carboxaldehyde (CBQCA). Residual protein content determined in a set of oils using the borate extraction−CBQCA assay was positively correlated with contents determined using a bicarbonate−total amino acid analysis method. Oil refining substantially reduced the oil protein content determined by the borate−CBQCA assay with neutralized/refined, bleached, and deodorized (fully refined) oils containing 62−265 ng/g oil, whereas crude un-degummed oils contained 86000−87900 ng/g of protein. These analyses and published data on cumulative threshold doses for soybean suggest that even the most sensitive individuals would need to consume at least 50 g of highly refined oil to experience subjective symptoms.

KEYWORDS: food allergy, protein, soybean oil, determination, refining

INTRODUCTION

Several commercially important fully refined vegetable oils are derived from the seeds of plants that are recognized as important food allergens (e.g., peanut, soy). Full refining of oils is a complex process and results in the almost complete removal of any residual protein (Figure 1).[1] Oil is extracted from crushed soybeans using a solvent (usually hexane) in a countercurrent process, which is removed subsequently by distillation. The resulting crude oil contains many impurities, including proteins, which are then removed during the refining process. First, the crude oil is degummed, a process that involves hydration and heating and removal of the resulting precipitate comprising proteins, carbohydrates, and phosphatides. The degummed oil is then neutralized using alkali to form a soap containing free fatty acids along with residual protein, carbohydrate, and phosphatides, which are then removed. These same residual impurities are reduced still further by the bleaching process, which involves the addition of adsorbents such as activated clay, silica, and in some cases activated charcoal. Finally, the oil is deodorized to remove volatile substances, including odors and off-flavors, pesticides, and light polycyclic aromatic hydrocarbons. This process is carried out under vacuum and at temperatures of between 180 and 270 °C using steam or nitrogen as a stripping agent.

Full refining should result in an oil virtually lacking any proteinaceous material, and because this is the component implicated in allergic reactions such oils should lack the capacity to provoke allergic reactions. Well-designed clinical challenge studies in patients allergic to the plants from which the oils were derived have shown this expectation to be well-founded for peanut and soybean oils.[2−4] However, some highly sensitive allergic individuals respond to allergens at very low doses, albeit at least 1 order of magnitude higher than the likely intake from a normal portion of edible oil [5]. Furthermore, some researchers have demonstrated occasional reactivity to oils designated as refined,[6] leading to a vigorous debate about the safety of refined oils and specifically whether to label each type of oil individually because of the possible risk to allergic consumers.

Demonstration of the safety of fully refined peanut oil[4] led to a Code of Practice, which defined the refining process to ensure the safety of all edible oils.[7] This Code of Practice was implemented in all European refineries by 2002. Interestingly the clinicians who had previously reported reactions to various refined oils concluded more recently that reactions now seemed to be confined to cold-pressed unrefined oils.[8] However, whereas the Code of Practice appears to be successful in assuring the absence of allergenicity, reliable benchmarks against which the final product could be judged are still lacking. Attempts to quantify and characterize the residual proteins of oils indicate that crude oils contain about 100−300 mg/kg, whereas fully refined oils contain at least 100-fold less.[1] However, current analytical methodology is inadequate and has not been fully validated for use with oils and aqueous extracts from oils. Little is known about the contribution of different processing steps to protein removal, although this information is crucial to risk assessment, particularly when considering process modifications. Crevel and co-workers[1] concluded that there was a need to standardize and validate methodology for measuring the protein content and immunoreactivity of edible oils so that these data can

Received: September 14, 2010
Revised: December 1, 2010
Accepted: December 2, 2010
Published: January 20, 2011

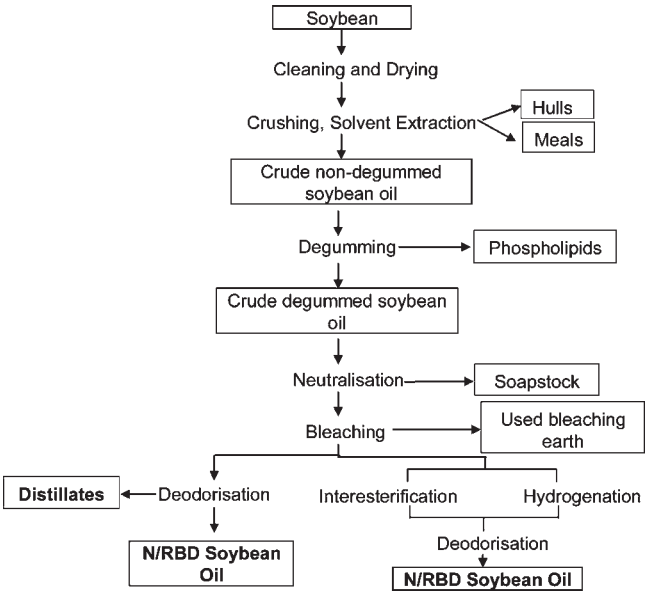

Figure 1. Simplified production process of neutralized, refined, bleached, and deodorized (N/RBD) soybean oil (adapted from FEDIOL, http://www.fediol.org/2/index2.php).

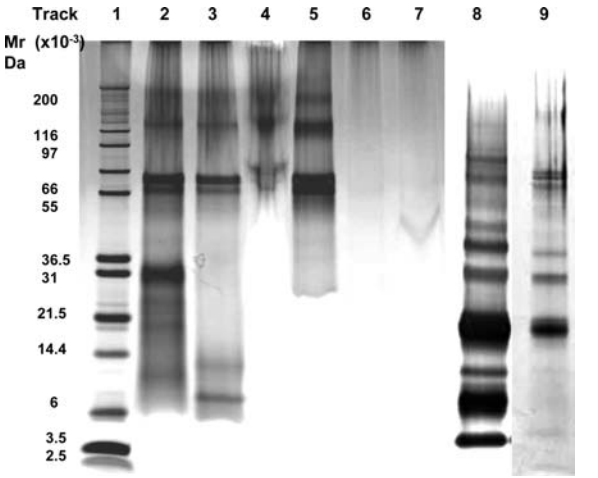

Figure 2. SDS-PAGE analysis of protein extracts of crude and highly refined N/RBD oils using three different extraction methods. Track 1 shows molecular weight markers. Extracts were from crude degummed oil (tracks 2, 4, 6), its counterpart N/RBD oil (tracks 3, 5, 7), and a crude non-degummed oil (tracks 8, 9). Extracts were prepared using the bicarbonate (tracks 2, 3, 8), phosphate-buffered saline (tracks 4, 5), cold acetone (tracks 6, 7), and borate (tracks 9) methods. Protein was stained using silver. The protein loaded per track and its equivalent in oil mass were as follows: track 2, 0.85 μg (4.9 g of oil); track 3, 1.1 μg (5.5 g of oil); track 4, 1.3 μg (7.5 g of oil); track 5, 1.5 μg (7.5 g of oil); track 6, 1.3 μg (7.5 g of oil); track 7, 1.5 μg (7.5 g of oil); track 8, 0.4 μg (0.25 g of oil); track 9, 2.5 ng (28 μg of oil). Staining intensity relates to length of staining, because tracks 8 and 9 were developed more extensively to allow visualization of protein in track 9.

be used to maintain process specifications. This paper describes extraction methodology, coupled with the application of a very sensitive protein assay, to quantify the levels of protein in commercial samples of fully refined soybean oil of diverse origins, as well as in samples of oil from different points of the refining chain.

MATERIALS AND METHODS

General Chemicals and Reagents. Acetone and hexane were purchased from Fisher Scientific (Leicester, U.K.). Sodium phosphate, sodium tetraborate, and 2% dimethyldichlorosaline in heptane were supplied by BDH (Poole, U.K.). Sodium bicarbonate was obtained from Sigma Chemical Co. (Poole, U.K.), trichloroacetic acid was from Fluka Analytical (Poole, U.K.), and NaCl was supplied by Riedel-de Haën (Poole, U.K.)

Soybean Oil Samples. Soybean oils of different types were obtained from several sources. Crude degummed and fully refined (neutralized, bleached, and deodorized; NBD) soybean oils were kindly provided by member companies of the EU Oil and Protein Meal Industry (FEDIOL) from different European refineries (Spain, Germany, Italy, France, The Netherlands). Other samples analyzed included a crude non-degummed and its counterpart fully refined oil, a physically refined oil, and a hydrogenated and an interesterified oil. The fully refined oils provided were representative of the edible soybean oils traded commercially in Europe, including oils sold to retail consumers or used in consumer products. Another set of oils was sourced from 29 worldwide suppliers in blind fashion with the assistance of the Institute of Shortening & Edible Oils, Washington, DC. Samples of these oils were available from each stage of refining. These oils had been used in a previous clinical and analytical study and showed evidence of oxidation but were analyzed because their protein contents had previously been determined using total amino acid analysis.

Extraction of Proteins from Soybean Oil. A number of different protocols, some of which have been reported previously, were used to extract proteins from the oils and prepare them for analysis.

Low-Temperature Acetone Precipitation[9]. Soybean oil (100) was frozen overnight with 100 mL of acetone at −80 °C and then left for 18 h at 4 °C. The protein and other precipitated material was recovered by filtration using a 0.2 μm membrane (RC 58, Schleicher & Schuell, VWR International Leicestershire, U.K.) and the filtration membrane washed three times with hexane (5 mL) at room temperature. The protein was recovered by washing the filtration membrane with a small volume (0.5−1 mL) of phosphate-buffered saline (PBS) solution (0.01 M sodium phosphate buffer, pH 7.4, containing 0.138 M NaCl; 0.5−1 mL), which was then lyophilized, dissolved in 0.5 mL of water, and stored frozen until required.

PBS Extraction[10]. Soybean oil (200 mL) was mixed with PBS (4:1, w/v) at 40 °C for 3 h and then centrifuged at 10000*g* for 30 min at 40 °C. The aqueous layer was dialyzed against deionized water four times over 48 h (Spectrum dialysis tubing 3.5 kDa cutoff, Sigma Chemical Co.). The dialyzed solution was lyophilized, dissolved in 0.5 mL of deionized water, and stored at −20 °C until required.

Bicarbonate Extraction[11]. Briefly, soybean oil (200 mL) was mixed with hexane-containing petroleum fraction (120 mL) and the mixture homogenized by stirring for 15 min at 4 °C An aqueous extract was prepared by adding 40 mL of 0.1 M sodium bicarbonate (pH 8.0) and stirring for 48 h at 4 °C. The mixture was centrifuged at 35000*g* for 45 min at 4 °C. Sodium bicarbonate (0.1 M, pH 8.0, 100 mL) was added again to the organic layer, mixed for 30 min, and centrifuged as above. The aqueous layers were pooled and stored at 4 °C. The protein was precipitated with a solution of cold trichloroacetic acid (TCA) as described by Mahuran et al.[12] prior to resuspension in 0.5 mL of water and stored at −20 °C until for protein. A smaller scale extraction was performed on oils that were analyzed for protein content using total amino acid analysis as follows. Briefly, 15 mL of oil was extracted with 7.0 mL of ammonium hydrogen carbonate (0.2 M, pH 7.8) on a vigorous wrist action shaker for 2 h at room temperature. The resulting emulsion was separated by centrifugation and the aqueous layer retained for amino acid analysis. Triplicate samples of 750 μL of protein extract were then dried in a vacuum centrifuge/concentrator, resuspended in 500 μL of distilled deionized water, and dried again. After this washing

Table 1. Analysis of Replicate Protein Extracts from Fully Refined (N/RBD) and Crude Degummed Soybean Oil Samples by the Bicarbonate Method Using Three Different Protein Determination Assays

sample	BCA method, ng/g	Bradford method, ng/g	CBQCA method, ng/g
N/RBD (fully refined) oil			
extract 1	255.5	218.4	259.4
extract 2	179.4	80.6	122.4
extract 3	185.8	533.8	262.0
extract 4	272.0	2031.0	231.6
extract 5	197.1	2393.7	*a*
extract 6	162.5	77.6	100.0
mean	208.7 (±44.4)	889.2 (±1044.6)	195.1 (±77.9)
crude degummed oil			
extract 1	196.2	91.7	210.2
extract 2	244.4	97.8	171.8
extract 3	234.2	127.8	141.2
extract 4	162.5	81.3	178.6
extract 5	143.2	81.3	156.2
extract 6	186.0	99.7	183.2
mean	194.4 (±39.5)	96.6 (±17.2)	173.5 (±23.7)

[a] Not analyzed due to insufficient material.

step had been repeated, the sample was neutralized with 100 μL of 88% (v/v) formic acid, dried, resuspended in 100 μL of deionized water, and finally dried again.

Borate Extraction. All glassware was silanized to prevent loss of protein by adsorption onto untreated silica surfaces. Glassware was rinsed with a solution of 2% (v/v) dimethyldichlorosilane in heptane (BDH) and allowed to dry before use. Soybean oil (2 mL) was mixed with hexane-containing petroleum fraction (1.2 mL) for 5 min at 20 °C. An aqueous extract was prepared by adding 1 mL of 0.1 M sodium tetraborate (pH 9.3) and wheel-mixed for 48 h at 20 °C prior to centrifuging at 3000*g* for 40 min. The resulting organic phase was removed and the aqueous phase back-extracted by addition of 3 mL of hexane and wheel-mixing for 1 h. After centrifuging at 3000*g* for 40 min, the organic phase was again removed and the aqueous phase analyzed immediately. As a consequence of their physical properties, unrefined soybean oils were initially subjected to two cycles of extraction. The first consisted of the addition of 4 mL of hexane and wheel-mixing for 1 h, followed by centrifugation at 3000*g*. Subsequently, the procedure was adapted by back-extracting the post-48 h extraction aqueous phase twice with 4 mL of hexane by wheel-mixing for 1 h. The resulting aqueous extract was diluted with 3 mL of 0.1 M sodium tetraborate (pH 9.3) prior to undergoing a further round of back-extraction with 2 mL of hexane to obtain a clear aqueous phase.

Protein Determination. Protein content was determined using bovine serum albumin (BSA) as a standard using the bicinchoninic acid (BCA),[13] Bradford[14] kit-form (Pierce, Rockford, IL) together with the 3-(4-carboxybenzoyl)quinolone-2-carboxaldehyde (CBQCA)[15] kit-form (ATTO-TAG, Invitrogen, Paisley, U.K.) methods. In addition, the protein content of bicarbonate extracts of selected oils was estimated using total amino acid analysis. Samples underwent hydrolysis for 16 h at 115 °C in 100 μL of 6 N HCl, 0.2% (w/v) phenol containing 2 nmol of norleucine. Each sample was then dried and resuspended in 100 μL of Beckman amino acid loading buffer (Beckman Instruments, now Beckman Coulter, Fullerton, CA). Amino acid analysis was performed on a Beckman model 6300 ion exchange instrument (Beckman Instruments), calibrated using a 2 nmol mixture of amino acids.

SDS-PAGE. Protein extracts from 20 g of soybean oil were resuspended in lithium dodecyl sulfate (LDS) sample buffer and separated by PAGE under reducing conditions using a 4−12% Bis-Tris gel in a NuPAGE system utilizing MES buffer (Invitrogen, Groningen, The Netherlands) according to the manufacturer's instructions. Proteins were visualized by either silver stain (SilverXpress staining kit, Invitrogen), Sypro Ruby stain (Bio-Rad, Hemel Hempstead, Herts, U.K.), or colloidal Coomassie (SimplyBlue safe stain, Invitrogen). The following polypeptides were used as molecular mass markers: myosin (M_r 200,000), β-galactosidase (M_r 116,300), phosphorylase *b* (M_r 97400), BSA (M_r 66300), glutamic dehydrogenase (M_r 55400), lactate dehydrogenase (M_r 36500), carbonic anhydrase (M_r 31000), soybean trypsin inhibitor (M_r 21500), lysozyme (M_r 14400), aprotinin (M_r 6000), insulin B chain (M_r 3500), and insulin A chain (M_r 2500).

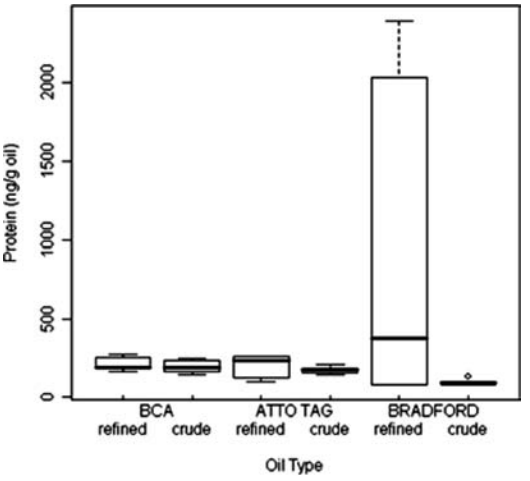

Figure 3. Box plot illustrating the variability of three different protein assays applied to the quantification of protein in bicarbonate extracts of soybean oil. Crude, crude degummed oil; refined, N/RBD. Bold lines represent the median value for six replicate extractions of each oil sample. Bars and dotted lines represent the upper and lower limits for the data sets. The upper and lower quartiles are shown by the upper and lower boundaries of the box.

Statistical Analysis. The precision and accuracy of the three different protein assays (BCA, Bradford, and CBQCA) were compared using repeated measures ANOVA and the *F* test on the ratios of

Table 2. Comparison of Replicate Variances for Different Protein Determination Methods

BCA versus CBQCA method	F = 6.3433	df[a] = 24	denom df = 22	p value = 4.775e−05
BCA versus Bradford method	F = 0.187	df = 24	denom df = 24	p value = 0.0001146
CBQCA versus Bradford method	F = 33.8765	df = 24	denom df = 22	p value = 4.578e−12

[a] Degrees of freedom.

variances. The correlation between the protein contents estimated by the total amino acid analysis method and the CBQCA method coupled with borate extraction was investigated as follows. First, an optimal sampling scheme was produced by running several simulations based upon varying the values of the following: (a) the "true" correlations between the two methods of measuring protein, (b) the number of different samples taken, and (c) the number of replicate extracts taken from each sample. After the data were collected, to estimate the strength of the association between the two methods of measuring protein content, a regression model was fitted. All analyses were done using the statistics package R.[16]

RESULTS

Extraction of Residual Protein from Soybean Oil. Four different methods for extracting proteins from oils were initially compared using samples of a crude non-degummed, a crude degummed, and a fully refined (N/RBD) oil. One was based on the low-temperature acetone extraction procedure of Paschke et al.,[9] a second on the PBS method of Errhali et al.,[10] and a third on the bicarbonate method of Olszewski et al.,[11] wheras the fourth method replaced the high-pH bicarbonate buffer with one containing sodium tetraborate. The latter method had the advantage of using a small volume of oil, allowing the extraction to be performed in a single tube, reducing the number of steps involved, whereas mixing (rather than stirring) resulted in the formation of a coarser emulsion that was more amenable to separation by centrifugation at 3000*g*. In addition, the borate buffer allowed direct analysis of protein by the CBQCA method, which had been validated only for use using borate buffer.

A qualitative comparison of protein extract composition using SDS-PAGE (Figure 2) showed streaking in the higher M_r region of the gel, which probably results from lipid contamination. The cold acetone extraction did not extract protein that could be resolved by SDS-PAGE. Whereas the PBS method proved able to extract protein from the N/RBD oil (track 5) that could be resolved into a number of discrete polypeptides of M_r > 100,000 together with major polypeptides of M_r ∼55000−66000, the PBS extract of crude degummed oil was very poorly resolved (track 4), possibly as a result of residual oil in the sample. Both the bicarbonate and borate methods extracted a greater complexity of polypeptides, including polypeptides of M_r > 100,000 together with other polypeptides of M_r ∼ 66000, 55000, 36000, and 30000. In general, a more complex pattern of polypeptides was observed in the bicarbonate extract of the crude non-degummed oil (track 8) compared with that from either the degummed (track 2) or the fully refined (N/RBD, track 3) oils, with a number of polypeptides of higher M_r (∼100,000 Da) and intermediate M_r ∼ 66000, 55000, 36000, and 30000 being common to both oil extracts. In addition, the bicarbonate extract of both the N/RBD (track 3) and non-degummed oils (tracks 8) contained lower M_r polypeptides of 6−10 kDa. This polypeptide was only faintly present in the borate extract of the crude non-degummed oil (track 9), although comparisons are difficult due to differences in loading resulting from the small scale of the borate extraction method, which allowed only 5 ng of protein to be loaded per track compared to 375 ng for the bicarbonate extract (track 8). These observations are consistent with the wide range of polypeptide sizes extracted from oils by others,[10, 11, 17] although there are clear methodological effects both in the method of extraction and in the detection of proteins, silver staining revealing a different spectrum of polypeptides from Coomassie Blue,[18] which makes comparisons difficult.

Table 3. Repeated-Measures ANOVA on Replicate Protein Extracts of Fully Refined (N/RBD) and Crude Degummed Soybean Oil Samples by the Bicarbonate Method Using Different Protein Determination Assays[a]

	response: 1/(protein)			
	sum sq	df	F value	Pr (>F)
method	5.3601e−05	2	2.9798	0.06552 (NS)
oil type	2.9640e−05	1	3.2955	0.07916 (NS)
residuals	2.7882e−04			

[a] Derived from data in Table 1.

Reproducibility of Protein Determination Methods. Qualitative analysis indicated that the high-pH extraction methods performed best in terms of the spectrum of proteins extracted. One of these, the bicarbonate method, was then used to determine the most effective protein determination method to use. Six replicate extractions on a sample of crude degummed oil and its N/RBD counterpart and the protein content of extracts determined using the BCA, Bradford dye-binding, and CBQCA fluorescence assays (Table 1). Box plot analysis of the data in Table 1 (Figure 3) illustrates the much wider range of values obtained using the Bradford assay, which was heavily influenced by three relatively large values. Consequently, the variances (i.e., the measurement errors) of replicates for each of the three protein determination assays were assessed. To undertake this, it was necessary to first scale the units of the CBQCA and Bradford replicate data so that these were on the same units as the average protein levels (and so directly comparable with each other and the BCA method). Replicate variances were then compared using a simple linear regression and from the resulting set of residuals (i.e., the difference for each "predicted" protein determination replicate from the "actual" mean of the three replicates of the same sample) and the F test employed to compare the variances from these linear regressions (Table 2). The CBQCA method had the smallest variance (i.e., the best "precision"), followed by the BCA method, with the Bradford method being the worst (largest variance, worst "precision"). From the F test, all of these differences were statistically significant at the 0.05 level. This is reflected in the fact that the BCA and CBQCA methods gave fairly similar results of 143.2−244.4 and 141.2−210.2 ng of protein/g of oil for crude degummed oil, respectively.

The lower limits of the working range for the three protein determination assays, as defined in the manufacturers' instructions, were 100 μg/mL for the Bradford dye-binding assay,

Table 4. Analysis of Protein in Soybean Oil Samples Using Bicarbonate and Borate Extraction Methods and the CBQCA Protein Determination Assay[a]

	protein content, ng/g of oil (mean)	
oil type and origin	bicarbonate	borate
N/RBD oil		
Spain, sample 1	143.0, 151.7 (147.4)	172.1, 253.1 (212.6)
Spain, sample 2	167.6, 136.3 (152.0)	94.4, 106.9 (100.6)
France[b]	139.0	165.0, 70.7 (117.8)
Italy, sample 1	225.1, 176.6 (200.9)	63.3, 70.7 (61.2)
Italy, sample 2	ND	265.1 ± 24.5
Italy, sample 3	ND	247.9, 237.5 (242.7)
Italy, sample 4	62.0, 63.2 (63.0)	260.4, 291.6, 243.3 (265.1)
Germany		
N/RBD, sample 1	221.5, 208.8 (215.2)	226.8, 213.5 (220.2)
N/RBD, sample 2	205.7, 204.2 (204.7)	223.5, 156.9 (190.2)
crude non-degummed oil		
Italy, sample 1	997.2, 465.4 (730.0)	83500, 91800, 85600 (86600)
Italy, sample 2	659.6, 1705 (1510)	87300, 89800, 86500 (87900)
Italy, sample 3	ND	87900 ± 1700
physically refined oil	ND	516.600 ± 79.9
partially hydrogenated soybean oil[b]	ND	421.7
interesterified soybean oil	ND	256.4 ± 92.7

[a] Protein extraction was performed in duplicate or triplicate. Mean values and standard deviations were calculated only for triplicate analyses. ND, not determined. Arithmetic means are given in parentheses. [b] Extraction procedure was modified by increasing hexane volume from 2 to 5 mL to dissolve the fat phase during extraction.

20 μg/mL for the BCA assay, and 75 ng/mL for the CBQCA assay. All of the oil protein extracts analyzed lay well within the range of calibration standards used for the protein assays, but were outside the assay protocol specification for the Bradford assay, a number of samples falling below the lower limit of quantification. Because the precision at the lower end of the calibration curve is much lower, this is the likely reason for the poor performance of the Bradford dye-binding protein assay. A further confounding factor is that this assay relies on the presence of binding sites on largely intact proteins to function and shows variability in responsiveness between proteins. It is also more susceptible to interference from other substances, such as surfactants used in protein extraction.[19] The greater precision of the CBQCA assay reflects its greater sensitivity, an important factor in quantifying the low levels of protein extracted from the oil, which were at the limit of detection of the BCA method. On this basis it was evident that the CBQCA protein determination assay was the most appropriate for determination of extracted protein, and therefore it was used for all further analyses.

The analytical results on each of the six replicate extractions for the fully refined (N/RBD) and crude degummed oils were then compared by ANOVA, after inverse transformation of the protein values to satisfy the requirement for normal distribution of the errors (Table 3). The initial ANOVA analysis showed significant differences between the methods and was repeated without the Bradford data. No significant difference was found between protein levels determined using the BCA and CBQCA methods. This lack of significant difference was confirmed by a repeated-measures ANOVA (Table 3). The poor reproducibility between different extracts of the same oils is due to technical problems encountered during centrifugation of the water−oil mixtures and the difficulty of removing the aqueous from the oil phase. The amount of protein that could be extracted from the crude degummed oil (179.4−272.0 ng of protein/g of oil) was very similar to that from the refined N/RBD oil (100.0−262.0 ng of protein/g of oil). The similarity in protein levels between these oils was initially surprising, but was consistent with the fact that the crude degummed oil was clear and bright in color, as a result of the removal of the bulk of the proteins with the phosphatides prior to neutralization (see Figure 1). The levels of protein obtained were in the same range as those found in other studies. Thus, Paschke et al.[9] extracted around 35−101 ng of protein/g of oil, as determined by the Bradford method, whereas Errahali et al.[10] obtained 0.32 and 1.8 μg/g from deodorized and cold-pressed soybean oil, respectively, although they do not report the protein determination method used.

Effect of Refining Process on Protein Content in Soybean Oil. The manipulations involved in the bicarbonate extraction procedure and the need to dilute extracts prior to analysis using the CBQCA protein determination assay were potential sources of error that could be overcome by employing the borate extraction procedure, because this buffer is compatible with the CBQCA, allowing direct assay of extracts without prior dilution. Consequently, a panel of oils from different refineries, including products from different types of refining process, was investigated using both extraction procedures (Table 4). The results showed that the reproducibilities of the two different extraction methods were similar overall, although the borate method proved superior at extracting proteins from non-degummed (crude) oils. These data confirmed that the refining process substantially reduced the protein contents of the oils as indicated by the fact that the protein content of N/RBD oil was substantially lower (242−265 ng/g oil) than that of crude non-degummed oil (86000−87900 ng/g; Table 4). There were subtle differences depending on the source of the N/RBD fully refined

Table 5. Protein Content of Soy Oils Analyzed by the Borate−CBQCA Method and Bicarbonate−Total Amino Acid Nitrogen Methods[a]

oil sample	country of origin	borate−CBQCA protein, μg/g of oil	bicarbonate−total amino acid nitrogen, ppm
crude degummed	Spain	0.3	18.642
crude degummed	Spain	0.38	ND
neutralized	Spain	0.083	5.385
neutralized, bleached	Spain	0.16	2.927
neutralized, bleached, deodorized	Spain	0.082	0.434
crude degummed	The Netherlands	1.31	2.195
neutralized	The Netherlands	0.14	0.100
neutralized, bleached	The Netherlands	0.25	0.078
neutralized, bleached, deodorized	The Netherlands	0.092	0.029
crude degummed	United States	0.79	0.952
neutralized	United States	0.38	0.443
neutralized, bleached	United States	0.14	0.044
neutralized, bleached, deodorized	United States	0.698	0.058
crude degummed	United States	16.18	7.005
neutralized	United States	0.064	0.110
neutralized, bleached	United States	0.046	0.034
neutralized, bleached, deodorized	United States	0.14	0.025
crude degummed	Germany	1.63	1.493
neutralized	Germany	0.37	0.507
neutralized, bleached	Germany	0.184	0.220
neutralized, bleached, deodorized	Germany	0.047	0.033
crude degummed	Bolivia	20.33	3.065
neutralized	Bolivia	0.25	0.180
neutralized, bleached	Bolivia	0.026	0.046
deodorized	Bolivia	0.14	0.057
crude degummed	Brazil	10.18	2.100
neutralized	Brazil	1.7	0.258
neutralized, bleached	Brazil	0.32	0.308
neutralized, bleached, deodorized	Brazil	0.33	0.045

[a] ND, not determined. Based on analyses of repeat analyses using the borate−CBQCA method simulation analysis, which indicated analysis of 29 samples with singlet extraction would be sufficient to establish the relationship (if any) between protein levels and those determined using the bicarbonate−total amino acid nitrogen methods.

oils, but all samples contained between around 62 and 220 ng/g of oil (Table 4). In addition, some more highly processed oils were analyzed, although the extraction procedure had to be modified for the partially hydrogenated soybean oil by increasing the hexane volume from 2 to 5 mL to dissolve the fat phase during extraction. The partially hydrogenated and the interesterified oils, produced from fully refined oils, also had less protein than the physically fully refined oil (where the neutralization phase is omitted), indicating that further refining systematically reduced the protein content.

Quantification of Soybean Oil Using Borate−CBQCA and Bicarbonate−Total Amino Acid Measurement Methods. There are concerns that colorimetric methods of protein determination may underestimate the protein contents of oils.[18] To address this issue, the protein content of a set of oils measured by total amino acid analysis following bicarbonate extraction was compared with results obtained using the borate extraction procedure followed by the CBQCA protein determination method (Table 5). Simple correlation analysis of the resulting data sets (Table 5) using Spearman's correlation (nonparametric) showed significantly nonzero positive correlations as the data were not normally distributed (Figure 4). This renders the Pearson's correlation invalid, and hence data were log-transformed so that it was now plausibly normally distributed. This gave a Pearson's (parametric) correlation of 0.598 and a Spearman's correlation of 0.615, both significantly nonzero and very similar. Fitting a model that takes the oil sample type and origin into account was also undertaken. Oil protein levels determined using both bicarbonate−total amino acid and the borate−CBQCA methods were log-transformed, to achieve acceptable regression diagnostics; regressing the log of the oil protein determined by the borate−CBQCA method on the log of the protein determined by bicarbonate−total amino acid gave a final ANOVA (Table 6). The ANOVA showed that both the sample type and the (log of) the oil protein level determined using the bicarbonate−total amino acid are significantly correlated with the protein content determined using the borate−CBQCA method. The sample type is weakly significantly correlated. The model coefficients for log (bicarbonate−total amino acid) indicate that, if all other variables are held constant, for each unit that the log of the oil (bicarbonate−total amino acid) protein content increases, the log of the oil (borate−CBQCA) protein content increases by about 1.05 units. This is close to a one-to-one relationship. The "strength" of the relationship

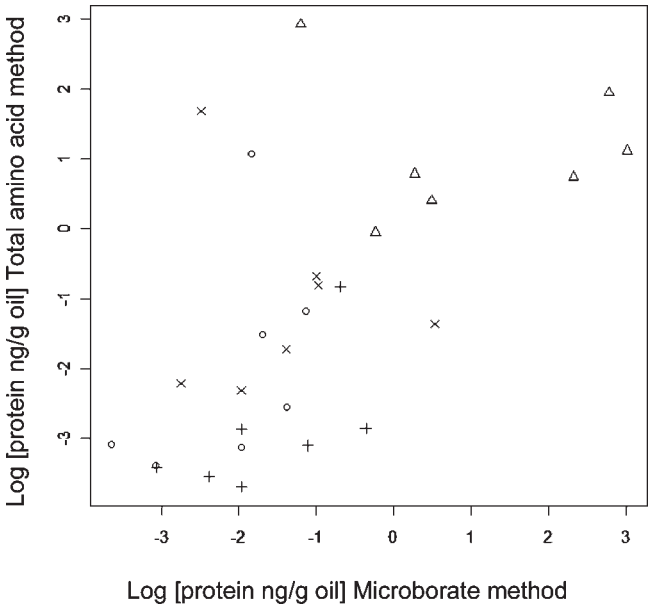

Figure 4. Correlation between analysis of protein content of soybean oil performed using total amino acid measurement or extraction of protein using the microborate method followed by CBQCA protein analysis. Symbols represent different types of oil as follows: (△) crude degummed; (○) bleached; (+) neutralized; (×) deodorized.

Table 6. ANOVA Analysis of Protein Contents of Soy Oils Determined Using the Borate−CBQCA and Bicarbonate−Total Amino Acid Nitrogen Methods[a]

	sum sq	df	*F* value	Pr (>*F*)
sample	16.93	6	2.93	0.0379 *
log (bicarbonate−total amino acid N method)	10.03	1	10.40	0.0050 **
oil type	8.16	3	2.82	0.0701
residuals	16.39	17		

[a] Data set is presented in Table 5. Response: log (CBQCA method).

between (logs of) the oil protein levels determined by the two methods can be measured using the "partial correlation", which is defined as the correlation between the two methods after taking into account the other variables (i.e., sample type and origin). This came out to r (partial) = 0.62. This analysis demonstrated that the oil protein levels determined in this study using the borate−CBQCA extraction method correlated directly with those determined previously using a total amino acid determination approach. However, the absolute levels of protein obtained by the different methods are different, consistent with results published by others, albeit on a much more limited panel of oils than that presented here.[18] This might be explained by the fact that the methods have such different bases and that the oils were older when reanalyzed using the borate−CBQCA method.

Several methods have been developed and used,[1, 10−12, 18] but have not completely met the requirements of robustness and sensitivity for undertaking allergenic risk assessment. The lack of appropriate methods has resulted in uncertainty over the true protein content of oils and difficulties in interpreting from a safety perspective studies reporting reactions to different edible oils. By adapting existing methodology for extracting and estimating the protein content of edible oils, a more robust method suitable for larger scale analysis of residual protein in oils has been developed. Employing a borate, rather than a bicarbonate, extraction buffer gave more reproducible results, particularly when used to extract proteins from crude non-degummed soybean oil and aged N/RBD soybean oil. Furthermore, because the extracts could be assayed directly without further treatments, such as dialysis, the preparation process was faster and extracts will include low molecular weight peptides unlike the phosphate-buffered saline extraction method of Ramazzotti et al.[18] By combining this more effective extraction process with a much more sensitive fluorescence-based method for protein detection using CBQCA, the protein content could be determined from as little as 10 mL of oil. The CBQCA assay also proved to be more robust than the Bradford dye-binding assay probably because the former is less prone to interference by the presence of trace amounts of fatty acids in the extracts.

Residual Oil Protein and Allergenic Risk Assessment. The existence of minimum eliciting doses (MEDs) or thresholds for allergenic proteins is now generally acknowledged,[1, 2, 20] and data have now been generated and analyzed for a number of allergenic foods including peanut[21] and soy.[19] Threshold studies in soybean allergic patients have demonstrated that cumulative threshold doses ranged from 10 mg to 50 g for subjective symptoms and from 454 mg to 50 g for objective symptoms.[20] These data make it possible to undertake quantitative assessments of the risk of allergic reactions. However, the robustness of these risk assessments depends critically on the quality of the residual (allergenic) protein estimation. This estimation is rendered more challenging for residual oil protein by the need to use an efficient extraction method, as well as the extremely low concentrations of residual protein in the oils.

On the basis of the analysis of residual protein in the highly refined oils presented in this paper, even the most sensitive individuals included in such studies would need to consume at least 50 g of highly refined oil to experience subjective symptoms. This is consistent with the observation in clinical studies that the allergenic activity of residual proteins in refined soybean oils is insufficient to elicit a reaction in oral challenge procedures.[3] The bulk of the protein in the crude non-degummed oil was removed during the degumming process, the first step in the refining process. Similarly, modified soybean oils, that is, partially hydrogenated and interesterified oils, and a physically refined soybean oil sample also contained very low levels of protein. This explains the lack of reaction observed to highly refined oils, unlike unrefined or partially refined culinary oils, which have been found to elicit allergic reactions in sensitized individuals,[8] and supports the decision of the European Food Safety Authority (EFSA) to grant a permanent exemption from allergen labeling normally required by Annex IIIa of Directive 2003/59/EC and the amendment 2006/142/EC. This was given for fully refined N/RBD soybean oil prepared in a defined process, as it was concluded such oils were unlikely to trigger an allergic reaction in susceptible individuals.[21]

Finally, it is worth noting that difficulties in interpreting studies on edible oils do not arise solely from analytical issues. The terminology used to designate oils that have undergone different degrees of refinement has also played a part. Thus, the term "refined oil" refers, in some parts of the world, to oil that has undergone degumming and neutralization steps, whereas in terms of traded commodities, the term "crude oil" designates an oil that has undergone degumming. We would therefore recommend that, for the purposes of clarity, "crude oil" be used only for oils that have undergone solvent extraction or other

steps to separate them from the seed solids, whereas an oil that has undergone degumming, but no further processing is designated "crude degummed". Finally, as the term "fully refined", although a useful shorthand, is not well-defined, oils that have undergone a complete refining treatment should be named neutralized/refined, bleached, deodorized (N/RBD) oils.

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Funding Sources

This study was funded by FEDIOL and IMACE. E.N.C.M. and A.I.S. were supported by the competitive strategic grant from the U.K. Biological and Biotechnological Sciences Research Council.

Abbreviations Used

NBD, neutralized, bleached, and deodorized; N/RBD, neutralized, refined, bleached, and deodorized; PBS, phosphate-buffered saline; BCA, bicinchoninic acid; CBQCA, 3-(4-carboxybenzoyl) quinolone-2-carboxaldehyde; MES, 2-(N-morpholino)ethanesulfonic acid; Bis-Tris, bis(2-hydroxyethyl)aminotris(hydroxymethyl) methane; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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