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Characterization and Quantification of Proteins in Lecithins

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Several methods for extraction and quantification of proteins from lecithins were compared. Extraction with hexane−2-propanol−water followed by amino acid analysis is the most suitable method for isolation and quantification of proteins from lecithins. The detection limit of the method is 15 mg protein/kg lecithin, and the quantification limit is 50 mg protein/kg. The relative repeatability limits for samples containing 0−500 and 500−5000 mg protein/kg sample were 12.6 and 7.5%, respectively. The protein recovery ranged between 101 and 123%. The protein content has been determined in different kinds of lecithins. The results were as follows: standard soy lecithins (between 232 and 1338 mg/kg), deoiled soy lecithin (342 mg/kg), phosphatydylcholine-enriched soy lecithins (not detectable and 163 mg/kg), sunflower lecithins (892 and 414 mg/kg), and egg lecithin (50 mg/kg). The sodium dodecyl sulfate−polyacrylamide gel electrophoresis protein patterns of the standard soy and sunflower lecithins are very similar to those of soy flour. The protein profile of the egg lecithin shows several bands with a broad range of molecular masses. The molecular masses of the main proteins of soy lecithins and soy flour have been determined by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and ranged from 10.5 to 52.2 kDa. Most of the major proteins from soy and sunflower lecithins identified by MALDI-MS and electrospray tandem MS belong to the 11*S* globulin fraction, which is one of the main fractions of soy and sunflower seeds. In addition, the seed maturation protein P34 from the 7*S* globulin fraction of soy proteins has also been identified in soy lecithins. This protein has been reported as the most allergenic protein in soybean.

KEYWORDS: Lecithin; protein; amino acid analysis; spectrophotometric methods; SDS−PAGE; MALDI-MS; ESI-MS/MS

INTRODUCTION

Lecithins are used in a wide variety of products including processed food, cosmetics, and pharmaceuticals. Commercial sources of lecithin are predominantly vegetable oils seeds (e.g., soybeans and sunflower seed); however, for pharmaceutical and some dietary applications, egg yolk is very important.

Lecithin has several functions in the body. Specifically, lecithin supplies choline to the body. Choline is a cofactor for the production of the hormone acetylcholine, the neurotransmitter that "activates" muscle contraction. Lecithin is also a source of ω-3 fatty acids and essential fatty acids typically undersupplied in most peoples' diets. A third function of lecithin is as an emulsifying agent within the digestive system. Indeed, lecithins are added to food products as emulsifiers and stabilizers. They are derived from the oil manufacturing and are mostly obtained by hexane extraction. Crude lecithins are separated from the oils by degumming and standardization, and its composition has a large variability. These standard lecithins are used for food and pharmaceutical applications. They mainly consist of phospholipids, glycolipids, and fatty acids, but they also contain residual proteins.

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Further fractionation forms of the lecithins are also produced such as deoiled lecithin (obtained by precipitation with acetone and used for dietary purposes); phosphatydylcholine (PC)-enriched fraction (ethanol soluble fraction), used for medical applications; and PC-depleted fraction (ethanol insoluble fraction), which is used as a special emulsifier. These products may also contain proteins but generally in lower amounts than the crude standard lecithins.

Although the available information is controversial, there are indications that the proteins present in lecithins may elicit allergic reactions in sensitive individuals. Evidence of adverse reactions to lecithins has been reported by Fine (*1*), Renaud et al. (*2*), Weidmann et al. (*3*), and Palm et al. (*4*). Because of their potential allergenicity problems, the quantification and characterization of the proteins present in lecithins are obviously very important for risk assessment.

Currently, no validated methods are available for quantification of proteins from lecithins. Usually, a first step of extraction is performed using aqueous/organic solvents, and afterward, the protein content is determined using different assays, such as Bradford (*5*) by Paschke et al. (*6*), Lowry (*7*) by Awazuhara et al. (*8*) and Gu et al. (*9*), and enzyme-linked immunosorbent assay (ELISA) by Porras et al. (*10*) and Müller et al. (*11*). However, these methods may not always give reliable results,

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since the Lowry and Bradford methods have not been evaluated in lipid matrices and the ELISA is only semiquantitative and requires specific antibodies for each kind of lecithin (i.e., soy, sunflower, and egg).

The aim of this work is to develop and validate a method for the quantification and characterization of proteins in lecithins. For that, several methods of extraction and quantification of proteins were compared. The most suitable method to achieve this purpose was validated and subsequently used to quantify proteins in several types of soy, sunflower, and egg lecithins. The characterization and identification of the proteins of the studied lecithins have been carried out by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS), and electrospray tandem mass spectrometry (ESI-MS/MS).

MATERIALS AND METHODS

Samples. The following commercial lecithins were analyzed in this study. Crude soy lecithin 1 was from brand A. Crude soy lecithin type 2, crude soy lecithin type 3, crude soy lecithin type 4, deoiled soy lecithin, PC-enriched fraction soy lecithin type 5, PC-enriched fraction soy lecithin type 6, and egg lecithin were from brand B. Crude sunflower lecithins 1 and 2 were from brands C and D, respectively. In addition, soybean flour (Sigma) was also analyzed.

Protein Extraction from Lecithins. Solvents were cooled to 4 °C before use, and centrifugation was performed at 7000*g* and 4 °C for 20 min. Three different methods for extractions of proteins from lecithins were tested. Precipitates obtained from each extraction were dried overnight in an oven at 40 °C and weighed.

Extraction with Acetone−Hexane (AH) (1:1). The isolation of proteins was performed using the method described by Paschke et al. (*6*) with some modifications. To 25 g of lecithin, 75 mL of AH (1:1) was added. The mixture was shaken vigorously, kept for 1 h at 4 °C, and shaken every 10 min. The mixture was then centrifuged, and the supernatant was discarded. The precipitate was washed twice with 20 mL of AH (1:1). After each washing, the mixture was centrifuged and the supernatant was discarded.

Extraction with Hexane−Isopropanol−Water (HIW) (3:2:1). The isolation of proteins was performed using the lipid extraction method described by Hara and Radin (*12*) and adapted for lecithins by Awazuhara et al. (*8*), with some modifications. To 25 g of lecithin, 150 mL of HIW (3:2:1) was added. The mixture was shaken vigorously, kept for 1 h at 4 °C, and shaken every 10 min. The mixture was then centrifuged. The precipitates that were formed between the aqueous and the nonaqueous layers and on the bottom of the tube, were collected together. The precipitate was washed three times with 20 mL of HIW (6:4:1) and once with 20 mL of hexane−2-propanol (3:2). After each washing the mixture was centrifuged and the supernatant was discarded.

Extraction with Chloroform−Methanol−Water (CMW) (2:1:1). The isolation of proteins was performed basically using the lipid extraction method described by Folch et al. (*13*), with some modifications. To 25 g of lecithin, 150 mL of CMW (2:1:1) was added. The mixture was shaken vigorously, kept for 1 h at 4 °C, and shaken every 10 min. The mixture was centrifuged. The precipitates that were formed between the two layers and on the bottom of the tube, were collected separately. The "interphase" precipitate was washed twice with 20 mL of chloroform−methanol (2:1). After each washing, the mixture was centrifuged and the supernatant was discarded. The "bottom" precipitate was washed three times with 20 mL of methanol−water (1:1). After each washing, the mixture was centrifuged and the supernatant was discarded.

Quantitative Determination of Protein. *Amino Acid (AA) Analysis.* Proteins were quantified by using AA analysis, with a Hitachi L-8500 system (Tokyo, Japan). The method corresponds to the AACC method 07-01 (*14*), with some modifications. Five to 10 mg of precipitate was dissolved in 8 mL of 6 M hydrochloric acid, and nitrogen was introduced for 2 min. The solution was hydrolyzed in an oven for 24 h at 110 °C. The hydrolyzed sample was filtered into a 50 mL volumetric flask and made up to the mark with deionized water; 30 mL of this solution was evaporated. The residue was then dissolved in 2 mL of 0.02 M hydrochloric acid and filtered through a membrane filter before injection on the AA analyzer. The protein content was calculated from the AA data. The AA tryptophan and the sulfur-containing AAs cysteine and methionine were not included in the quantification.

Spectrophotometric Methods. The protein fraction of lecithins (10 mg) was suspended in 100 mM NaOH (15 mL), incubated for 5 min at 50 °C, and sonicated for 15 min. The protein content of the extracts was determined by three different methods: micromethod of Bradford (*5*) using the Coomassie Protein assay reagent kit from Pierce Chemical Co. (Rockford, United States); Micro Bicinchoninic Acid (BCA) Protein Assay reagent kit from Pierce Chemical Co., and 2D Quant Kit from Amersham Biosciences (San Francisco, CA). Soy flour calibration standards were used for soy and sunflower lecithins, and bovine serum albumin (fraction V, Pierce) was used for egg lecithin. All protein analysis by an AA analyzer and spectrophotometric methods were carried out in duplicate.

SDS−PAGE. SDS−PAGE was performed using the Xcell II Mini-Cell system from Novex. Isolated fraction samples from lecithins were diluted in Lämmli sample buffer from Bio-Rad with β-mercaptoethanol to obtain about 25 mg precipitate/mL or 2 mg protein/mL. Afterward, samples were heated for 15 min at 95 °C and centrifuged for 2 min at 10000*g*, before loading 20 μL on the gel. Electrophoresis was carried out on a Bis-Tris-HCl polyacrylamide gel NuPAGE 4−12% with NuPAGE MES−SDS running buffer from Invitrogen. The migration conditions were based on those recommended by Invitrogen. Proteins were visualized by Coomassie brilliant blue G-250 staining. A low molecular mass (LMW) proteins calibration kit (Amersham Biosciences) was used as a reference.

Mass Spectrometry Analysis. *MALDI-MS on Intact Proteins Extracted from Soybean.* Proteins from lecithins extracted with different procedures were analyzed by MALDI-MS. Mass spectra were recorded on an Autoflex (Bruker, Bremen, Germany) MALDI time-of-flight mass spectrometer operating in delayed extraction linear positive ion mode. Dihydroxybenzoic acid was used as matrix with a saturated solution of acetonitrile (30%) and 0.1% trifluoroacetic acid (TFA) in water (70%). Samples were resuspended in H_2O/acetic acid (50/50). Typically, 1 μL of the saturated matrix solution was mixed with 1 μL of the lecithin samples. The resulting mixture was then deposited on a "ground steel" (Bruker) target and allowed to dry at room temperature. Ions formed upon irradiation by a pulsed UV laser beam (nitrogen laser, 337 nm) were accelerated at 20 kV. Each mass spectrum was produced by averaging from 70 to 100 laser shots spread all over the spot surface. External calibration was performed with a protein mixture containing bovine insulin, ubiquitin, cytochrome C, and myoglobin (Bruker).

Identification of Proteins by MALDI-MS and ESI-MS/MS after SDS−PAGE Electrophoresis. After protein separation by SDS−PAGE, the protein band was excised using a gel picker (Gelpix, Genetix Ltd., United Kingdom) subjected to automated trypsin digestion (Proteam Digest, Tecan, Maennedorf, Switzerland) according to the manufacturers protocols, and the resulting peptides were analyzed by MALDI-MS and LC-ESI-MS/MS. Peptide mass fingerprint spectra were recorded on an Autoflex (Bruker) MALDI time-of-flight mass spectrometer operating in delayed extraction reflectron positive ion mode. α-Cyano-4-hydroxy-cinnamic acid (HCCA) solubilized at a concentration of 0.18 g/L in acetonitrile (90%) and TFA (0.01%) was used as the matrix. The desalted (ZipTip μC18) peptides mixture was automatically deposited on an Anchorchip (600 μm) target plate. External calibration was performed with a standard peptide mixture supplied by Bruker. The peptide fingerprints were processed using Biotools software (Bruker) in combination with Mascot database searching (SwissProt/Trembl database).

LC-ESI-MS/MS was performed on a LCQ classic ion trap mass spectrometer (ThermoFinnigan, United States) equipped with a NanoESI source (ThermoFinnigan). Protein digests were reconstituted in 0.1% formic acid and injected in trap (0.3 mm × 5 mm) and analytical (0.18 mm × 150 mm, PepMap C18 3100) columns. The high-performance liquid chromatography (HPLC) system consists of a Rheos 2000 pump with CPS-module (Flux Instruments, Germany) and a PAL HTC autosampler (CTC Analytics, Switzerland). Peptides were eluted with

Table 1. Protein Content in Soy and Sunflower Lecithins after Extraction of Lipids with AH, HIW, and CMW Measured by Different Protein Tests: AA Analysis, Coomassie Protein Assay, Micro BCA Protein Assay (BCA), and 2D Quant Kit (2D Quant)

		protein (mg/kg)[b,c]		
lecithin	protein test	AH	HIW	CMW
soy 1	AA analysis	1264 ± 3.54 a	1338 ± 37.1 a	1089 ± 54.5 b
	Coomassie[a]	1304 ± 75.6 a	1330 ± 60.1 a	1189 ± 6.36 b
	BCA[a]	1778 ± 67.9 a	1689 ± 71.4 a	1288 ± 73.5 b
	2D Quant[a]	1395 ± 62.2 a	1196 ± 39.6 a	1012
sunflower 1	AA analysis	599 ± 8.49 a	892 ± 12.0 c	
sunflower 2	AA analysis	285 ± 4.95 a	414 ± 7.07 c	

[a] Soy flour calibration standards have been used. [b] Mean values and standard deviation of duplicate determination. [c] For letters a and b, values in the same row without a common superscript letter were significantly different: $p \leq 0.05$. For letters a and c, values in the same row without a common superscript letter were significantly different: $p \leq 0.01$.

a linear gradient of 0.5% (v/v) acetic acid/0.05% (v/v) TFA/80% acetonitrile into a nanoelectrospray needle. Full scan MS and MS/MS data acquisition and analysis were performed with Xcalibur software V1.2 (ThermoFinnigan), including Bioworks V2.0 software package for SEQUEST database (SwissProt and Trembl) searches.

Validation of the Method for Protein Quantification. *Repeatability of Duplicates and Simple Repeatability.* The repeatability of duplicates was determined in the ranges 0−500 and 500−5000 mg protein/kg lecithin, by analyzing seven and eight samples loaded in duplicate, respectively. Analyses were performed on several days by different technicians. The simple repeatability was determined by analyzing on three consecutives days, by the same technician, one sample six times.

Recovery. Soy lecithin containing about 1000 mg/kg protein was spiked by adding a known amount of soy flour. Spike concentrations used were 1000, 3000, and 8000 mg soy protein/kg lecithin. Analyses were performed in duplicate.

Limit of Detection (LOD) and of Quantification (LOQ). A mix of PC-enriched fraction soy lecithin type 5 (protein free) and PC-enriched fraction soy lecithin type 6 (163 mg protein/kg) (1:1) was analyzed six times, on two different days, by the same technician. The LOD and LOQ were defined as three and 10 times the robust standard deviation, respectively.

Statistical Analysis. The significance of the differences was evaluated using one-way analysis of variance. The Tukey HSD test was used to identify significant differences (when the number of samples is $k = 2$, the analysis of variance is equivalent to a Student test). The statistical data for the validation of the method were calculated according to EURACHEM (*15*).

RESULTS AND DISCUSSION

Protein Extraction Procedure and Protein Quantification. To determine which is the most suitable extraction method for proteins in lecithins, three procedures were compared. In addition, several methods for quantification of proteins in lecithins were studied with the aim to determine which method suffers less from interferences with the residual lipids and organic solvents present in the samples.

Table 1 shows the protein content measured by different protein tests in crude soy lecithin 1 and in sunflower lecithins 1 and 2, after applying the three extraction procedures. The protein content determined by AA analysis in soy lecithin 1, after extraction using CMW, was significantly lower than that found with the other two extraction procedures studied. On the other hand, the protein content found after extraction with AH and with HIW was very similar. However, the results obtained for the two samples of sunflower lecithins studied showed that the protein content was much higher after extraction with HIW than with AH, which suggest that the extraction with AH in

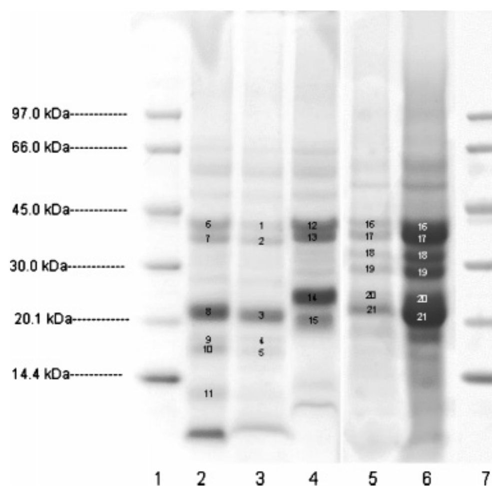

Figure 1. SDS–PAGE (4–12%) of proteins from soy lecithin 1, after extraction with HIW (lane 2), AH (lane 3), and CMW (lane 4); proteins from sunflower lecithin 1 after extraction with AH (lane 5) and HIW (lane 6). The LMW calibration kit (Amersham Bioscience) is shown in lanes 1 and 7.

sunflower lecithin strongly underestimates the content in proteins. These results were confirmed by SDS−PAGE (**Figure 1**, lanes 5 and 6). Therefore, from the different procedures studied, the extraction with HIW was chosen as the most suitable for the isolation of the proteins from lecithins.

The quantification of proteins in soy lecithin has been carried out using AA analysis, Coomassie, Micro BCA, and 2D Quant protein kits (**Table 1**). The AA analysis was taken as the reference method, since it does not have interferences with residual lipids and organic solvents. Discrepancies between the AA analysis and some of the other procedures studied can be observed. The Micro BCA method gave higher values of proteins than the AA analysis, which was attributed to interferences of this method with residual lipids. The results found with the 2D Quant kit were relatively close to those ones found by AA analysis. However, this test has several disadvantages: The detection limit of the method is relatively high because a high amount of buffer is required for the dilution of the sample, and it is also time-consuming and cumbersome. Therefore, it was discarded as a candidate for the final procedure.

The protein contents determined with Coomassie method were close to those found by AA analysis for samples containing protein levels within the range of the calibration curve (7−20 μg/mL), but relatively high variability was found with samples whose protein levels were outside this range (data not shown). With this restriction in mind, this method could be used as an alternative method to the AA analysis for quantification of proteins in this kind of lecithin.

Method Validation. The validation of this method has been carried out using the AA analysis for the determination of proteins. However, in some cases, this determination was also performed using the Coomassie protein kit with the aim to check if this test gives reliable results.

Recovery. The results of the spiking experiment with soy flour in crude soy lecithin 1 from brand A and HIW extraction are shown in **Table 2**. For quantification, AA analysis and Coomassie protein kit have been used. It can be observed that the results with both methods compare very well and the recoveries are also very good (ranged between 101 and 114% and 99 and 118%, when analyses were performed with AA analysis and Coomassie kit, respectively). In addition, the recovery was determined for samples with lower amounts of

Table 2. Recovery of Soy Protein in Soy Lecithin Spiked Prior Extraction[a]

sample	spiking soy protein (mg/kg)	measured protein (mg/kg)[b] AA	measured protein (mg/kg)[b] CO	recovery (%) AA	recovery (%) CO
soy lecithin 1	0	1208 ± 109	1344 ± 115		
	1000	2355 ± 0	2531 ± 28.9	114	118
	3000	4285 ± 18.3	4554 ± 34.7	103	107
	8000	9278 ± 82.0	9260 ± 137	101	99
PC-enriched soy lecithin, type 5	0	ND			
PC-enriched soy lecithin, type 5 + type 6 (1:1)	82	101 ± 4.9[c]		123	

[a] Quantification of proteins was performed by AA analysis and Coomassie protein kit (CO). ND, not detectable. [b] Mean values and standard deviation of duplicate determination. [c] Mean values and standard deviation of six replicates.

Table 3. Protein Content of the Commercial Lecithins Analyzed[a]

lecithin	supplier	protein content (mg/kg)[b,c] AA	protein content (mg/kg)[b,c] CO
soy lecithin 1	A	1338 ± 27.6 a	1330 ± 60.1 a
soy lecithin, type 2	B	692 ± 35.4 a	981 ± 17.7 c
soy lecithin, type 3	B	232 ± 5.66 a	333 ± 15.6 b
soy lecithin, type 4	B	276 ± 14.1 a	421 ± 18.4 b
deoiled soy lecithin	B	342 ± 20.5 a	480 ± 30.4 b
PC-e soy lecithin, type 5	B	ND	
PC-e soy lecithin, type 6	B	163 ± 2.83 a	257 ± 2.83 c
egg lecithin	B	50 ± 4.24 a	49 ± 1.41 a
sunflower lecithin 1	C	892 ± 12.0 a	929 ± 14.1 a
sunflower lecithin 2	D	414 ± 7.07 a	423 ± 25.5 a

[a] Quantification of proteins was performed by AA analysis and by Coomassie protein kit (CO). ND, not detectable; PC-e, PC-enriched. [b] Mean values and standard deviation of duplicate determination. [c] For letters a and b, values in the same row without a common superscript letter were significantly different: $p \leq 0.05$. For letters a and c, values in the same row without a common superscript letter were significantly different: $p \leq 0.01$.

protein, by mixing PC-enriched fraction soy lecithin types 5 and 6 as indicated in the Materials and Methods. The recoveries found in this case were also good for this kind of determination (123%).

Repeatability of Duplicates and Simple Repeatability. The relative repeatability limits at 95% for samples containing proteins in the range 0−500 mg/kg and in the range 500−5000 mg/kg were 12.6 and 7.5%, respectively. The simple repeatability determined in a standard soy lecithin sample containing 1338 mg/kg was also very good (the relative repeatability limit at 95% is 6.5%).

LODs and LOQs. The detection limit determined as defined in the Materials and Methods was 15 mg of protein/kg of sample. The quantification limit was 50 mg protein/kg sample. Taking into account that the lecithins used in food contain a relatively high amount of proteins, the obtained detection limit was considered as acceptable. However, if necessary by optimizing the conditions of the method, it should be possible to achieve a much lower detection limit.

Lecithin Sample Analysis. The protein content of different lecithins after extraction with HIW and quantification by AA analysis and by Coomassie protein assay is reported in **Table 3**.

The protein content determined by AA analysis of the crude soy lecithins analyzed ranged between 232 mg/kg for crude soy lecithin type 3 from brand B to 1338 mg/kg for the soy lecithin 1 from brand A. The deoiled lecithin contains 342 mg protein/kg. The reason that the protein content of this product is higher than in the soy lecithins types 3 and 4 can be explained by the fact that acetone extraction is used during processing of the deoiled lecithin, in which the proteins are insoluble and may be increased by deoiling. Regarding the quantification of the mentioned samples with the Coomassie protein kit, the protein content was significantly higher than the one found with the AA analysis, with the exception of the soy lecithin 1. These results could be attributed to some interferences of this method with lipid matrices. Data derived from the literature also show a wide variability of protein levels in lecithins: Müller et al. (*11*), using ELISA for quantification of proteins, found more protein in soy lecithin type 2 and less in soy lecithin type 4 and in deoiled lecithin (3100, 118, and 65 mg/kg, respectively). Paschke et al. (*6*) found a higher protein amount in crude soy lecithins (between 2303 and 2689 mg/kg), using the Bradford method (*5*). Similar results (2800 mg/kg) also were reported by Awazuhara et al. (*8*), using the Lowry method (*7*). Gu et al. (*9*) determined protein contents in commercial lecithins with the Lowry method and found values close to those of this study (between 115 and 1402 mg/kg). Porras et al. (*10*) used ELISA for protein quantification and found a wide variability (between 1000 and 27000 mg/kg).

The ethanol soluble PC-enriched fractions, types 5 and 6, which are of importance for medical applications, are expected to be protein free because proteins are insoluble in ethanol. We were indeed unable to detect proteins in PC-enriched fraction type 5, but surprisingly, a relative high amount of proteins was found in PC-enriched fraction type 6 (163 mg/kg by AA analysis). The protein content determined by the Coomassie protein kit was significantly higher (257 mg/kg). Müller et al. (*11*) did not find proteins in similar PC-enriched fractions analyzed using ELISA.

The egg lecithin analyzed contains about 50 mg of protein/kg by AA analysis. The results of the AA analysis showed a very high peak with the same retention time as tyrosine, which corresponded to ca. 60% of the total amount of AAs. This peak was not taken into account for the protein quantification. Similar results were found by the Coomassie assay (49 mg/kg).

These results show that the samples PC-enriched fraction type 6 and egg lecithin do not meet the specifications, because these products should not contain any proteins at all.

In the two samples of sunflower lecithins from different suppliers, the amount of proteins was in the same range as those found in standard crude soy lecithins (892 and 414 mg/kg by AA analysis, for sunflower lecithins 1 and 2, respectively). No significant differences were found by the Coomassie assay (929 and 423 mg/kg, for sunflower lecithins 1 and 2, respectively). To our knowledge, no literature data exist on the protein content of egg and sunflower lecithins.

Regarding the use of the Coomassie protein kit as an alternative method to the AA analysis for the determination of proteins in lecithins, it can be concluded that the mentioned kit does not always give reliable results, probably due to interferences of this method with lipid matrices.

SDS−PAGE. The two fractions obtained from soy lecithin 1, after extraction with CMW, interphase, and pellet (see details in Materials and Methods), were analyzed separately by SDS−PAGE. The results showed several bands of proteins in the interphase fraction and an absence of protein bands in the pellet (not shown). Therefore, only the interphase fraction was further investigated in the frame of this study.

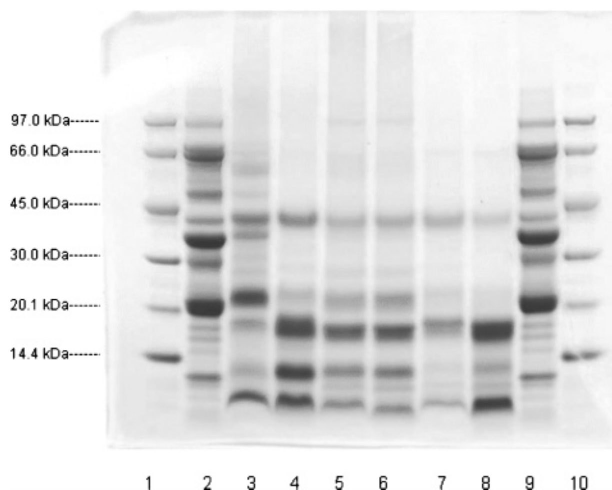

Figure 2. SDS–PAGE (4–12%) of proteins from crude soy lecithin 1 (lane 3); crude soy lecithins types 2 (lane 4), 3 (lane 5), and 4 (lane 6); deoiled lecithin (lane 7); and PC-enriched soy lecithin type 6 (lane 8). For comparison, soy flour proteins are shown in lanes 2 and 9. The LMW calibration kit (Amersham Bioscience) is shown in lanes 1 and 10.

Figure 1 shows the SDS−PAGE results for soy lecithin 1 and sunflower lecithin 1. The protein profiles obtained with the three extraction methods studied are very similar for soy lecithin (**Figure 1**, lanes 2−4), showing several bands of proteins with a molecular mass ranging between 70 and lower than 14 kDa. A higher intensity of the bands is showed in the regions around 40 and 20 kDa. Sunflower lecithin 1 also gave similar protein profiles for the two extraction procedures assayed (**Figure 1**, lanes 5 and 6), but the intensity of the bands is clearly higher with the HIW extraction method (lane 6), indicating that this extraction method is more suitable for sunflower lecithins than the AH method.

Taking into account the results of protein determination as reported above and the results obtained by SDS−PAGE, it can be concluded that the HIW extraction procedure is the most suitable for the extraction of proteins from lecithins.

The protein profile obtained by SDS−PAGE for the different soy lecithins analyzed after extraction with HIW is shown in **Figure 2**. For comparison, soy flour was also included (**Figure 2**, lanes 2 and 9), and its electrophoretic profile shows several bands between 70 and lower than 14 kDa. The standard crude soy lecithins analyzed (lanes 3−6) exhibit a similar band pattern, in which there are five bands of higher intensity, with a molecular mass of about 40, 20, 18, 12, and 8 kDa. The deoiled lecithin and the theoretically protein-free PC-enriched lecithin type 6 show three bands of high intensity with molecular masses of about 40, 18, and 8 kDa (**Figure 2**, lanes 7 and 8). Comparing the results with the literature, Müller et al. (*11*) reported for crude soy lecithins type 2 and type 4 only one strong protein band at 39−40 kDa and a weaker background between 25 and 70 kDa using SDS−PAGE after India ink staining. However, when these samples were analyzed by tricine-SDS−PAGE, many protein bands above and below 17 kDa were detected. On the other hand, Gu et al. (*9*) found protein profiles similar to those reported in our study in soy lecithins used in health supplements and in soy lecithins used as food ingredients, with four major bands of 39, 20, 12, and 7 kDa.

Figure 3 shows the protein profile obtained for the two sunflower lecithins analyzed from different suppliers (lanes 3 and 4) and egg lecithin (lane 5). For comparison, a sample of soy flour is also shown (lane 2). The protein profile of the sunflower lecithins analyzed is similar. However, the intensity of the bands is higher in the sunflower lecithin 1 (lane 3), indicating that the protein content of this lecithin is higher than that of the sunflower lecithin 2 (lane 4). This result is in agreement with the protein content reported above. Both samples show several bands with high intensity with molecular masses ranging between 40 and 20 kDa. The egg lecithin (lane 5) shows several bands representing a broad range of molecular mass, which is characteristic for the protein profile of egg yolk proteins and thereby confirming the presence of residual proteins as reported above.

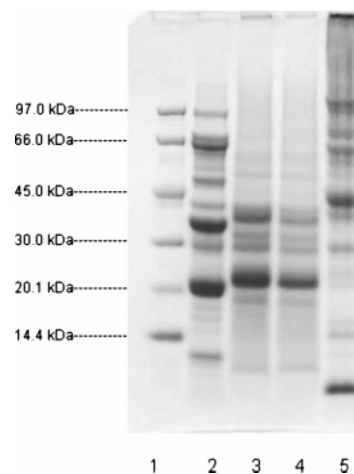

Figure 3. SDS–PAGE (4–12%) of proteins from sunflower lecithin 1 (lane 3), sunflower lecithin 2 (lane 4), and egg lecithin (lane 5). For comparison, soy flour proteins are shown in lane 2. The LMW calibration kit (Amersham Bioscience) is shown in lane 1.

MALDI-MS and LC-ESI-MS/MS. The MALDI mass spectra obtained from soy flour and crude soy lecithin 1 after extraction with AH and with HIW are shown in **Figure 4a** (from 10 to 25 kDa) and **4b** (from 20 to 60 kDa). Many of the proteins present in soybean flour are also present in the two soy lecithin extracted fractions. For these fractions, the two major proteins are observed at *m*/*z* about 15.5 and 26.5 kDa. The comparison between the two extractions shows that the extraction of lecithin with HIW permitted to obtain more proteins than the extraction with AH. Indeed, the proteins at *m*/*z* about 20 and 52 kDa are only present in the fraction extracted with HIW.

Table 4 summarizes the results of identification by MALDI-MS and ESI-MS/MS of the major protein bands obtained after SDS−PAGE from soy lecithin 1 after extraction with AH, HIW, and CMW and from sunflower lecithin 1 after extraction with AH and HIW (analyzed bands are numerated in **Figure 1**). Most of the identified proteins in soy lecithin 1 belong to the 11*S* globulin fraction, which have been implicated as major allergens (*16*, *17*). These proteins correspond to glycinin A acid subunits (35 kDa) (**Figure 1**, numbers 1, 6, and 12), glycinin B basic subunits (18−20 kDa) (**Figure 1**, numbers 3, 8, and 14; 4 and 9; 15; and 5 and 10); and glycinin A5 subunit (10 kDa) (**Figure 1**, number 11). The seed maturation protein P34 (32 kDa) from the 7*S* globulin fraction of soy proteins has also been identified (**Figure 1**, numbers 2, 7, and 13). This protein has been reported as the most allergenic protein in soybean (*18*, *19*). By N-terminal analysis, Gu et al. (*9*) have identified in lecithins a 12 kDa band as a methionine-rich protein from the 2*S* albumin class of soy proteins and a 20 kDa band as the soybean Kunitz trypsin inhibitor. These proteins were not identified in the present study.

Sunflower lecithin protein bands extracted with AH and HIW were also analyzed by MALDI-MS and LC-ESI-MS/MS (**Figure 1**, numbers 16−21). The identified major proteins

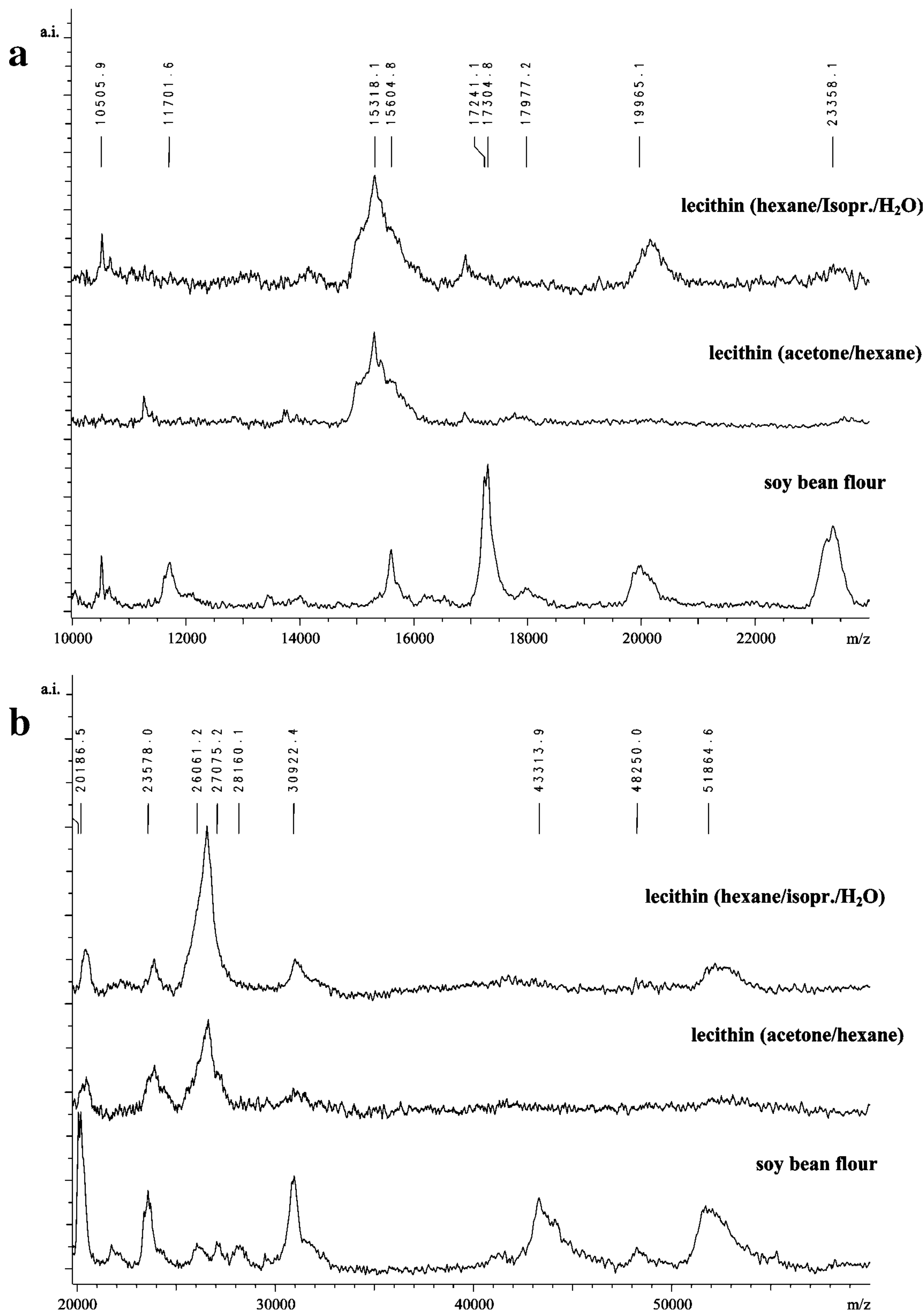

Figure 4. MALDI mass spectra of soy flour and soy lecithin 1 after extraction with AH and with HIW. From (**a**) 10 to 25 kDa and (**b**) 20 to 60 kDa.

correspond to the acidic and basic chains of the 11*S* globulin seed storage protein G3 (**Table 4**), which is one of the main protein fractions of sunflower seeds (*20*).

In conclusion, the extraction with HIW followed by AA analysis is a reliable quantitative method for determination of proteins from lecithins. The protein content has been determined in different kinds of lecithins. The results ranged between not detectable in PC-enriched soy lecithin type 5 to 1338 mg/kg in crude soy lecithin 1.

The spectrophotometric methods micro BCA, Coomassie, and 2D Quant protein kits are not suitable for the quantification of proteins in lecithins. The SDS−PAGE protein patterns of the standard soy and sunflower lecithins are very similar to that of soy flour. The protein pattern of the egg lecithin shows several bands with a broad range of molecular masses. Most of the major proteins from soy and sunflower lecithins identified by MALDI-MS and LC-ESI-MS/MS belong to the 11*S* globulin fraction, which is one of the main fractions of soy and sunflower

Table 4. Identification by MALDI-MS and LC-ESI-MS/MS of the Major Protein Bands Obtained after SDS–PAGE Electrophoresis from Soy Lecithin 1 after Extraction with AH, HIW, and CMW and from Sunflower Lecithin 1 after Extraction with AH and with HIW[a]

sample	numbers from SDS–PAGE gel (**Figure 1**)	identified proteins
soy lecithin 1	bands 1, 6, and 12	glycinin A, acidic subunits (35 kDa)
	bands 2, 7, and 13	7*S* globulin seed maturation protein P34 (32 kDa)
	bands 3, 8, and 14	glycinin B, basic subunits (18–20 kDa)
	bands 4 and 9	
	band 15	
	bands 5 and 10	
	band 11	glycinin A5 subunit (10 kDa)
sunflower lecithin 1	bands 16 and 17	11*S* globulin seed storage protein G3, acidic chain (36–38 kDa)
	bands 18 and 19	11*S* globulin seed storage protein G3, acidic chain (30 kDa)
	bands 20 and 21	11*S* globulin seed storage protein G3, basic chain (20–22 kDa)

[a] Analyzed bands are numerated in **Figure 1**.

seeds. In addition, the seed maturation protein P34 from the 7*S* globulin fraction of soy proteins has also been identified in soy lecithins. This protein has been reported as the most allergenic protein in soybean.

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