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Determination of Proteins in Refined and Nonrefined Oils

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Five methods using aqueous/organic solvents for the separation of proteins from oils were compared. The extraction with acetone−hexane followed by amino acid analysis was found to be the most suitable method for isolation and quantification of proteins from oils. The detection limit of the method was 0.18 mg protein/kg oil, and the quantification limit was 0.6 mg protein/kg. The relative repeatability limit for samples containing 1−5 mg protein/kg sample was 27%. The protein recovery ranged between 68 and 133%. Using this method, the protein content of 14 refined and nonrefined oils was determined. In none of the refined oils were proteins detected, whereas the protein content of the unrefined oils ranged between undetectable in extra virgin olive oil to 11 mg/kg in rapeseed oil. With sodium dodecyl sulfate−polyacrylamide gel electrophoresis in combination with silver staining, many protein bands were visible in the unrefined soy, olive, peanut, and rapeseed oil samples. Proteins bands were not obtained from the refined fish oil. In the other refined oil samples, a few proteins bands could be visualized. Two protein bands with apparent molecular molecular masses of 58 and 64 kDa were always observed in these oils.

KEYWORDS: Oil; protein; amino acid analysis; SDS-PAGE

INTRODUCTION

Oils are used in a wide variety of products including processed food, cosmetics, and pharmaceuticals. The presence of peptides and proteins has been reported in several kinds of crude oils and in refined oils as well [see reviews of Crevel et al. (*1*) and Hidalgo and Zamora (*2*)].

Although the available information is not conclusive, there are indications that the proteins present in oils may elicit allergic reactions in sensitive individuals. Moneret-Vautrin et al. (*3*, *4* and *5*) have reported that peanut oil could provoke allergic reactions, but two other studies showed that peanut oil was unable to elicit a reaction in sensitive individuals (*6, 7*). Few data exist on the potential allergenicity of other edible vegetable oils. Most publications suggest that the major refined oils, soy, maize, sunflower, olive, and palm, do not provoke reaction in sensitive individuals [see reviews of Hefle and Taylor (*8*), Crevel et al. (*1*), and Hidalgo et al. (*2*)], but (again) Moneret-Vautrin et al. (*9*) have reported adverse reactions in an infant to soy oil, and Errahali et al. (*10*) have shown the presence of allergens in cold-pressed and refined soy oil.

Nevertheless, it is clear that reliable analytical methods for the detection and characterization of proteins in oils are necessary to allow for a proper safety assessment. Unfortunately, methods that have been described thus far are generally not properly validated. Information about the efficiency of the various extraction protocols is also lacking. Usually, a first step of extraction is performed using aqueous/organic solvents (*11, 12*), and afterward, the protein content is determined using different assays, such as Bradford (*13*), Lowry (*14*), or bicinchoninic acid (BCA) assays (*15*). However, these methods do not give always reliable results in lipid matrices (*16*). The protein content can also be determined by enzyme-linked immunosorbent assay (ELISA) (*17, 18*), but this method is only semiquantitative and requires specific antibodies for each kind of oil (i.e., soy and sunflower). Hidalgo et al. (*19*) have developed a method for determining the residual proteins in oils, based on acetone precipitation followed by filtration and quantification with amino acid analysis. However, the efficiency of the extraction procedure has not been compared with other methods.

In a previous work, several methods of extraction and quantification of proteins in lecithins were compared (*16*). The extraction with hexane−isopropanol−water (HIW) followed by quantification of proteins with amino acids analysis was found to be the most suitable method for this type of matrix.

The objective of the present work was to develop and validate a sensitive method for the quantification and characterization of proteins in oils. For that, several methods of extraction followed by amino acid quantification of proteins were compared. The selected method to achieve this purpose was validated and subsequently used to quantify proteins in several types of commercial refined and unrefined oils. In addition, the extracted proteins from the studied oils were characterized using sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) after silver staining.

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MATERIALS AND METHODS

Samples. Commercial refined and unrefined oils analyzed in this study are described in **Table 3**. In addition, soybean flour (Sigma) was also analyzed.

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

Extraction with Acetone−Hexane (AH). The isolation of proteins was performed using the method described by Paschke et al. (*20*) for lecithin with some modifications. To 100 g of oil, 250 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 10 mL of AH (1:1). After each washing, the mixture was centrifuged, and the supernatant was discarded.

Extraction with Acetone−Methanol (AM). This extraction was similar to the extraction of AH described above, but the precipitate was washed twice with 5 mL of AM (1:1) and once with 5 mL of acetone.

Extraction with Acetone Followed by Filtration (AF). The isolation of proteins was performed basically using the method described by Hidalgo et al. (*19*). To 100 g of oil, 250 mL of acetone was added. The mixture was shaken vigorously, kept for 1 h at 4 °C, and shaken every 10 min and then filtered through Whatman no. 1 filter paper using a Buchner funnel. Proteins from the paper were extracted with 5 mL of tetrahydrofurane and 5 mL of dioxane as described by Hidalgo et al. (*19*).

Extraction with Acetone (A). This extraction was similar to the extraction for AF but without the filtration step. The mixture was kept at 4 °C and was centrifuged, and the precipitate was washed twice with 5 mL of acetone.

Extraction with HIW. The isolation of proteins was performed using the lipid extraction method of Hara and Radin (*21*), adapted for oils. To 100 g of oil, 250 mL of HIW (3:2:1) was added. The mixture was shaken vigorously, kept for 1 h at 4 °C, shaken every 10 min, and centrifuged. The precipitate was washed once with 5 mL of HIW (6:4:1) and once with 5 mL of hexane−isopropanol (3:2). After each wash, the mixture was centrifuged, and the precipitated was discarded.

Quantitative Determination of Protein. *Amino Acid Analysis.* Proteins were quantified by using amino acid analysis, with a Hitachi L-8500 system (Tokyo, Japan). The method corresponds to AACC method 07-01 (*22*), with some modifications described previously (*16*). Dried precipitates obtained from 100 g of oil were dissolved in 10 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; afterward, 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 (75 μL) on the amino acid analyzer.

SDS-PAGE. SDS-PAGE was performed using the Xcell II Mini-Cell system from Novex. Isolated fraction samples from oils were diluted in Lämmli sample buffer from Bio-Rad with β-mercaptoethanol to obtain between 10 and 20 mg precipitate/mL, with the exception of the refined maize and rapeseed oils, which were diluted to 4 and 2 mg/mL, respectively. Furthermore, the unrefined peanut and rapeseed oils were further diluted to obtain 2 and 0.45 mg/mL, repectively. 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 using a silver staining technique [adapted from Blum et al. (*23*) and Shevchenko et al. (*24*)]. A low molecular mass (LMW) proteins calibration kit (Amersham Biosciences) was used as reference.

Validation of the Method for Protein Quantification. *Repeatability of Duplicates.* The repeatability of duplicates was determined for samples with protein concentrations in the range of 0.9−5.0 mg protein/kg oil, by analyzing seven samples in duplicate. Analyses were performed on several days by the same technician. The difference between duplicates should not exceed the relative repeatability limit, *r*, at the 95% confidence level.

Recovery. The recovery was determined for samples with protein concentrations within the range 0.2−2 mg protein/kg oil, by mixing unrefined rapeseed oil (3.28 mg protein/kg) and chemically refined rapeseed oil (protein free) at different concentrations. Analyses were performed in triplicate on several days.

Limit of Detection (LOD) and of Quantification (LOQ). The LOD and the LOQ were determined by analyzing six oil samples, containing low amounts of protein (most of the amino acid peaks were detectable but under the limit of linearity). The LOD and LOQ were defined as three and 10 times the robust standard deviations of repeatability, respectively, of the samples analyzed in duplicate.

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. The statistical data for the validation

Table 1. Protein Content in Unrefined Rapeseed Oil after Extraction of Lipids with AH, AM, and AF and Measured by Amino Acid Analysis

extraction method	protein (mg/kg)[a]
AH	3.3 ± 0.96 a[b]
AM	1.0 ± 0.36 b[c]
AF	0.7 ± 0.20 b[c]

[a] Values without a common letter were significantly different: $p \leq 0.01$. [b] Mean values and standard deviations of nine replicates. [c] Mean values and standard deviations of triplicate determinations.

Table 2. Recovery of Protein in a Mix of Unrefined and Refined Rapeseed Oils[a]

rapeseed oil	dilution	theoretical protein (mg/kg)	measured protein(mg/kg)[b]	recovery (%)
unrefined			3.28 ± 0.96[c]	
refined			ND	
dilution of unrefined	2×	1.64	2.18 ± 0.18[b]	133
with refined oil	3×	1.09	1.07 ± 0.31[b]	98.5
	5×	0.66	0.44 ± 0.051[b]	68.0
	10×	0.33	0.26 ± 0.013[b]	79.4
	15×	0.22	0.21 ± 0.038[b]	97.3

[a] Quantification of proteins has been performed by amino acid analysis. ND, not detectable. [b] Mean values and standard deviation of triplicate determinations. [c] Mean values and standard deviations of nine replicates performed on several days.

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

type	description	protein content (mg/kg)[b]
olive	extra virgin oil	>0.2 and <0.6
olive	refined	<0.2
soy	biological, cold-pressed oil	1.44 ± 0.06
hazelnut	refined (85%), cold-pressed (15%) hazelnut oil	<0.2
maize	refined oil	<0.2
fish	refined oil (high DHA)	<0.2
LC-PUFA	70% refined palm oil, 20% refined fish oil, and 10% ARASCO	<0.2
DHActive	refined DHA from microalgae	<0.2
ARASCO	refined vegetable oil from fungi with 40% arachidonic acid	<0.2
DHASCO	refined vegetable oil from microalgae with 40% DHA	<0.2
rapeseed	refined oil	<0.2
rapeseed A	cold-pressed unrefined oil	3.28 ± 0.96[c]
rapeseed B	unrefined oil	11.1 ± 0.11
peanut	cold-pressed unrefined oil	1.32 ± 0.25

[a] DHA, docosahexanoic acid. [b] Mean values and standard deviations of duplicate determinations. [c] Mean values and standard deviations of nine replicates performed on several days.

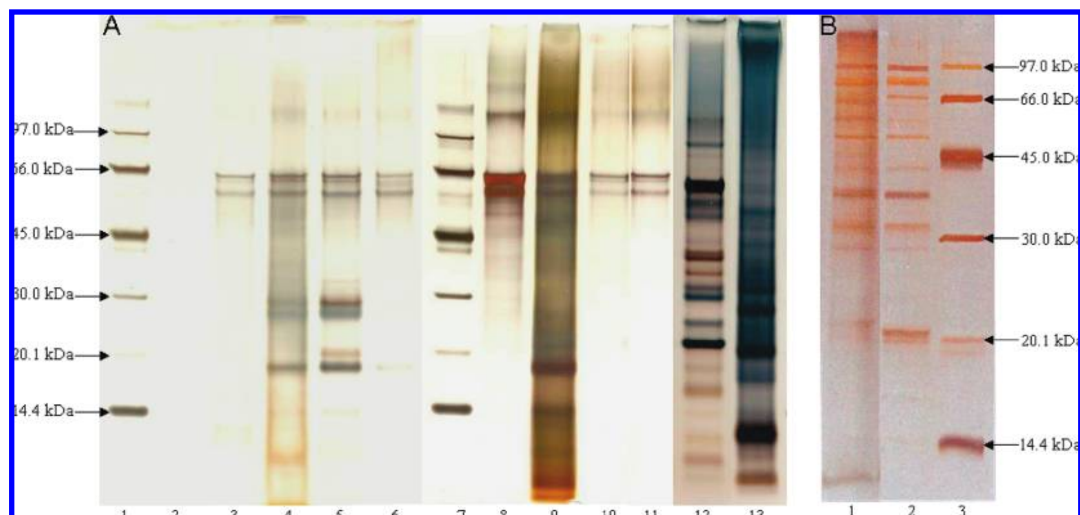

Figure 1. SDS-PAGE (4−12%) after silver staining of proteins from (**A**) fish oil (lane 2), LC-PUFA (lane 3), DHASCO (lane 4), DHActive (lane 5), ARASCO (lane 6), refined maize oil (lane 8), extra virgin olive oil (lane 9), refined hazelnut oil (lane 10), refined rapeseed oil (lane 11), cold-pressed peanut oil (lane 12), and unrefined rapeseed oil (lane 13); and (**B**) cold-pressed soy oil (lane 1). For comparison, soy flour proteins are shown in gel **B**, lane 2. The LMW calibration kit (Amersham Bioscience) is shown in gel **A**, lanes 1 and 7, and in gel **B**, lane 3.

of the method were calculated using the ESTER software, according to EURACHEM (*25*).

RESULTS AND DISCUSSION

Protein Extraction Procedure and Protein Quantification. Five different procedures were used to extract proteins from unrefined rapeseed oil, after which the proteins were quantified by amino acid analysis. The results (**Table 1**) show that the protein content obtained after extraction with AH was significantly higher than that found with the procedures of AM and AF. The extraction using acetone, without a filtration step (A), gave a much larger extracted fraction (pellet) than the other procedures, but this method was rejected due to the presence of artifacts in the chromatograms, which hampered the performance of accurate protein determination. The extraction procedure HIW was not suitable, because no protein could be detected by SDS-PAGE after silver staining (data not shown). In view of these results, the extraction with AH was chosen as the most appropriate for the isolation of the proteins from oils.

Method Validation. *Recovery.* The protein recovery was determined in the concentration range 0.2−2 mg protein/kg, by diluting unrefined with refined rapeseed oil (**Table 2**). The recoveries were calculated by comparing the protein content determined in the diluted samples with that of the unrefined oil. Recoveries ranged between 68 and 133%, which is good for this kind of determination, taking into account that there is a very small amount of proteins in the oils.

Repeatability. The difference between duplicates should not exceed 27% of their average for oils in the range of 1−5 mg protein/kg oil, which corresponds to the relative repeatability limit, *r*, at the 95% confidence level.

LOD and LOQ. The LOD determined as defined in the Materials and Methods was 0.18 mg of protein/kg oil and the LOQ was 0.6 mg protein/kg oil.

Protein Content in Commercial Oils. The protein content of different oils after extraction with AH and quantification by amino acid analysis is shown in **Table 3**. The amount of protein found in the refined oils analyzed was under the LOD (<0.2 mg/kg). On the other hand, the extra virgin olive oil showed a protein content under the LOQ (>0.2 and <0.6 mg/kg). The protein content found in the rest of the unrefined oils analyzed ranged between 1.32 mg/kg for cold-pressed peanut oil and 11.1 mg/kg for unrefined rapeseed oil. As compared with the data reported in the literature, there is a wide variability, depending on the type of oil and the methods used for extraction and for quantification of the proteins. Olszewski et al. (*12*), using the BCA method, found values of 3.4 mg protein/kg in crude peanut oil and between 0.1 and 0.2 mg/kg in refined peanut oil. On the other hand, Skinner and Haynes (*26*) reported a substantially higher value for crude peanut oil (187 mg/kg) with the Micro BCA protein kit. Using ELISA for protein quantification, Porras et al. (*17*) found 110−3300 mg protein/kg in three unspecified soy oils, whereas in five other soy oils, no protein could be detected. Awazuhara et al. (*27*) showed very low protein values in soy oils (0.04−0.17 mg/kg). Hidalgo et al. (*19, 28*) observed values between 0.1 and 1.8 mg/kg in refined olive oil and 0.7 and 5 mg/kg in unrefined oil.

SDS-PAGE. The electrophoretic pattern obtained by SDS-PAGE after silver staining of the different oil samples analyzed after extraction with AH is shown in **Figure 1**. With the exception of the fish oil (**Figure 1**, gel **A**, lane 2) in which no protein bands could be detected, the extracts of the refined oils, LC-PUFA (**A**, lane 3), DHASCO (**A**, lane 4), DHActive (**A**, lane 5), ARASCO (**A**, lane 6), maize (**A**, lane 8), hazelnut (**A**, lane 10), and rapeseed (**A**, lane 11) all show a few protein bands. In each of these samples, two protein bands at about 58 and 64 kDa were always observed. In addition, in DHASCO and in DHActive, other protein bands at about 29, 27, and 18 kDa were visible. In the refined maize oil, a protein band with a molecular mass higher than 97 kDa is clearly visible.

As expected, the unrefined oils showed a more intense background than the refined oils, due to the higher amount of proteins loaded for these samples. The virgin olive oil (gel **A**, lane 9) shows an electrophoretic pattern similar to that found in the DHASCO (gel **A**, lane 4) but with higher intensity and some extra proteins bands with a molecular mass lower than 14 kDa. Previous studies of virgin and refined olive oils have only reported the presence of a polypeptide with an apparent molecular mass of 4.6 kDa (*19*).

The electrophoretic pattern of the unrefined peanut oil (**A**, lane 12) revealed many protein bands of different intensity, ranging from higher than 97 and lower than 14 kDa. The proteins of about 65, 40, 31, and 23 kDA were the most

intensive bands of this oil. In addition, a weak band with an apparent molecular mass of about 18 kDa was clearly detected. Olszewski et al. (*12*) have reported a close protein profile in refined and unrefined peanut oils, including as well the weak band of 18 kDa. This protein was found to be allergenic by Western blot (*12*). The unrefined rapeseed oil (**A**, lane 13) shows a complex electrophoretic pattern with many protein bands, among the most prominent at 31, 28, 20, and 10 kDa.

In addition, the protein profile of the cold-pressed soy oil is very similar to that of soy flour (gel **B**, lanes 1 and 2, respectively), with many bands over a broad molecular range. The protein pattern of the soy oil that we investigated is close to what was previously found in unrefined and refined soy oil (*10*) and in soy lecithins (*16*). In the present study, a protein band with an apparent molecular mass of 35 kDa is visualized. This protein was identified previously in soy lecithin (*16*) as the seed maturation protein P34 from the 7 S globulin fraction, which has been reported as the most allergenic protein in soybean. We also found a protein band with a molecular mass of 56 kDa, which is similar to the molecular mass of the allergenic protein detected in soy oil by Errahali et al. (*10*). On the contrary, Awazuhara et al. (*27*) visualized in soy oil only three bands with a molecular mass that ranged between about 58 and 68 kDa, but it was not specified whether these oils were refined or not, while Paschke et al. (*20*) found similar protein profiles in refined and unrefined soy oil, with seven main bands between 94 and 14 kDa.

In conclusion, five procedures for the extraction of proteins from oils have been compared. The extraction procedure with AH followed by amino acids analysis gave the highest recoveries. Using this method, different types of refined and unrefined commercial oils were analyzed. The results ranged between not detectable in the refined oils and 11 mg/kg in unrefined rapeseed oil. The SDS-PAGE protein patterns of the oils analyzed confirmed that generally only few proteins bands were found in the refined oils, while many protein bands were detected in the unrefined oils.

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