Low Molecular Weight Polypeptides in Virgin and Refined Olive Oils

Francisco J. Hidalgo, Manuel Alaiz, and Rosario Zamora*

Instituto de la Grasa, CSIC, 41012 Sevilla, Spain

ABSTRACT: Twenty-eight virgin olive oils—from different regions of Spain and prepared from olive drupes of different varieties—and six refined olive oils were analyzed to determine the presence of proteins in these oils. All oils studied showed the presence of proteins in the range of 7–51 μg/100 g of oil. There were no significant differences in protein content in oils from different varieties or between virgin or refined oils. In addition, all oils exhibited analogous amino acid patterns, suggesting a similarity among protein fractions obtained from different oils. A polypeptide with an apparent M.W. of 4600 Da was common to the isolated protein fractions. These results suggest that this polypeptide is a previously unknown minor component in olive oils. No clear influence of this component on oil stability was observed when oil stabilities were estimated as a function of phenol, tocopherol, phosphorus, and protein contents of the oils.

Paper no. J10072 in *JAOCS 79,* 685–689 (July 2002).

KEY WORDS: Amino acid analysis, minor components, oil stability, phenols, proteins, refined olive oil, tocopherols, virgin olive oil.

Olive oil is composed primarily by TAG and secondarily (less than 5%) by FFA and some other glyceridic and nonglyceridic constituents (1–3). These minor constituents are important for the stability and flavor of olive oil, and their quantitative analysis is a major determinant defining olive oil genuineness (detection of adulterations with seed oils or solvent-extracted oils) and quality grade (extra virgin, virgin, "lampante," "refined," "pure," etc.) (4,5).

The various classes of minor constituents can be divided into two groups. The first group consists of FA derivatives such as MAG and DAG, phosphatides, waxes, and esters of sterols. The second group includes classes of compounds not related chemically to FA, such as hydrocarbons, aliphatic alcohols, free sterols, tocopherols, chlorophylls, carotenoids, and polar phenolic compounds. All these different classes of compounds are present in a broad range of concentrations, and some of them may be reduced or eliminated during refining (1–3).

The presence of different enzymes in virgin olive oils has been described as an impurity (6,7). No previous reports have indicated that the proteins are usual components of olive oils. Recent studies from this laboratory have developed a methodology that allows for the determination of peptides and proteins in fats and oils (8). As a continuation of that study, the present investigation was undertaken for the systematic screening of olive oils from different origins and degrees of processing, to analyze if the presence of proteins was accidental in some oils or if proteins should be considered minor components of these oils. In addition, the influence of these compounds on the stability of the oils was also studied.

*To whom correspondence should be addressed at Instituto de la Grasa, Avenida Padre García Tejero 4, 41012 Sevilla, Spain.
E-mail: rzamora@cica.es

EXPERIMENTAL PROCEDURES

Materials. Twenty-eight virgin olive oils and six refined olive oils were analyzed in this study (Table 1). Virgin olive oil samples were obtained from different olive varieties in different extraction plants located in several regions of Spain. They were selected as representative of the different virgin olive oils consumed in Spain. For this reason some of oils were monovarietals and others were a mixture of several varieties. In addition, both unfiltered and filtered oils were analyzed. The refined oils were prepared in industrial plants by both chemical and physical processing.

Isolation of peptides and proteins from the oils. Peptides and proteins were isolated according to a previously described procedure (8). Briefly, the oil (40 g) was maintained at 18°C for at least 90 min prior to treatment with acetone (98 mL), which was previously cooled at 4ºC. The resulting mixture was maintained at 4ºC for 30 min and then filtered through Whatman Grade 1 filter paper using a Büchner funnel. The paper was extracted by shaking it first in the presence of 5 mL of THF and then with 5 mL of dioxane. The extracts were combined and taken to dryness with nitrogen.

Quantification of protein content. Peptides and proteins contained in the obtained residues were quantified by using amino acid analysis. The residues plus D,L-α-aminobutyric acid, which was added as internal standard, were dissolved in 1 mL of 6.0 M hydrochloric acid and hydrolyzed for 20 h at 110ºC. The hydrolyzed samples obtained were taken to dryness, dissolved in 3 mL of 1 M sodium borate buffer (pH 9.0), and derivatized with diethyl ethoxymethylenemalonate. Protected amino acids were fractionated by RP-HPLC with UV detection at 280 nm using a previously described gradient (9). Protein content was calculated from amino acid data.

Electrophoretic analysis of the residues obtained from olive oils. Residues obtained from olive oils were studied by electrophoresis. Tricine-SDS-PAGE was performed according to Schägger and Jagow (10) with 16.5% total acrylamide

TABLE 1
Olive Oils Used in This Study and Protein Content Determined

Sample	Quality	Origin	Variety[a]	Filtration	Protein[b]
1	Virgin	Andalusia	ARB	No	21.68 ± 1.48
2	Virgin	Catalonia	ARB	No	11.43 ± 1.13
3	Virgin	Catalonia	ARB	No	21.43 ± 0.63
4	Virgin	Catalonia	ARB	No	26.35 ± 6.23
5	Virgin	Catalonia	ARB	No	13.78 ± 0.65
6	Virgin	Andalusia	HBL	No	18.50 ± 5.45
7	Virgin	Andalusia	HBL	No	31.25 ± 2.00
8	Virgin	Andalusia	HBL	No	19.33 ± 2.03
9	Virgin	Andalusia	HBL	Yes	10.33 ± 2.45
10	Virgin	Andalusia	KOR	No	22.93 ± 2.88
11	Virgin	Andalusia	PCL	No	50.83 ± 2.75
12	Virgin	Andalusia	PCL	No	13.50 ± 2.13
13	Virgin	Andalusia	PCL	No	15.50 ± 0.25
14	Virgin	Andalusia	PCL	No	47.25 ± 1.98
15	Virgin	Andalusia	PCL	No	8.95 ± 1.53
16	Virgin	Andalusia	PCL	No	11.13 ± 0.95
17	Virgin	Andalusia	PCL	No	9.05 ± 0.65
18	Virgin	Andalusia	PCL	Yes	14.58 ± 5.30
19	Virgin	Andalusia	ARB/HBL	Yes	14.38 ± 2.38
20	Virgin	Andalusia	HBL/PCL	No	16.25 ± 2.85
21	Virgin	Andalusia	HBL/PCL	Yes	11.08 ± 2.38
22	Virgin	Andalusia	HBL/PCL/PCD	No	7.25 ± 3.25
23	Virgin	Andalusia	UNK	No	9.00 ± 3.43
24	Virgin	Andalusia	UNK	No	9.00 ± 0.88
25	Virgin	Andalusia	UNK	Yes	12.43 ± 1.53
26	Virgin	Castile	UNK	No	21.18 ± 1.88
27	Virgin	Extremadura	UNK	Yes	22.83 ± 4.70
28	Virgin	Extremadura	UNK	Yes	11.55 ± 4.53
29	Refined	Chemical	UNK		11.50 ± 2.13
30	Refined	Chemical	UNK		13.93 ± 1.75
31	Refined	Chemical	UNK		17.65 ± 2.55
32	Refined	Chemical	UNK		15.38 ± 1.90
33	Refined	Physical	UNK		8.33 ± 2.38
34	Refined	Physical	UNK		8.80 ± 1.65

[a]Abbreviations: ARB, Arbequina; HBL, Hojiblanca; KOR, Cornicabra; PCD, Picudo; PCL, Picual; UNK, unknown.
[b]Values are mean ± SD of three experiments and are given as μg/100 g of oil.

gels containing 3% of crosslinker. Gels were silver stained according to Morrissey (11). Briefly, gels were treated successively with glutaraldehyde, dithiothreitol, silver nitrate, formaldehyde/sodium carbonate, citric acid, water, Farmer's reducer, and new silver staining. A typical calibration curve ($r = -0.987$, $P = 0.018$) was obtained with BSA (66.0 kDa), chicken egg albumin (45.0 kDa), bovine erythrocyte carbonic anhydrase (29.0 kDa), chicken egg white lysozyme (14.3 kDa), and bovine insulin chain B (3.5 kDa).

Oil analysis. Oil stabilities were determined by the Rancimat method as described previously (12). Briefly, oil samples (2.5 g) were heated at 110ºC in a Metrohm Rancimat apparatus (Metrohm AG, Herisau, Switzerland). A continuous stream of air (15 L/h) was passed through the heated sample, and the volatiles were absorbed in a conductivity cell. Conductivities were continuously monitored until a sudden rise signified the end of the induction period (IP). IP were determined (in hours) by the method of tangents to the two parts of the kinetic curve.

Total phenols were determined colorimetrically at 765 nm using the Folin–Ciocalteau reagent (13). Briefly, 10 mL of a solution of methanol/water (80:20 vol/vol) plus Tween 20 (2% vol/wt) was added to 10 g of olive oil and mixed with an UltraTurrax (IKA-Werke GmbH, Staufen, Germany) for 1 min at the maximum speed and then centrifuged at 2000 × *g* for 10 min. The extraction was repeated two times. The extracts were combined and evaporated to a final volume of 1 mL. A known volume of this concentrate (20–100 μL) was diluted to 1 mL with water and treated with 500 μL of Folin–Ciocalteau reagent. After 3 min, 1 mL of 30% sodium carbonate was added, and the resulting mixture was incubated for 1 h. At the end of the incubation period, 7.5 mL of water was added, the mixture obtained was centrifuged at 2000 × *g* for 10 min, and the absorbance of the mixture was measured at 765 nm.

Tocopherols were determined directly by HPLC using a fluorescence detector (14).

Total phosphorus content was determined by the method of Bartlett as described by Kates (15) after extraction of the phospholipids with methanol (16). Briefly, 0.7 g of the oil was extracted three times with 7 mL of methanol using an UltraTurrax for 1 min at the maximum speed and then centrifuged at 2000 × *g* for 10 min. The extracts were combined and taken to dryness. The residue was suspended in 1 mL of water, treated with 2 mL of perchloric acid, and digested for 90 min at 220–230ºC. The cooled digest was then diluted with 9.5 mL of water and treated with 2.0 mL of amidol solution (1% 2,4-diaminophenol dihydrochloride and 20% sodium metabisulfite in water) and 1 mL of 8.4% ammonium molybdate in water. The mixture was allowed to develop the blue color for 20 min, and the absorbance at 680 nm was measured.

Statistical analysis. All results are expressed as mean values ± SD of three experiments unless otherwise indicated. Statistical comparisons between two groups were made using Student's *t*-test. With several groups, ANOVA was used. When significant *F* values were obtained, group differences were evaluated by the Student–Newman–Keuls test (17). Statistical procedures were carried out either using Primer of Biostatistics: The Program (18) or the SPSS for Windows (v. 10.0.6; Chicago, IL) statistical package. Significance level was $P < 0.05$ unless otherwise indicated.

RESULTS AND DISCUSSION

Presence of proteins in virgin and refined olive oils. The precipitation of proteins with acetone and their later hydrolysis and quantification of the amino acids produced by HPLC showed that the 34 olive oils studied contained proteins in the range 7–51 μg/100 g of oil (Table 1). Although there were significant differences among the quantities of proteins found in the different oils studied, there was no clear relationship with the variety of the olive drupe, the origin of the oil, or whether the oil was filtered or refined.

The mean protein content of oils obtained from olives of the variety Arbequina was 18.93 ± 6.16 μg/100 g of oil ($n = 5$); location of cultivation, i.e., northern (Catalonia) or southern (Andalusia) part of Spain, did not affect protein content.

A similar protein content, 19.85 ± 8.62 µg/100 g of oil (n = 4), was found in olives of the variety Hojiblanca, and also in olives of the variety Picual, 21.35 ± 17.28 µg/100 g of oil (n = 8). Other virgin olive oils from different regions and mixtures of varieties exhibited a variable protein content.

Refining the oils seemed to decrease the protein content [17.96 ± 10.62 µg/100 g of oil (n = 28) for virgin oils vs. 12.60 ± 3.71 µg/100 g of oil (n = 6) for refined oils], but the difference was not significant (P = 0.236). Only the analyzed oils submitted to physical refining exhibited a clear decrease in protein content. In fact, the two refined oils obtained by physical refining had two of the three lowest protein contents among the 34 oils analyzed. The protein content for oils obtained by chemical refining was 14.61 ± 2.58 µg/100 g of oil (n = 4), and 8.57 ± 0.33 µg/100 g of oil (n = 2) for oils obtained by physical refining. This difference was significant (P = 0.036).

Although filtration of the oils seemed to decrease the protein content, 19.32 ± 11.79 µg/100 g of oil (n = 21) in unfiltered oils vs. 14.13 ± 4.61 µg/100 g of oil (n = 6) in filtered oils, this difference was not significant (P = 0.307).

Amino acid composition of protein residues obtained from olive oils. Figure 1 shows the amino acid pattern of protein residues obtained from three virgin olive oils of the varieties Arbequina, Hojiblanca, Picual, and one refined olive oil. The four chromatograms were very similar, suggesting that protein residues were similar for the different oils analyzed. The only appreciable difference was the presence of some unknown peaks, which were considerably higher in the refined oils.

This similarity among HPLC patterns was confirmed when amino acid compositions were compared (Table 2). The compositions determined for the protein residues obtained from virgin olive oils of different varieties as well as refined oils were very similar, with only two amino acids, arginine and leucine, exhibiting small significant differences, therefore suggesting that the protein fraction present in olive oils is always similar and is independent of the variety of the olive drupes employed in the elaboration of the oil. This composition is similar to the previously described amino acid composition for the 4.6 kDa-polypeptide found in olive drupes (19), therefore confirming that this polypeptide is the major component of the protein fraction isolated. The only difference was a decrease in cysteine, methionine, and tyrosine, which are likely decomposed during the acid hydrolysis as a consequence of other components present in the oils, and an increase in the content of glycine and serine. However, the ratio among the other amino acids was almost identical to that previously described (19).

Electrophoretic analysis of protein residues obtained from olive oils. An additional confirmation of the similarity observed by HPLC among the protein fractions was obtained by tricine-SDS-PAGE. All the oils analyzed in the present study exhibited a main spot corresponding to a polypeptide of apparent 4.6 kDa M.W., and there were only very small differences in the mobility of the spot among the different oils assayed (data not shown). This polypeptide, which was previously found in both the mesocarp and the seeds of olive drupes and was partially soluble in organic solvents (8,19), should pass to the oil during olive oil extraction, constituting a previously unknown minor component in these oils.

Influence of these new minor components on the stability of olive oils. In an attempt to investigate whether these new

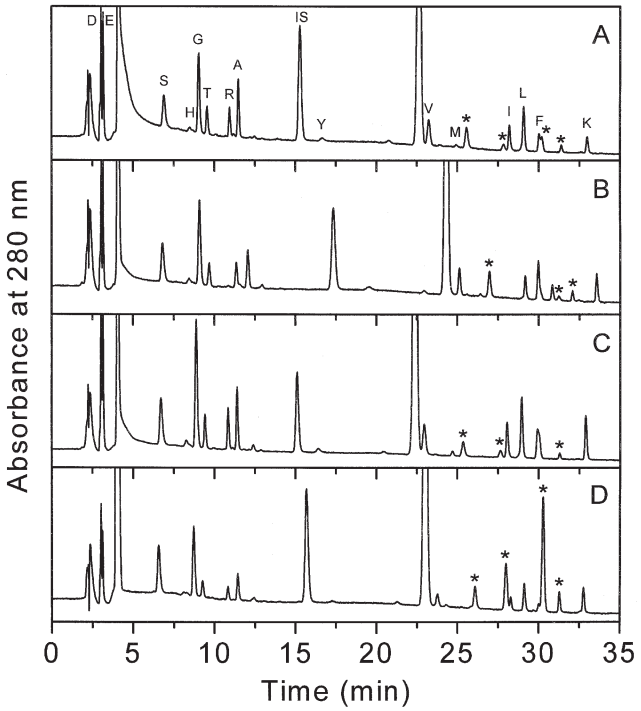

FIG. 1. Amino acid profiles obtained by HPLC after acid hydrolysis and derivatization with diethyl ethoxymethylenemalonate of proteins obtained by acetone precipitation of (A) virgin olive oil, cv. Arbequina; (B) virgin olive oil, cv. Hojiblanca; (C) virgin olive oil, cv. Picual; (D) refined olive oil. Peaks are labeled with single-letter notations for amino acids; IS, internal standard; *, unknown peaks.

TABLE 2
Amino Acid Composition of Protein Residues Obtained by Acetone Precipitation of Virgin and Refined Oils[a]

Amino acid[b]	Arbequina	Hojiblanca	Picual	Refined
Ala	7.09 ± 1.21	6.54 ± 0.60	6.70 ± 0.72	5.81 ± 0.22
Arg	4.12 ± 0.14[a]	4.36 ± 0.56[a]	3.65 ± 0.74[a,b]	3.21 ± 0.21[b]
Asx	8.45 ± 3.46	11.26 ± 3.06	10.94 ± 2.17	7.15 ± 3.20
Cys	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Glx	12.84 ± 0.86	13.03 ± 0.41	10.07 ± 3.32	10.82 ± 1.19
Gly	20.11 ± 2.79	20.44 ± 2.82	20.15 ± 2.36	23.39 ± 1.35
His	2.47 ± 0.24	2.61 ± 0.83	3.08 ± 1.08	3.37 ± 0.68
Ile	4.88 ± 1.19	5.10 ± 1.17	4.56 ± 1.00	5.60 ± 2.06
Leu	8.35 ± 0.82[a]	7.22 ± 1.49[a,b]	7.62 ± 0.65[a,b]	6.24 ± 1.54[b]
Lys	3.14 ± 1.28	2.94 ± 0.44	3.23 ± 0.55	4.02 ± 0.37
Met	0.00 ± 0.00	0.34 ± 0.46	0.20 ± 0.37	0.00 ± 0.00
Phe	3.51 ± 1.41	2.78 ± 0.26	2.82 ± 1.80	2.42 ± 0.30
Ser	14.18 ± 3.00	13.52 ± 1.96	16.48 ± 4.54	18.20 ± 1.09
Thr	5.52 ± 0.62	5.42 ± 0.41	5.78 ± 1.07	5.81 ± 0.66
Tyr	0.29 ± 0.49	0.25 ± 0.51	0.32 ± 0.38	0.00 ± 0.00
Val	5.02 ± 1.14	4.20 ± 0.52	4.42 ± 0.82	3.97 ± 0.44

[a]Mean values in the same row with different roman superscripts are significantly different (P < 0.05)
[b]Asx, aspartic acid + asparagine; Glx, glutamic acid + glutamine.

TABLE 3
Stability and Phenol, Tocopherol, and Phosphorus Contents of the Assayed Oils

Sample	Stability[a] (h)	Phenol[a] (ppm)	Tocopherol[b] (ppm)	Phosphorus[b] (ppm)
1	9.35 ± 0.09	29.8 ± 1.7	137	7.0
2	28.90 ± 0.75	74.0 ± 1.6	128	5.4
3	16.67 ± 0.15	46.1 ± 2.5	135	5.5
4	13.83 ± 0.41	41.7 ± 3.1	150	7.5
5	13.93 ± 0.32	39.2 ± 2.3	162	1.9
6	16.33 ± 0.15	36.1 ± 3.5	180	0.3
7	17.33 ± 0.47	46.9 ± 2.8	237	6.1
8	21.67 ± 0.38	65.4 ± 4.5	185	5.8
9	15.00 ± 0.10	44.7 ± 0.8	156	2.7
10	22.00 ± 0.40	66.6 ± 3.5	164	2.4
11	31.23 ± 0.87	89.1 ± 5.8	167	1.1
12	14.63 ± 0.15	41.8 ± 5.2	179	1.8
13	25.23 ± 0.31	45.2 ± 2.9	238	4.1
14	20.67 ± 0.51	40.7 ± 0.6	182	1.2
15	36.46 ± 1.04	68.9 ± 4.3	164	7.5
16	34.53 ± 1.17	68.7 ± 0.8	144	11.8
17	44.70 ± 0.26	97.4 ± 1.8	164	2.7
18	51.47 ± 1.76	84.2 ± 8.5	222	3.9
19	11.13 ± 0.06	34.5 ± 1.7	136	5.7
20	28.90 ± 0.10	86.0 ± 5.1	204	3.4
21	18.13 ± 0.25	53.1 ± 0.5	188	4.3
22	18.90 ± 0.20	51.4 ± 2.6	190	6.0
23	16.63 ± 0.21	46.7 ± 3.5	32	0.7
24	25.10 ± 0.26	154.3 ± 7.9	77	0.6
25	44.90 ± 0.35	94.9 ± 7.3	205	4.7
26	32.40 ± 0.87	131.6 ± 4.2	124	2.5
27	9.70 ± 0.35	63.1 ± 4.4	39	1.1
28	6.43 ± 1.04	102.1 ± 4.0	10	1.2
29	21.23 ± 0.12	14.8 ± 1.7	155	2.5
30	8.12 ± 0.15	8.9 ± 0.3	111	0.3
31	6.90 ± 0.50	2.2 ± 1.1	145	2.0
32	19.63 ± 0.55	10.8 ± 0.6	169	0.3
33	12.13 ± 0.21	9.1 ± 0.3	127	2.9
34	21.70 ± 0.46	8.6 ± 0.7	159	0.9

[a]Values are mean ± SD of three experiments.
[b]Values are mean of two experiments.

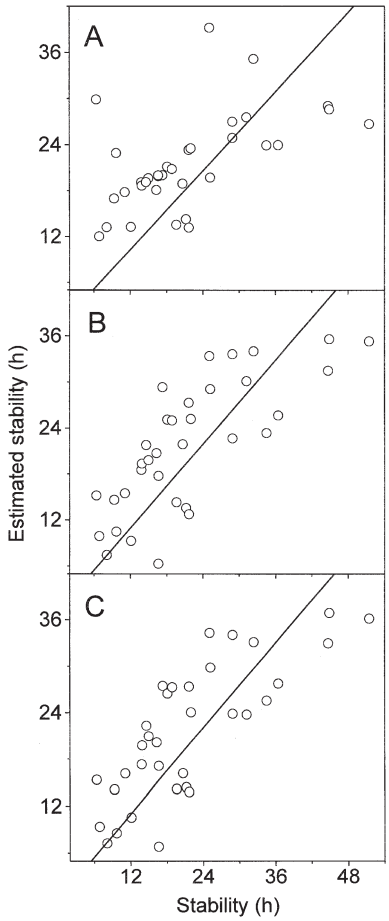

FIG. 2. Correlation between determined and estimated stabilities calculated when using (A) phenol; (B) phenol and tocopherol; and (C) phenol, tocopherol, phosphorus, and protein contents determined in the 28 virgin and the 6 refined olive oils analyzed in the present study.

minor components play a role in the stability of olive oils, the stabilities of the different oils as well as their phenol, tocopherol, and phosphorus contents were analyzed (Table 3). The assayed olive oils exhibited a broad range of stabilities as determined by the Rancimat method at 110ºC. Virgin olive oils studied had a moderate stability of 23.08 ± 11.54 h (n = 28). As expected, refined oils had lower (P = 0.108) stabilities (14.95 ± 6.73, n = 6), although some oils were freshly prepared and their stabilities were high (see, for example, oil numbers 29, 32, and 34).

Phenol content in the assayed virgin olive oils ranged from 29.8 to 154.3 ppm (n = 28) and was significantly higher than the phenol content determined in the refined oils (9.1 ± 4.1 ppm, n = 6). On the contrary, tocopherol content was very similar in virgin (154 ± 57 ppm, n = 28) and refined (144 ± 22 ppm, n = 6) oils. Analogous to phenols, refining also decreased phospholipids in the oils, and the assayed virgin olive oils had a phosphorus content (3.9 ± 2.7 ppm, n = 28) that was significantly higher (P < 0.05) than the phosphorus content of refined oils (1.5 ± 1.1 ppm, n = 6).

When phenol, tocopherol, phosphorus, and protein contents were correlated with the stabilities of the assayed oils, the best correlation was obtained with the phenol content (r = 0.560, P = 0.00058), followed by the tocopherol content (r = 0.421, P = 0.013). No significant correlation was observed with either phosphorus (r = 0.238, P = 0.175) or protein (r = 0.047, P = 0.790) contents (Table 4). Nevertheless, the correlation increased significantly if phenol and tocopherol contents were considered simultaneously (r = 0.761, P < 0.0001). The best results were obtained by using phenol, tocopherol, phosphorus and protein contents simultaneously (r = 0.779, P < 0.0001), but the regression line obtained was not significantly different from that obtained using phenol and tocopherol contents. Plots of correlations between determined and estimated stabilities calculated when using (A) phenol, (B) phenol and tocopherol, and (C) phenol, tocopherol, phosphorus, and protein contents are shown in Figure 2.

Proteins have been described to have antioxidant properties and to react with lipid oxidation products, producing endogenous antioxidants in food systems (20–22). However, at the concentrations present in these oils, they do not seem to play a clear role in oil stability.

TABLE 4
Correlations Between Stability and Phenol, Tocopherol, Phosphorus, and Protein Contents of the Assayed Oils[a]

Measurement	Equation	Correlation	Significance
Phe	Sta = 11.666 + 0.179·Phe	0.560	0.00058
Toc	Sta = 7.909 + 0.0904·Phe	0.421	0.013
Pho	Sta = 18.179 + 1.000·Phe	0.238	0.175
Pro	Sta = 22.553 – 5.348·Phe	0.047	0.790
Phe/Toc	Sta = –6.802 + 0.205·Phe + 0.112·Toc	0.761	<0.0001
Phe/Toc/Pho	Sta = –6.967 + 0.203·Phe + 0.108·Toc + 0.249·Pho	0.763	<0.0001
Phe/Toc/Pro	Sta = –4.817 + 0.208·Phe + 0.119·Toc – 18.887·Pro	0.778	<0.0001
Phe/Toc/Pho/Pro	Sta = –4.966 + 0.207·Phe + 0.117·Toc + 0.134·Pho – 18.313·Pro	0.779	<0.0001

[a]Stabilities were determined by the Rancimat method at 110ºC and calculated in hours. Phenol, tocopherol, phosphorus, and protein contents were expressed in ppm. Abbreviations: Phe, phenol content; Toc, tocopherol content; Pho, phosphorus content; Pro, protein content; Sta, stability.

ACKNOWLEDGMENTS

We are indebted to Koipe S.A. for the gift of many of the oils used in this study, to Jose L. Navarro for the technical assistance, and to Dr. Antonio Garrido for his help in the statistical analysis of the results. This study was supported in part by the Comisión Interministerial de Ciencia y Tecnología of Spain (Project OLI96-2124) and the Junta de Andalucía (Project AGR 0135).

REFERENCES

1. Kiritsakis, A., Chemistry of Olive Oil, in *Olive Oil*, edited by A.K. Kiritsakis, American Oil Chemists' Society, Champaign, 1990, pp. 25–55.
2. Boskou, D., Olive Oil Composition, in *Olive Oil: Chemistry and Technology*, edited by D. Boskou, AOCS Press, Champaign, 1996, pp. 52–83.
3. Kiritsakis, A., and W.W. Christie, Analysis of Edible Oils, in *Handbook of Olive Oil*, edited by J. Harwood and R. Aparicio, Aspen Publishers, Gaithersburg, MD, 2000, pp. 129–158.
4. European Communities, Regulation 2568/91, *Off. J. Eur. Communities*: L248 (1991).
5. International Olive Oil Council. COI/T. 15/NC n.2/rev. 4 (June 6), Madrid, 1996.
6. Georgalaki, M.D., T.G. Sotiroudis, and A. Xenakis, The Presence of Oxidizing Enzyme Activities in Virgin Olive Oil, *J. Am. Oil Chem. Soc. 75*:155–159 (1998).
7. Georgalaki, M.D., A. Bachmann, T.G. Sotiroudis, A. Xenakis, A. Porzel, and I. Feussner, Characterization of a 13-Lipoxygenase from Virgin Olive Oil and Oil Bodies from Olive Endosperms, *Fett/Lipid 100*:554–560 (1998).
8. Hidalgo, F.J., M. Alaiz, and R. Zamora, Determination of Peptides and Proteins in Fats and Oils, *Anal. Chem. 73*:698–702 (2001).
9. Alaiz M., J.L. Navarro, J. Girón, and E. Vioque, Amino Acid Analysis by High-Performance Liquid Chromatography After Derivatization with Diethyl Ethoxymethylene Malonate, *J. Chromatogr. 591*:181–186 (1992).
10. Schägger, H., and G. von Jagow, Tricine–Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis for the Separation of Proteins in the Range from 1 to 100 kDa, *Anal. Biochem. 166*:368–379 (1987).
11. Morrissey, J.H., Silver Stain for Proteins in Polyacrylamide Gels: A Modified Procedure with Enhanced Uniform Sensitivity, *Anal. Biochem. 117*:307–310 (1981).
12. Alaiz, M., R. Zamora, and F.J. Hidalgo, Natural Antioxidants Produced in Oxidized Lipids/Amino Acids Browning Reactions, *J. Am. Oil Chem. Soc. 72*:1571–1575 (1995).
13. Montedoro, G., M. Servili, M. Baldioli, and E. Miniati, Simple and Hydrolyzable Phenolic Compounds in Virgin Olive Oil. 1. Their Extraction, Separation, and Quantitative and Semiquantitative Evaluation by HPLC, *J. Agric. Food Chem. 40*: 1571–1576 (1992).
14. American Oil Chemists' Society, *Official Methods and Recommended Practices of the American Oil Chemists' Society*, 5th edn., AOCS Press, Champaign, 1999, Method Ce 8-89.
15. Kates, M., *Techniques in Lipidology: Isolation, Analysis and Identification of Lipids,* North-Holland, Amsterdam, 1972, p. 355.
16. Ramesh, B., S.S. Adkar, A.V. Prabhudesai, and C.V. Viswanathan, Selective Extraction of Phospholipids from Egg Yolk, *J. Am. Oil Chem. Soc. 56*:585–587 (1979).
17. Snedecor, G.W., and W.G. Cochran, *Statistical Methods*, 7th edn., Iowa State University Press, Ames, 1980.
18. Glantz, S.A., *Primer of Biostatistics*, 2nd edn., McGraw-Hill, New York, 1987.
19. Zamora, R., M. Alaiz, and F.J. Hidalgo, Influence of Cultivar and Fruit Ripening on Olive *(Olea europaea)* Fruit Protein Content, Composition, and Antioxidant Activity, *J. Agric. Food Chem. 49*:4267–4270 (2001).
20. Zamora, R., M. Alaiz, and F.J. Hidalgo, Feed-back Inhibition of Oxidative Stress by Oxidized Lipid/Amino Acid Reaction Products, *Biochemistry 36*:15765–15771 (1997).
21. Alaiz, M., F.J. Hidalgo, and R. Zamora, Effect of Initial Slight Oxidation on Stability of Polyunsaturated Fatty Acid/Protein Mixtures Under Controlled Atmospheres, *J. Am. Oil Chem. Soc. 75*:1127–1133 (1998).
22. Hidalgo, F.J., and R. Zamora, The Role of Lipids in Nonenzymatic Browning, *Grasas Aceites 51*:35–49 (2000).

[Received August 27, 2001; accepted April 30, 2002]