Pheophytin α Degradation Products as Useful Indices in the Quality Control of Virgin Olive Oil

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ABSTRACT: In previous studies we reported the presence of compounds with spectral characteristics similar to pheophytin α (Pheo α), which often accompany the Pheo α peak in the chromatographic profile of virgin olive oils (VOO) at 410 nm under normal-phase HPLC conditions. The occurrence and levels of these compounds were found to be affected by storage conditions of the oil samples. In the present study we investigated whether the major Pheo a degradation products, identified as pyropheophytin α (coeluting with the respective epimer) and 13^2-OH-pheophytin α, could be used as estimates of VOO history. The content of Pheo α and its degradation products was determined for a great number of authentic olive oil samples of unknown history. Results are discussed in comparison with other quality indices (e.g., antioxidant content) when necessary. High amounts of the pyro form (20–30% of total pheophytins) were related to thermal abuse or lengthy storage. The presence of allomers indicated oxygen availability. The levels of these products, 0–20% of the total pheophytin content for 62% of the samples, seemed to be influenced by the presence of pro- and antioxidants. When low levels of Pheo α are not accompanied by other degradation products, light exposure for a certain period of storage can be assumed.

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KEY WORDS: Chemical indices, 13^2-OH-pheophytin α, pheophytin α, pheophytin α degradation products, pyropheophytin α, virgin olive oil.

In previous studies (1–4) we have reported the presence of products with spectral characteristics similar to pheophytin α (Pheo α), which often accompany the Pheo α peak in the chromatographic profile at 410 nm under normal-phase HPLC conditions. The occurrence and levels of these compounds were found to be related to the oxidative status of the oil samples as a result of storage conditions and the extent of storage. Indeed, in recently produced virgin olive oil (VOO) samples, such degradation products were absent or found in only minute amounts. However, in commercial samples analyzed near their expiration date, significant levels of these compounds were found in relation to those of Pheo α. The conditions that favor their formation are storage in the dark (3), since under light exposure Pheo α bleaching prevails (4). In autoxidation studies of VOO samples at different temperatures and oxygen availability conditions, these derivatives were formed to different degrees. At higher temperatures, the levels of products eluting before Pheo α, tentatively identified as epimers and/or pyro forms, were increased. Oxygen availability gave rise to more polar derivatives, which could be assigned to allomeric forms (5). In a recent paper (6) the presence of Pheo α degradation products was also evidenced for Spanish VOO samples stored under mild conditions for 1 yr (15°C, dark, 3% headspace). These products, eluted under RP-HPLC conditions after extraction of pigments with *N*,*N*,dimethylformamide, were identified as 15^1-OH-lactone pheophytin α, 13^2-OH-pheophytin α (OH-Pheo α), and pyropheophytin α (pyro-Pheo α) on the basis of previously reported findings of the research group. The authors suggested that "the content and class of pigments present in virgin olive oil are authentic indicators of its history prior to marketing."

VOO "history" can be defined as all the events that occurred in the period that elapsed from production to analysis, and the consequences to the overall quality of the product. The latter are expected to be undesirable in the case of improper handling and to affect several chemical indices (e.g., total polar phenol and tocopherol contents, K_{232}, PV) of the oil.

In this study we investigated whether the presence and levels of Pheo α degradation products could be used as estimates of VOO history. For this reason, the contents of Pheo α and its degradation products were determined for a great number of authentic olive oil samples of unknown history and are discussed in comparison with other quality indices when necessary.

EXPERIMENTAL PROCEDURES

Samples. Olive oil samples (n = 40) that had been purchased in bulk were collected from households throughout Greece (August–September 2003), according to a certain protocol, during a campaign sponsored by SEVITEL (Greek Association of Industries and Processors of Olive Oil, Athens, Greece) within the time frame of a European project for the promotion of olive oil consumption. Sampling from sealed, 16-L tinplate containers was carried out by properly trained personnel. Consumers were asked to fill in a questionnaire at the time of sampling. Samples were kept in a freezer until analysis (October 2003). The authenticity of the samples was tested on the basis of provisions of EEC Regulation No. 1989 by the Food Industrial Research and Technological Development Company (ETAT) S.A. (Athens, Greece). Two additional samples, O1 and O2 (ELAIS

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S.A., Piraeus, Greece), were used for shelf-life studies. Samples O1 and O2 were kept at room temperature in a series of tinplate containers (250 mL, 0% head space) stored in the dark for different periods of time (0, 4, 8, and 12 mon).

Solvents and standards. The solvents (HPLC grade) were used without further purification. *n*-Hexane 95% and 2-propanol were from Panreac Quimica SA (Barcelona, Spain), and the diethyl ether was from Riedel-de Haën AG (Seelze, Germany). Chlorophyll a was from Sigma Chemical Co. (St. Louis, MO).

Apparatus. The solvent delivery system consisted of an HPLC device with a gradient pump equipped with a diode array detector in series with a fluorimetric detector. A Rheodyne injection valve (Model 7125) with a 20-μL fixed loop (Rheodyne, Cotati, CA) was used. Data from the detector were processed with the chromatographic software EZChrom 6.6 (Sci. Software, Inc., San Ramon, CA). Absorbance measurements were taken by a double-beam UV spectrophotometer in 1-cm matched quartz cells.

HPLC determination and characterization of VOO chlorophyll pigments. n-Hexane/2-propanol (98:2, vol/vol) (A) and 2-propanol (B) were used as eluents. The gradient used was: 0% B for 10 min, 0–5% B in 4 min, 5% B for 6 min, 5–0% B in 4 min, and 0% B for 6 min. Separation was achieved on a 250 × 4 mm i.d. LiChrospher-100 Si, 5 μm, column (Analyzentechnik, Mainz, Germany) at a 1.0 mL min^{-1} flow rate. The injection volume was 10 μL. Chlorophyll standards were prepared in diethyl ether. Pheo α was obtained by acid treatment of the solution of chlorophyll α (7). Partial epimerization of Pheo α was performed according to the method of Watanabe *et al.* (8). Because of the lack of availability of a pyro-Pheo α standard, the following procedure was chosen. A VOO sample was heated at 120°C for several hours and was periodically chromatographed using the above-mentioned protocol. When the Pheo α peak was minimal, the sample was subjected to fractionation using semipreparative HPLC. The isolated pigment was characterized as pyro-Pheo α using MS. Samples (8% wt/vol) were prepared in *n*-hexane and filtered through a 0.45-μm membrane filter (Schleicher & Schuell, Dassel, Germany) just before HPLC analysis. Care was taken to avoid exposure of samples and standard solutions to sunlight throughout the analytical procedure. Peak identification and purity control were based on photodiode array spectroscopic data, retention time, and peak spiking with authentic standards as previously described (1). Quantification was carried out at 410 nm (Pheo α). Standard curves (concentration vs. peak area, 0.3–1.9 mg/L) were calculated by linear regression analysis ($y = 4 \cdot 10^6 x - 5347.4$, $R^2 = 0.9915$). Repeatability for a standard solution was satisfactory ($C_{\cdot} = 0.6$ mg/L, CV% = 7.5, $n = 5$). Each sample solution was then tested once. Control of standard chlorophyll solution concentrations was carried out through spectrometry using extinction coefficients from the literature (8). The calibration curve was tested daily.

Spectrometric estimation of total chlorophyll pigment content. The equation C (mg kg^{-1} as Pheo α) = $345.3[A_{670} + (A_{630} + A_{710})/2]/L$, where A_λ is the absorbance of the oil at the respective wavelength and L is the cell thickness (mm), was applied to determine the content of total chlorophyll pigments (9).

Other quality characteristics of olive oil samples. Evaluation of the quality of the samples was based on measurements of acidity, PV, and absorbance at 232 and 270 nm (10). Determinations of total polar phenol and α-tocopherol contents were based on published methods (11,12).

RESULTS AND DISCUSSION

The 40 authentic olive oil samples were of unknown history since data for the cultivar, extraction conditions, year of production, and chemical composition were not certified at the time of sampling. According to the questionnaires, samples were bought between November 2002 and the sampling time. Samples were used to examine whether the chlorophyll pigment profile and chlorophyll levels might be useful chemical indices of the history of the oils. Ranges for quality characteristics and the FA composition of the samples are given in Table 1.

The chromatographic profile for all samples at 410 nm revealed the presence of three peaks having typical Pheo α UV-vis spectra (Fig. 1). The assignment of peak 1 to Pheo α was based on spiking with a standard solution. The assignment of peak 2 to a single derivative was not feasible, since under the chromatographic conditions, Pheo α′ and pyro-Pheo α coelute. This was verified by spiking with the respective standards. Peak 3 corresponded to the allomerization product of Pheo α, and specifically to OH-Pheo α. This was confirmed by co-chromatography with authentic standards and by electrospray ionization-MS spectra after isolation by semipreparative HPLC.

Table 2 presents the content of chlorophyll pigments as estimated spectrometrically along with the levels of Pheo α and its degradation products (actual amounts and relative quantities). The content of chlorophyll pigments varied within a range similar to that reported for commercial VOO samples (2). Obviously, such a descriptive parameter cannot be used as an estimate of the history of the oil. On the contrary, determination of the levels of individual pigments gave meaningful information. Chlorophylls α and *b* as well as pheophytin *b* were not detected in any of the samples. Chlorophyll α is expected to be present only in recently produced VOO since the replacement of the

TABLE 1
Ranges for Quality Characteristics and FA Composition of 40 ($n = 40$) Authentic Olive Oil Samples

Quality characteristic	Range
Acidity (% oleic acid)	0.3–1.9
PV (meq O_2 kg^{-1})	6.8–27.8
K_{232}[a]	1.60–3.15
K_{270}[a]	0.10–0.24
α-Tocopherol content (mg kg^{-1})	50–283
Total phenols (mg kg^{-1}, as caffeic acid)	22.3–217.5
FAME (% peak area)	
18:1	64.1–80.3
18:2	4.3–12.8
18:3	0.5–0.8

[a]K_{232} and K_{270}, extinction coefficients at 232 and 270 nm, respectively.

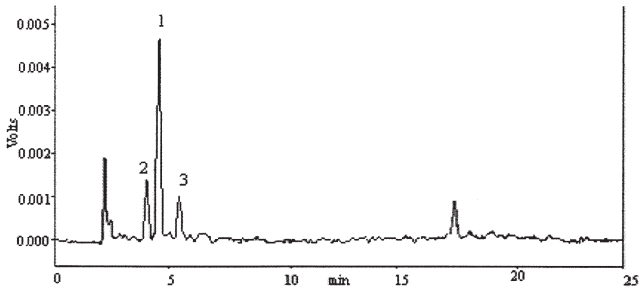

FIG. 1. Typical HPLC profile of virgin olive oil at 410 nm: 1, pheophytin α; 2, pheophytin α′ and/or pyropheophytin α; 3, 13^2-OH-pheophytin α. Chromatographic conditions are given in the text. Nonassigned peaks correspond to carotenoids.

central magnesium atom by two protons seems to occur even after a short storage period (2,6). On the other hand, type *b* derivatives usually exist in insignificant amounts, so their presence could not easily be confirmed under normal-phase chromatographic conditions (i.e., without the pigment isolation and preconcentration step). For this reason, the absence of chlorophyll *b* could not be directly related to VOO history.

Pheo α was the major pigment in most of the samples, ranging from 4.0 to 18.5, with a mean value of 9.2 mg/kg. Only one-third of the samples contained levels higher than 10 mg/kg Pheo α. These levels are lower than those reported for fresh VOO samples, 70% of which contained >10 mg/kg Pheo α (2). Pheo α derivatives were found at levels comparable to those of the remaining Pheo α.

TABLE 2
Total Chlorophyll Content (measured spectrometrically), Total Pheophytin α (Pheo α) Content, and Levels of Pheo α and Derivatives (measured by HPLC) of Olive Oil Samples

				Peak 2[c]		Peak 3[d]	
Samples	Total chlorophylls[a]	Total Pheo α[a,b]	Pheo α[a]	Content[a]	% of total Pheo α	Content[a]	% of total Pheo α
1	18.5	13.1	7.6	2.7	20.3	2.9	21.8
2	44.1	23.9	15.0	6.3	26.3	2.6	10.8
3	23.6	14.3	9.9	4.3	30.4	Traces	0.0
4	29.7	17.1	10.8	3.5	20.2	2.9	16.9
5	44.1	24.4	16.2	5.8	24.0	2.3	9.4
6	12.2	9.8	5.2	2.4	24.9	2.2	22.0
7	32.1	19.0	10.8	3.4	17.6	4.9	25.9
8	38.1	18.6	14.1	4.5	24.0	Traces	0.0
9	48.3	27.0	17.3	7.1	26.2	2.6	9.7
10	39.3	21.4	13.2	5.8	27.3	2.3	10.9
11	33.5	18.0	10.7	4.6	25.4	2.8	15.4
12	32.9	10.9	4.7	3.2	29.5	2.9	26.8
13	21.6	12.7	5.1	5.4	42.2	2.3	18.0
14	11.7	7.5	4.9	2.5	34.0	Traces	0.0
15	37.9	18.5	7.7	6.9	37.4	3.9	21.0
16	21.9	11.5	7.7	3.7	32.5	Traces	0.0
17	34.1	20.0	11.1	4.9	24.6	3.9	19.7
18	26.0	15.2	6.7	5.4	35.4	3.1	20.3
19	28.2	15.7	8.8	4.6	29.5	2.3	14.6
20	15.5	11.2	6.3	2.6	23.6	2.3	20.7
21	30.0	18.4	8.5	4.3	23.6	5.6	30.1
22	19.9	13.9	8.0	3.7	26.7	2.2	15.6
23	32.8	17.8	8.7	5.8	32.4	3.4	19.0
24	21.1	12.5	6.5	3.1	24.5	3.0	23.6
25	32.0	17.9	9.7	5.8	32.5	2.4	13.6
26	31.9	16.5	8.4	5.7	34.4	2.4	14.6
27	39.7	21.4	11.3	5.2	24.2	4.9	22.8
28	16.1	8.7	5.1	3.7	41.9	Traces	0.0
29	32.3	17.4	10.6	4.1	23.6	2.7	15.3
30	13.5	10.8	6.3	2.5	23.1	2.1	19.0
31	39.4	20.9	10.4	5.0	24.0	5.5	26.3
32	68.3	30.3	18.1	6.7	22.0	5.5	18.3
33	13.9	9.3	4.3	2.6	28.0	2.4	26.3
34	8.6	8.2	4.0	2.1	25.6	2.1	25.6
35	28.6	17.5	9.8	4.5	25.7	3.2	18.3
36	60.6	33.8	18.5	7.9	23.3	7.4	21.9
37	11.4	6.7	4.2	2.5	37.1	Traces	0.0
38	19.8	15.3	4.8	5.5	35.7	5.0	32.7
39	38.6	17.1	9.9	4.2	24.8	2.9	17.1
40	12.2	7.7	4.9	2.8	36.2	Traces	0.0

[a]Expressed as mg Pheo a kg^{-1} oil.
[b]Total Pheo α = Pheo α + Pheo α′/pyro-Pheo α + OH-Pheo α. pyro-Pheo α, pyropheophytin α; OH-Pheo α, 13^2-OH-pheophytin α.
[c]Pheo α′ and/or pyro-Pheo α.
[d]OH-Pheo α.

TABLE 3
Changes in Lipid Substrate (expressed in PV, K_{232}) and Pheo α Content of Olive Oil Samples O1 and O2 Stored in the Dark at Room Temperature

Sample	Storage time (mon)	PV (meq O_2 kg^{-1})	K_{232}	Pheo α ($mg\ Kg^{-1}$)		
				Pheo α	Peak 2[a]	Peak 3[a]
O1	0	4.7	2.42	7.2	0.8	ND[b]
	4	7.5	2.76	4.7	2.3	ND
	8	9.9	2.80	4.5	2.9	ND
	12	9.1	2.81	4.0	2.7	ND
O2	0	9.7	1.89	14.3	ND	ND
	4	11.0	2.16	12.7	2.4	ND
	8	13.4	2.56	9.8	2.6	Traces
	12	13.2	2.42	8.3	4.8	Traces

[a]Peak assignment as in Figure 1.
[b]ND, not detected; for other abbreviation see Table 2.

Degradation products that corresponded to peak 2 (Pheo α′ and/or pyro-Pheo α) ranged from 2.1 to 7.9 mg/kg. The allomer OH-Pheo α (peak 3) was found at similar levels (2.1–7.4 mg/kg). In 87.5% of the samples, the derivatives corresponding to peak 2 were present at higher levels than those observed for OH-Pheo α. Analogous levels of Pheo α degradation products were reported for commercial samples analyzed 1 or 2 mon before the stated expiration date (2). In the latter study, these derivatives were absent from the majority of the fresh samples or occurred at very low levels (<1.5 mg/kg).

These observations are better illustrated in terms of relative quantities (percentage Pheo α′/pyro-Pheo α and percentage OH-Pheo α of the total Pheo α content) given in the same table. Thus, 65% of the samples had amounts of Pheo α′ and/or pyro-Pheo α representing 20–30% of the total Pheo α content, whereas another 32.5% of the samples had even higher levels (>30% of total Pheo α content). Our previous experience (1) pointed out a mean value of 10% for these components for oils with a short history. The amounts of pyro forms are expected to depend on the initial Pheo α concentration, storage temperature, and the length of heat treatment (6,13). As far as OH-Pheo α is concerned, its levels represented approximately 12% of the total pigment content for the majority of samples (67.5% of the samples). For the rest of the samples, the mean value was twofold higher (approx. 25%). In previous work (2) this derivative was absent or present at minute amounts in the majority of the fresh oils examined.

Pheo α degradation products in the samples of the present study offer the opportunity to investigate their use as indices of oxidative deterioration and loss of freshness on storage, allowing insight into the history of the oils. The occurrence of Pheo α′ and/or pyro-Pheo α at appreciable levels is indicative of extended storage of the oil in the dark and/or exposure at elevated temperatures. Pyro formation by demethoxycarbonylation as a result of heating may be spontaneous and is practically irreversible. This is the main transformation that Pheo a suffers on heating of VOO, although its formation also has been reported during extended storage at low temperature (6). On the other hand, Pheo a epimerization is a procedure that leads to an equilibrium in which $[\text{Pheo } \alpha']_{eq}$ ranges from 0.13 to 0.20 of [Pheo α] (14). Therefore, appreciable levels corresponding to peak 2, compared with those of Pheo α, can be mainly attributed to the pyro derivative. The fact that under normal-phase HPLC conditions, these two derivatives coelute does not undermine the importance of the presence of the corresponding peak in estimating the quality of the oil with respect to the handling conditions. In the case of RP-HPLC, although these derivatives can be separated and evaluated individually, the prerequisite for pigment extraction not only adds to the overall analysis time but also could lead to artifact formation (14).

The formation of OH-Pheo α, the main oxidation product, is affected by parameters that influence a reaction mechanism *via* free radicals (5). As proposed in a previous work (3) and as also evidenced by Gallardo-Guerrero *et al.* (6), oxygen availability is a critical factor for allomer formation. In the latter study, the formation of allomeric derivatives was observed only during the initial period of storage, whereas after 3 mon their levels remained unchanged, possibly because of the depletion of available oxygen. This is further illustrated in the results shown in Table 3 for two olive oil samples (O1 and O2) stored

TABLE 4
Levels of Polar Phenols, α-Tocopherol, PV, and K_{232} of Selected Samples Having the Highest Percentages of OH-Pheo α

Samples[a]	Total phenol content[b]	α-Tocopherol content[c]	PV[d]	K_{232}
7	106.7	186	17.0	2.62
12	58.0	163	19.3	2.35
21	69.8	213	23.9	3.15
31	93.2	133	27.8	2.64
33	47.2	50	20.2	3.00
34	22.3	96	17.2	2.68
38	46.4	159	17.6	2.89

[a]Sample numbering as in Table 2.
[b]Expressed as mg caffeic acid kg^{-1} oil.
[c]Expressed as mg kg^{-1} oil.
[d]Expressed as meq O_2 kg^{-1}. For abbreviations see Tables 1 and 2.

in metal containers with no headspace. The insignificant increase (traces) in the levels of the allomer even after 12 mon of storage at ambient temperature could be due to limited oxygen availability. On the other hand, the considerable levels of Pheo α allomers found for commercial samples in our published work (2) could be attributed to the oxygen permeability of the polymeric packaging material.

Allomerization of chlorophyll pigments, in solution, is considered to follow a free radical mechanism that is inhibited by radical scavengers (α-tocopherol, β-carotene) (5). The influence of radical scavengers on the formation of allomerization products is not clear in the case of olive oil and needs further study. This is supported by the results of our previous studies, where no clear trend was found for the formation of OH-Pheo α in VOO stored in the dark at room temperature for 24 mon (3).

From the 40 samples shown in Table 2, those with the highest percentages of Pheo α allomer were characterized by low levels of total polar phenols as well as an appreciable degree of oxidation based on PV and K_{232} values (Table 4). Thus, it can be presumed that during storage of these oils, no precaution was taken to exclude oxygen, thus advancing autoxidation and the consumption of chain-breaking polar phenols. Samples having traces of OH- Pheo α, did not follow a certain pattern in relation to oxidative status and/or antioxidant content. This is shown in the examples of the selected samples in Table 5, where oils differing in the content of antioxidants or PV/K_{232} values were found to be almost free of this specific compound. It is obvious that a single correlation does not exist, since autoxidation is a complicated process, and the concomitant presence of and possible interrelations among different pro-oxidant and antioxidant factors perplex the situation. Moreover, we could not ensure that some of the samples shown in Table 5 had not been exposed to light during the handling process (4). For example, sample no. 40, although oxidized and almost depleted of antioxidants, contained only traces of the allomer.

The chromatographic profile at 410 nm of an olive oil sample conclusively indicates the conditions of oil storage history prior to analysis. The extent of Pheo α transformation and the type of degradation products provide useful information for quality control of the oil. The use of the sum of the epimer and pyro derivative contents (as a percentage of the total Pheo α content)–determined either by normal-phase or RP-HPLC—is thus proposed as a qualitative index of the storage length of authentic olive oil, thereby ensuring good manufacturing practice. The use of the percentage of the allomer is an indication of the storage conditions under which autoxidaton prevails. When low levels of Pheo α are not accompanied by the formation of other degradation products, light exposure for some time during storage cannot be precluded.

TABLE 5
Levels of Polar Phenols, α-Tocopherol, PV, and K_{232} of Selected Samples Having the Lowest Percentages of OH-Pheo α

Samples[a]	Total phenol content[b]	α-Tocopherol content[c]	PV[d]	K_{232}
3	80.1	250	8.7	1.70
8	128.3	263	6.8	1.60
14	134.3	179	9.0	2.02
16	150.8	213	15.2	2.79
28	154.0	158	13.7	2.12
37	69.6	191	14.3	2.46
40	57.4	74	19.0	3.05

[a]Sample numbering as in Table 2.
[b]Expressed as mg caffeic acid kg^{-1} oil.
[c]Expressed as mg kg^{-1} oil.
[d]Expressed as meq O_2 kg^{-1}. For abbreviations see Tables 1 and 2.

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