Simultaneous HPLC Determination of Tocopherols, Carotenoids, and Chlorophylls for Monitoring Their Effect on Virgin Olive Oil Oxidation

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The oxidative stability of virgin olive oil is related to triacylglycerol composition and the presence of polar phenolic and other components. The present paper presents an HPLC methodology for the simultaneous determination of tocopherols, major carotenoids, and chlorophylls suitable to monitor their fate during virgin olive oil oxidation. The separation of the above classes of components was carried out using an *n*-hexane/2-propanol gradient within 20 min. Detection was accomplished using a UV detector in series with a diode array system. The method was validated and applied to oil samples subjected to autoxidation or photo-oxidation. The results indicated that the method could be used to highlight the effect of these components on virgin olive oil oxidative stability.

Keywords: *Virgin olive oil; HPLC; tocopherols; carotenoids; chlorophylls; oxidation*

INTRODUCTION

Recognition of the nutritional value of virgin olive oil, which is rich in monounsaturated fatty acids, has revitalized interest in studies of its oxidative stability.

Virgin olive oil is obtained from the olive fruit by mechanical means (pressure, centrifugation, or selective filtration), being thus the olive juice. Its oxidative stability, considered a priori as high due to triacylglycerol composition, was also related to the presence of antioxidants and other minor components. The levels of minor components in the oil depend on olive cultivar and degree of ripeness and also on olive fruit processing and storage conditions of the oil.

In recent years, research has been focused on the relationship of total phenol content or individual phenols, for example, 3,4-dihydroxyphenylethanol, with the oxidative stability of the oil (Montedoro and Cantarelli, 1969; Vázquez Roncero et al., 1973; Gutfinger, 1981; Tsimidou et al., 1992; Catalano and Caponio, 1996; Baldioli et al., 1996). Less attention has been paid to the role of α-tocopherol (Papadopoulos et al., 1993; Blekas et al., 1995; Servili et al., 1996; Baldioli et al., 1996). Besides, information on pigment contribution is limited (Kiritsakis and Dugan, 1984, 1985; Fakourelis et al., 1987; Rahmani and Saad, 1989; Gutiérrez-Rosales et al., 1992; Servili et al., 1996).

The present paper is part of a larger study on the role of α-tocopherol, carotenoids, and chlorophylls in the oxidative stability of virgin olive oil. Its objective is the development of an HPLC methodology for the simultaneous monitoring of the above components during oxidative stability studies of the oil. This objective was based on our opinion that the monitoring of antioxidant or pro-oxidant factors would add to the knowledge of mechanism and interactions occurring during autoxidation or photo-oxidation of virgin olive oil. Although many papers exist on the HPLC analysis of each category of components separately or of carotenoids in the presence of either tocopherols or chlorophylls in edible oils (Fraser et al., 1985; Zonta and Stancher, 1987; Stancher et al., 1987; Rahmani and Csallany, 1991; Endo et al., 1992; Mínguez-Mosquera et al., 1992; Gandul-Rojas et al., 1996), no reference was found for their parallel monitoring in olive oil or in other edible oils. Normal phase liquid chromatography was selected to avoid extra sample treatment (e.g., saponification). Detection was through a diode array detector on line with a dual-wavelength UV−vis system. The method was validated and applied to virgin olive oils stored in the dark or under diffused light.

MATERIALS AND METHODS

Samples. Two sets of virgin olive oil samples were examined. One set (30 oil samples) was obtained from various olive oil mills all over Greece. The other set of samples was produced in a pilot scale at the Institute of Subtropical Plants and Olive Tree (Chania, Crete, Greece). This set consisted of eight oil samples from Koroneiki cultivar, harvested at two different stages of fruit ripeness on the basis of color (green and black) and oil content. The olive fruits were processed just after harvest at the Institute using a "two-phase" and a "three-phase" horizontal decanter. The olive paste was subjected to malaxation at two different temperatures, namely 30 and 45 °C.

Solvents and Standards. The solvents, of HPLC grade, were used without further purification. *n*-Hexane was a Baker Analyzed HPLC 95% reagent (Deventer, Holland), 2-propanol (Chromasolv) was from Riedel de-Häen (Seelze, Germany), and the diethyl ether was from Labscan, Analytical Science (Dublin, Ireland). *dl*-α-Tocopherol (99% for biochemistry) (α-T), a set of four tocopherol species (α, *β*, *γ*, and *δ*-T, for biochemistry), and *β*-carotene (for biochemistry) were purchased from Merck (Darmstadt, Germany). Chlorophylls *a* and *b* (Chl *a* and *b*) and ubiquinone Q10 were from Sigma Chemical Co. (St. Louis, MO).

Apparatus. The solvent delivery system consisted of two Marathon IV Series HPLC pumps (Rigas Labs, Thessaloniki,

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Greece) and a Rheodyne injection valve (model 7125) with a 20 μL fixed loop (Rheodyne, Cotati, CA). The liquid chromatograph was equipped with a UV−vis spectrophotometric detector SPD-10AV (dual wavelength) Shimadzu (Kyoto, Japan) connected in series with a diode array linear UVIS-206 multiple-wavelength system (Linear Instruments, Fremont, CA). Fluorescence detection was accomplished by an SSI 502 programmable fluorescence detector (Scientific Systems Inc., State College, PA) set at 294 nm (excitation) and 330 nm (emission). Computer software, Linear UV−vis 206 (Linear Instruments), was used to obtain and store absorption spectral chromatographic data. The data from the UV−vis SPD-10AV detector were stored and processed with the chromatographic software EZChrom (Scientific Software, Inc., San Ramon, CA). In all other cases an HP 3396 Series II integrator (Hewlett-Packard, Avondale, PA) was employed. Absorbance measurements were recorded by a Hitachi U-2000 spectrophotometer (Hitachi Ltd., Tokyo, Japan) in 1 cm quartz cells.

HPLC Separation, Identification, and Quantification of Tocopherols, Carotenoids, and Chlorophylls of Virgin Olive Oil. *(a) Elution Protocol.* The gradient elution system was designed to achieve adequate separation of α-tocopherol, β-carotene, chlorophylls *a* and *b*, pheophytins *a* and *b*, and lutein within a reasonable time length. *n*-Hexane/2-propanol (99:1, v/v) (A) and 2-propanol (B) were used as eluents. The gradient 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-Si 60, 5 μm, column (Analyzentechnik, Mainz, Germany) at a 1.2 mL/min flow rate. The injection volume was 20 μL.

(b) Preparation of Standards and Samples. α-Tocopherol standard solutions were prepared in solvent A. The chlorophyll standards were dissolved in diethyl ether and injected in small volumes (5 μL) to avoid HPLC peak distortion and production of artifacts (Khachik et al., 1988). β- Carotene was first dissolved in a small quantity of tetrahydrofuran and then in solvent A. Pheophytins *a* and *b* (Pheo *a* and *b*) were obtained by acid treatment (one drop of 1 N HCl) of the solution of chlorophylls *a* and *b* (Sievers and Hynninen, 1977). Partial epimerization of pheophytin *a* was performed according to the method of Watanabe et al. (1984). Control of chlorophyll conversion to the respective pheophytin was via TLC and HPLC. Separation of chlorophyll pigments was carried out on silica plates (0.1 mm) using a spinach extract as a reference material. The latter was prepared according to the method of Johansson and Appelqvist (1984). The developing system was *n*-hexane/acetone/diethylamine (10:4:1, v/v). Oil samples (8% w/v) were prepared in solvent A and filtered through a 0.45 μm membrane filter (Schleicher & Schuell, Dassel, Germany) just before HPLC analysis. Care was taken to exclude sunlight exposure of samples and standard solutions throughout the analytical procedure.

(c) Detection and Identification. Tocopherols were detected at 294 nm by the UV−vis detector. Spectral data in the region 380−700 nm were recorded using the diode array detector. Detection wavelengths for pigments in ascending order of chromatographic elution were as follows: *all-trans* β-carotene, 453 nm; pheophytin *a*, 410 nm; pheophytin *b* and chlorophyll *a*, 430 nm; chlorophyll *b* and lutein, 453 nm. Peak identification and purity were based on photodiode array spectroscopic data, retention time, and peak spiking with authentic standards. Pigments were identified from their spectral characteristics—both λ_{max} and peak ratios. For chlorophyll pigments, the characteristic peak ratio was that between the absorbance of the Soret band (I) and the absorption maximum in the red region (II). For carotenoids, the height of the largest wavelength absorption band (III) was expressed as a percentage of that of the middle absorption band (II). The baseline was drawn at the valley minimum between these two peaks (Davies, 1975). The position of lutein was also confirmed by spiking the oil sample with the corresponding fraction of a spinach extract.

(d) Quantification. Standard curves (concentration versus peak area) were calculated by linear regression analysis. Injections in duplicate were made at each concentration for both standards and samples. Due to the lack of availability of standard lutein, its quantification relied on the similarity of extinction coefficient to that of β-carotene. Control of standard solutions was carried out spectrometrically. The concentrations of tocopherol standard solutions were calculated from absorbance values at 292 nm divided by a correction factor of 0.0076 as suggested by Pocklington and Dieffenbacher (1988). Concentrations of chlorophyll pigment solutions in diethyl ether were measured at their λ_{max} using the extinction coefficients for this solvent found in the literature (Watanabe et al., 1984). Method validation included study on specificity, linearity, precision, range, and detection limits.

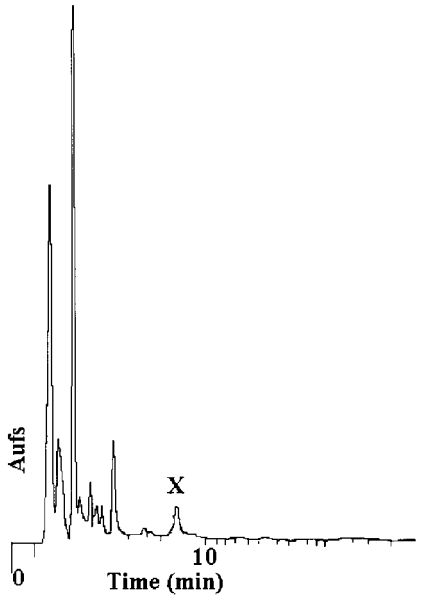

Figure 1. HPLC chromatogram of a virgin olive oil sample at 294 nm. Eluent: *n*-hexane/2-propanol (99:1, v/v).

"Apparent" Chlorophyll Content. "Apparent" chlorophyll content was estimated using the following equation: C (pheophytin *a*, mg/kg) = $345.3[A_{670} - (A_{630} + A_{710})/2]/L$, where A_λ is the absorbance of the oil at the respective wavelength and L is the cell thickness (mm) (Pokorny et al., 1995).

Studies of Olive Oil Autoxidation and Photo-oxidation. Virgin olive oil samples as lipid substrates were selected on the basis of their quality characteristics and apparent chlorophyll content. Evaluation of the quality of olive oil samples was based on fatty acid composition, acidity, peroxide value, absorbance at 232 nm (IUPAC, 1987), total phenol content (Gutfinger, 1981), and induction period determined according to the Rancimat test at 120 °C (Servili et al., 1996). Virgin olive oil samples were stored in the dark at 22 ± 2 °C and under diffused light at 25 ± 2 °C. Transparent glass bottles were completely filled with oil and sealed. No headspace was left in the bottles. Peroxide values and absorbance at 232 nm were periodically measured to follow the degree of oxidation. Samples stored in the dark were covered with aluminum foil and kept in a carton box. Samples stored under diffused light were periodically rearranged to equalize exposure.

RESULTS AND DISCUSSION

Simultaneous HPLC Monitoring of Tocopherols, Carotenoids, and Chlorophylls. *Preliminary Work.* During tocopherol determination by normal phase HPLC with *n*-hexane/2-propanol (99:1, v/v) and UV detection, the presence of several unknown peaks was observed (Figure 1). Careful literature review as well as scanning of the unknown peaks in the visible region revealed that some of them could be attributed to carotenoid or chlorophyll pigments (Fiksdahl et al., 1978; Watanabe et al., 1984; Weber, 1987). According to Rahmani and

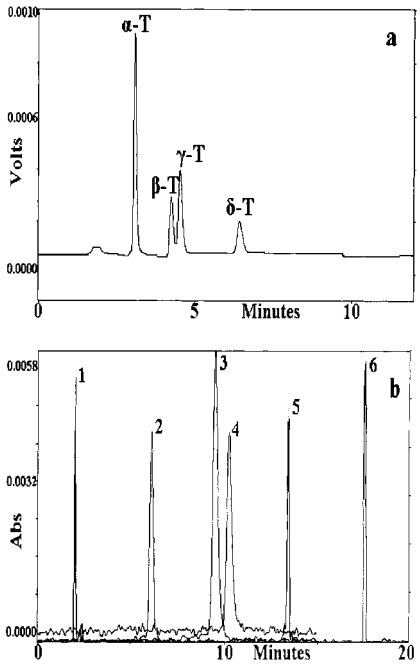

Figure 2. Normal phase HPLC separation of (a) a tocopherol standard mixture and (b) carotenoid and chlorophyll standards; peak 1, *β*-carotene; peak 2, pheophytin *a*; peak 3, chlorophyll *a*; peak 4, pheophytin *b*; peak 5, chlorophyll *b*; peak 6, lutein. Chromatographic conditions were as reported under Materials and Methods.

Csallany (1991) the determination of chlorophylls, pheophytins, and *β*-carotene in vegetable oils could be accomplished by isocratic normal phase HPLC using hexane/2-propanol (98.5:1.5, v/v). However, lutein, present in virgin olive oils (Zonta and Stancher, 1987; Stancher et al., 1987; Mínguez-Mosquera et al., 1990; Gandul-Rojas et al., 1996) was not eluted by that system. These observations indicated that, to determine other important antioxidant and pro-oxidant factors simultaneously with α-tocopherol, gradient elution was inevitable.

Development of Elution Protocol—Peak Detection and Identification. The elution system was designed to achieve adequate separation of α-tocopherol, *β*-carotene, chlorophylls *a* and *b*, pheophytins *a* and *b*, and lutein within a reasonable time period. Figure 2 illustrates the separation of all standards detected at their maximum wavelength. The position of lutein was located after the analysis of the respective fraction of a spinach extract. To our knowledge this is the first time that separation of all tocopherol analogues, chlorophylls, pheophytins, *β*-carotene, and lutein, is reported. In the case of oil samples, multiwavelength detection by diode array was employed to check peak purity and resolution from the nearest eluting peak. Table 1 gives the k' values together with spectral characteristics for each analyte.

The retention factor k' values given in Table 1 were within the optimum range ($1 \leq k' \leq 10$) for satisfactory chromatographic elution with the exception of *β*-carotene (Snyder and Kirkland, 1979). Chlorophyll pigments eluted in the order pheophytin a' < pheophytin *a* < chlorophyll *a* < pheophytin *b* < chlorophyll *b* in agreement with previous reports (Watanabe et al., 1984; Canjura and Schwartz, 1991).

Fluorometric detection at 294 nm (excitation) and 330 nm (emission) was applied to clarify the position of

Table 1. Chromatographic and Spectral Characteristics of α-Tocopherol, Carotenoids, and Chlorophylls

		spectral data for the HPLC eluent					
		max wavelength, nm					peak height
analyte	k'^a	I	II	III	IV	V	ratio[b]
β-carotene	0		450	478			25
α-tocopherol	0.75	294					
pheophytin a' (A)	1.05	405	508	536	608	670	1.97
pheophytin *a*	2.05	408	506	536	608	670	1.84
peak B	3.10	410	500	540	618	672	1.88
chlorophyll *a*	3.70	430	578	617		660	1.20
pheophytin *b*	4.05	436	525	600		656	4.62
chlorophyll *b*	5.65	452	595	642			2.53
lutein	8.40	422	448	476			61

[a] Retention factor $(k') = (t - t_c)/t_c$, where t is the retention time of the pigment peak and t_c is the retention time of *β*-carotene. [b] Peak ratio I/V for chlorophylls and 100III/II for carotenoids (Davies, 1975).

tocopherols in the chromatogram of the oils. Interest was focused on α-tocopherol, the main tocopherol species of virgin olive oil. In the chromatograms of some oils at 294 nm the presence of an unknown peak (Figure 1, peak X) was observed. Its spectra, scanned with a diode array detector, was similar to that of α-tocopherol (λ_{max} = 294 nm), but no fluorescence response was recorded. A search was also made for the presence of ubiquinone species, the antioxidant activity of which is generally accepted. Coenzymes Q10 and Q9 at about 100 and 10 ppm level, respectively, have been found in olive oil (Pregnolato et al., 1994). In this study, coenzyme Q10 was eluted before α-tocopherol, as expected (Lang and Packer, 1987). Detection was at 294 nm. Identification of ubiquinone species in real samples was not always feasible due to coelution of compounds less polar than α-tocopherol. Hence, the analysis of Q10 in the oil samples was not further investigated.

The specificity of the method was studied for 30 virgin olive oil samples. All of the samples showed similar chromatographic profiles for tocopherols (Figure 3a) and pigments (Figure 3b). The major pigment constituents of all samples belonged to three classes of compounds, namely, carotenes, xanthophylls, and chlorophylls.

A peak that could be attributed either to other carotenes, for example, phytofluene and *ζ*-carotene (Garrido-Fernández et al., 1990a,b; Perrin, 1992), or to a *β*-carotene cis isomer (Pettersson and Jonsson, 1990) accompanied the *β*-carotene one. That peak was not separated well under the current chromatographic conditions. Thus, the two peaks were coquantified in a subsequent analysis.

Lutein eluted within 18 min under these experimental conditions. In all samples the peak of lutein was followed by another minor peak of a carotenoid that was not clearly identified.

The peak identified as pheophytin *a* was the main chlorophyll derivative present in the oils studied. Two other peaks (A and B), with absorption characteristics similar to that of pheophytin *a*, were also detected at 410 nm (Figure 3b; Table 1). Peak A was tentatively identified as pheophytin a', the C-10 epimer of pheophytin *a*. The chlorophyll group also included pheophytin *b* and in some cases chlorophyll *b*. Chlorophyll *a* was not detected in any oil sample. The results were in agreement with those presented in previous papers for chlorophyll measurement by HPLC (Rahmani and Csallany, 1991; Gandul-Rojas et al., 1996).

Quantification and Method Validation. Quantification of pheophytin a' (peak A) and of the unidentified

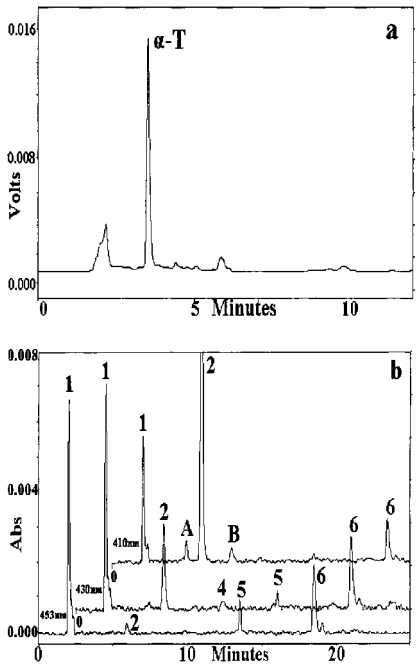

Figure 3. Typical HPLC profile of virgin olive oil (a) at 294 nm and (b) in the visible region (453, 430, 410 nm); peaks 1−6 are the same as in Figure 2; peak A, pheophytin *a*′; peak B, unidentified. Chromatographic conditions were as reported under Materials and Methods.

Table 2. Linearity and Detection Limit Data

analyte	range (ng/μL)	R^2	$y = a + bx^a$	detection limit (ng)
β-carotene	0.05−5	0.999	$y = 4187.3 + 175284x$	0.5
α-tocopherol	0.5−25	0.997	$y = -919.32 + 3469x$	10
chlorophyll *a*	0.6−10	0.979	$y = 8588.3 + 1729.5x$	2.5
chlorophyll *b*	0.6−10	0.999	$y = 1702.7 + 2994.7x$	2.5
pheophytin *a*	0.6−10	0.998	$y = -6562.9 + 3101.4x$	2.5
pheophytin *b*	0.6−10	0.987	$y = 5381.3 + 4472.6x$	2.5

[a] y = peak area, x = concentration (ng/μL).

chlorophyll derivative (peak B) was based on the calibration curve of pheophytin *a*. The concentrations of each standard working solution used for the construction of HPLC calibration curves were corrected on the basis of spectrometric data. Linearity for the standard curves within the ranges shown in Table 2 was very good, as indicated by correlation coefficients. Acceptable fit of the data to the regression line was seen in all cases.

Detection limits are also included in the same table. As detection limit was considered a signal-to-noise ratio of 3. The repeatability (CV%) of injection volume within the same day was 2.3% for α-tocopherol, 1.3% for β-carotene, and 4.6% for pheophytin *a*. The higher CV% value for the pheophytin *a* standard could be due to the lower injection volume (5 μL) used in this case. To determine measurement precision, an oil sample was injected six times and the CV for each analyte was calculated. The CV values were 5.0% for α-tocopherol, 7.2% for β-carotene, and 4.3% for pheophytin *a*. Precision data were considered satisfactory for the requirements of monitoring these components during oxidative stability studies.

To establish natural variability, the method was applied to 30 virgin olive oil samples. The concentration of α-tocopherol varied from 100 to 365 mg/kg, whereas two-thirds of the samples had a value ≥200 mg/kg. Tocopherol content of Greek origin olive oils seems to be higher compared with values published for Italian or Spanish oils (Ranalli and Angerosa, 1996; Cert et al., 1996). β-Carotene ranged from 0.3 to 4.4 mg/kg (mean value of 1.9 mg/kg), in agreement with values reported in the literature (Stancher et al., 1987; Zonta and Stancher, 1987; Rahmani and Csallany, 1991; Gandul-Rojas et al., 1996). The concentration of lutein varied from traces to 1.4 mg/kg (mean value of 0.7 mg/kg). Stancher et al. (1987) have reported similar levels for lutein content of commercial oils, whereas much higher values have been given for fresh Spanish virgin olive oils (Gandul-Rojas et al., 1996). Natural variability for pheophytin *a*, the main pigment of the samples examined, was wide, ranging from 3.3 to 34.0 mg/kg (mean value of 14.3 mg/kg). Pheophytin *a* content from traces up to 40 mg/kg has been reported. Only traces of pheophytin *b* and chlorophyll *b* were detected in one-third of the samples. Chlorophyll *a* was not found in any of the samples in accord with published data (Rahmani and Csallany, 1991; Gandul-Rojas et al., 1996). Because the oils under study were extracted from fruits of different cultivars and degrees of ripeness, the results may be considered indicative for the possible range of natural pigments in virgin olive oil. Moreover, at this stage the span of the levels of the various pigments due to olive fruit ripeness was examined for Koroneiki, the main Greek cultivar. Oils from green and black fruits were obtained using a two-phase and a three-phase centrifugal system. Malaxation of olive paste was carried out at 30 and 45 °C. The results are given in Table 3. No chlorophyll *a* was detected, even in samples obtained from green olives just after collection. It is worth mentioning that chlorophyll *a* also did not occur in eight of the nine Spanish varieties examined by Gandul-Rojas et al. (1996). Pigment content decreased with degree of ripeness. For example, the concentration of pheophytin *a* was almost double in oils produced in the beginning of the harvest compared with the levels found in oils at the end of the season. Natural variability studies for both sets of samples indicated that tocopherol and pigment levels were within the linear response ranges presented in Table 2.

Apparent chlorophyll content was almost 2-fold the concentration of pheophytin *a* measured by HPLC. Apparent chlorophyll content correlated significantly with the pheophytin *a* content measured by HPLC ($R^2 = 0.90$). In contrast, other authors (Ward et al., 1994) have reported higher values for HPLC measurement of pheophytin *a* compared with its spectrometric determination according to a different procedure (AOCS, 1989). Thus, it can be concluded that an objective measurement of chlorophylls is necessary. Our opinion is supported by Perrin (1992), who stated that "in order to evaluate the photo-oxidative stability of virgin olive oil there is a need to consider the concentration of each chlorophyll pigment and not only their total level". HPLC offers a reliable alternative, as was confirmed in the present study.

Application of HPLC Methodology To Monitor the Effect of Tocopherols and Pigments on Virgin Olive Oil Oxidation. Extra virgin olive oil samples were selected for oxidative stability studies on the basis of similarities in their fatty acid compositions and differences in phenol and apparent chlorophyll contents (Table 4). The induction periods estimated for the four samples indicated that phenol content could not be the only factor affecting the stability of the oil samples. The contents of α-tocopherol, β-carotene, lutein, and total

Table 3. Pigment Content of Virgin Olive Oils Obtained from Olives of Different Degrees of Ripeness (Milligrams of Pigment per Kilogram of Oil)

sample, degree of ripeness[a,b]/ extraction system	β-carotene	lutein	Chl *b*[c]	Pheo *a*	Pheo *a*′ [c]	peak B	Pheo *b*
green olives[a]/two-phase, 30 °C	4.0	1.3	0.9	21.6	2.3	2.7	0.4
green olives[a]/two-phase, 45 °C	4.7	1.9	2.3	29.7	7.1	2.8	0.4
green olives[a]/three-phase, 30 °C	3.6	1.3	0.4	18.2	2.4	2.8	0.1
green olives[a]/three-phase, 45 °C	4.4	1.7	1.2	23.6	2.7	1.6	0.2
black olives[b]/two-phase, 30 °C	2.1	1.3	0.4	14.5	2.3	2.3	
black olives[b]/tw0-phase, 45 °C	2.3	1.6	1.3	16.8	2.1	1.9	
black olives[b]/three-phase, 30 °C	2.2	1.2	0.2	13.8	2.3	2.3	
black olives[b]/three-phase, 45 °C	2.3	1.6	1.1	18.4	2.2	2.1	

[a] November 1996, green olives, oil content 18%. [b] January 1997, black olives, oil content 21.3% (A. Koutsaftakis, Institute of Subtropical Plants and Olive Tree, personal communication, 1996). [c] Chl *a* was not detected; **peak A.

Table 4. Quality Characteristics, α-Tocopherol, Apparent Chlorophyll, and Pigment Content of Virgin Olive Oil Samples Used in Oxidative Stability Studies

	sample			
quality characteristic	1	2	3	4
peroxide value (mequiv of O_2/kg of oil)	9.8	10.5	13.3	13.7
K_{232}[a]	1.95	1.20	2.09	2.55
acidity (% oleic acid)	0.44	0.34	0.44	0.39
total phenols (mg/kg, caffeic acid)	190	339	78	132
induction period (h)	10.6	14.7	13.8	5.5
"apparent" chlorophylls (mg/kg, Pheo *a*)	22.5	29.5	43.4	15.0
α-tocopherol (mg/kg)	233	184	265	284
β-carotene (mg/kg)	1.3	1.3	2.5	1.0
lutein (mg/kg)	1.0	0.5	1.9	0.8
total pheophytin *a* (mg/kg)	15.0	19.1	24.5	12.1
fatty acid methyl esters (% peak area)				
16:0	13.2	11.5	12.2	14.3
16:1	0.8	0.7	1.1	1.2
18:0	1.2	1.4	1.2	1.1
18:1	77.6	79.3	78.1	71.9
18:2	6.8	6.6	6.8	10.8

[a] K_{232} = absorbance at 232 nm.

pheophytin *a* as measured by HPLC differed from sample to sample (Table 4). These differences could be partially responsible for the unexpected high stability of sample 3 compared to the stability of sample 2. The oil samples were exposed to different storage conditions. Measurements for the samples stored in the dark were taken after 12 and 18 months. The respective measurements for changes during light exposure were taken after 3 months. The alterations in the lipid substrate and the changes in α-tocopherol and pigment level, expressed as percentage retention, are shown in Table 5.

A significant decrease was observed in α-tocopherol content during autoxidation for all samples (Table 5). This decrease was accompanied by a concomitant increase in the two earlier eluted peaks attributed to the formation of more polar oxidation products as illustrated by chromatograms b and c in Figure 4 for sample 3. Similarly, α-tocopherol decrease under diffused light was followed by a parallel increase of the same peaks, though in a different way (Figure 4d). As exemplified for samples 1 and 2 in Table 5, for similar peroxide values, the degradation of α-tocopherol was more rapid during photo-oxidation.

β-Carotene and lutein content retentions after 12 months of storage in the dark were similar for all samples. Variation in their retention was observed after 18 months of storage (Table 5), probably due to differences in the initial level of phenols and α-tocopherol of the samples. β-Carotene and lutein contents remained almost unchanged during storage under diffused light for 3 months (Table 5). This could be attributed to the protective effect of α-tocopherol, as suggested by its above-mentioned dramatic reduction. An analogous behavior has been reported for γ-tocopherol in the

Table 5. Alterations in the Lipid Substrate and Changes (Percentage of Retention) in α-Tocopherol and Pigment Content (Milligrams per Kilogram) of Oil Samples Stored in the Dark or under Diffused Light at Room Temperature

	sample			
	1	2	3	4
peroxide value[a]				
t = 12 months, dark	25.5	20.9	40.1	59.2
t = 18 months, dark	30.9	23.7	49.4	73.3
t = 3 months, light	20.9	21.9	18.7	24.9
K_{232}				
t = 12 months, dark	3.45	2.88	4.88	5.62
t = 18 months, dark	4.00	3.37	5.98	7.54
t = 3 months, light	1.96	1.97	2.18	2.86
α-tocopherol				
t = 12 months, dark	78.1	93.0	64.5	43.7
t = 18 months, dark	44.6	79.4	38.5	34.9
t = 3 months, light	48.5	49.0	44.5	54.9
β-carotene				
t = 12 months, dark	77.0	77.0	72.0	70.0
t = 18 months, dark	53.9	77.0	52.0	60.0
t = 3 months, light	100.0	92.3	76.0	100.0
lutein				
t = 12 months, dark	80.0	80.0	73.7	62.5
t = 18 months, dark	0.0	80.0	42.1	25.0
t = 3 months, light	80.0	100.0	79.0	87.5
total pheophytin *a*[b]				
t = 12 months, dark	86.0	97.4	82.9	90.9
t = 18 months, dark	73.3	94.8	46.5	64.5
t = 3 months, light	30.0	31.4	24.9	26.5

[a] mequiv of O_2/kg of oil. [b] Total pheophytin *a** = the sum of Pheo *a*, Pheo *a*′, and peak B content.

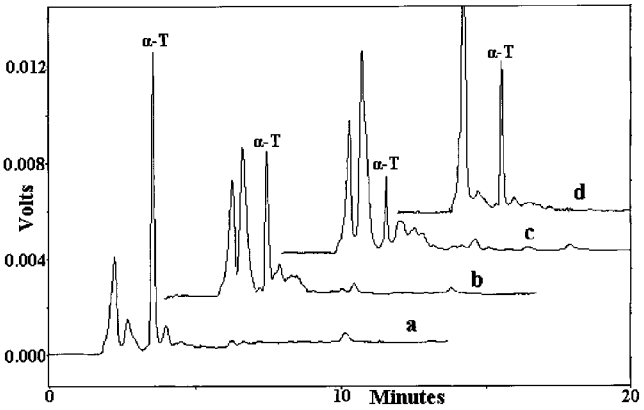

Figure 4. Changes in the chromatographic profile at 294 nm for sample 3 during storage: (a) $t = 0$; (b) $t = 12$ months in the dark; (c) $t = 18$ months in the dark; (d) $t = 3$ months under diffused light. Chromatographic conditions were as reported under Materials and Methods.

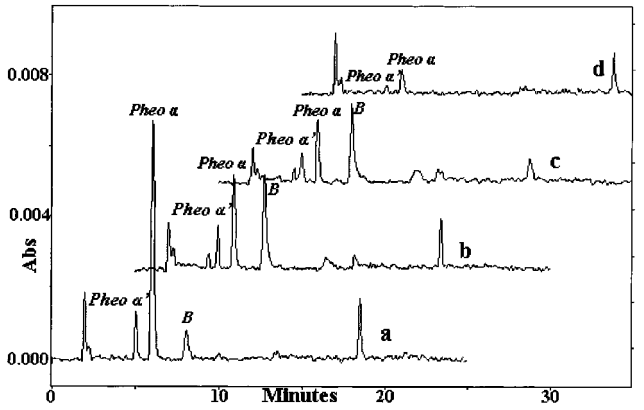

Figure 5. Changes in the chromatographic profile at 410 nm for sample 3 during storage: (a) $t = 0$; (b) $t = 12$ months in the dark; (c) $t = 18$ months in the dark; (d) $t = 3$ months under diffused light. Chromatographic conditions were as reported under Materials and Methods.

presence of β-carotene during rapeseed photo-oxidation (Haila and Heinonen, 1994).

The apparent chlorophyll content for all oil samples stored in the dark showed less than a 10% decrease. This slight decrease did not reflect the interconversion of chlorophylls as monitored by HPLC (Figure 5b,c). As shown in Figure 5c, the size of the pheophytin *a* peak became almost half after storage for 18 months, whereas the pheophytin *a′* peak and peak B increased considerably. The increase of peak B is an extra piece of evidence that this component is chemically related to pheophytin *a*. Such details on the fate of chlorophylls in the dark were not detectable in studies when absorbance measurements were used for their determination (Endo et al., 1985; Gutiérrez-Rosales et al., 1992). The effect of diffused light was destructive for total pheophytin *a* (Table 5; Figure 5d) after 3 months of storage, whereas the respective changes to the lipid substrate, expressed as peroxide values and K_{232}, were not so significant. Among pigments, pheophytins were more severely photodegraded. This should be attributed to their widely accepted role as photosensitizers.

The application of the method to samples stored under different conditions showed that this HPLC procedure could be used to monitor the fate of antioxidant and prooxidant factors during virgin olive oil oxidation.

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