# Chlorophyll and β-Carotene Pigments in Moroccan Virgin Olive Oils Measured by High-Performance Liquid Chromatography

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**Chlorophyll and β-carotene concentrations were determined by high-performance liquid chromatography (HPLC) in virgin olive oils, which were press-extracted from green and semi-black olives. Pheophytin A was found to be the major chlorophyll isomer in all oil samples. The occurrence of this pigment at higher concentrations in oil extracted from green olives is a possible indication of its time-related destruction during olive ripening. Some evidence for the *in vivo* existence of pheophytin A is also presented. Beta-carotene concentration in oils was found to decrease during olive ripening.**

KEY WORDS: β-carotene, chlorophylls, chlorophyll degradation, chlorophyll pigments, Morocco, olive, olive oil, oxidative degradation.

The chlorophylls (CHL) A and B and their oxidation products, pheophytins (PHY) A and B, are naturally occurring in vegetable oils and are responsible for the greenish color of these oils. Except for virgin olive oil where a greenish color is tolerated, an excessive amount of chlorophyll (> 20 μg/g) is considered undesirable as it is difficult to remove by conventional bleaching processes (1). Chlorophyll pigments are known to act as photosensitizers of edible oils, accompanying the production of singlet oxygen. However, β-carotene, which also occurs in vegetable oils, acts as a singlet oxygen quencher (2,3). The quantity of chlorophyll and β-carotene pigments in virgin olive oil depends on a number of factors such as: the variety, the degree of maturity of the olives, method of oil extraction, and several other biological and technical factors (4). In virgin olive oils produced from mature olives, the concentrations of β-carotene have been reported to vary from 0.33 μg/g to 3.69 μg/g; whereas, those of chlorophylls and pheophytins range, respectively from 1.0 to 10.0 μg/g and from 0.2 to 24.0 μg/g (5). Among the chlorophyll pigments, PHY A was found predominantly in virgin olive oils as well as in other edible plant oils (4,6,7). The question whether pheophytins are oxidation products of chlorophylls formed during extraction of the oil (6) or preexist already in oily seeds and fruits, as suggested by Usuki *et al.* (7,8), is not well established.

In the present study, chlorophyll and β-carotene concentrations were determined by high-performance liquid chromatography (HPLC) in Moroccan virgin olive oils that were press-extracted from green and semi-black olives. Some evidence supporting the *in vivo* existence of PHY A is presented.

## MATERIALS AND METHODS

*Olive samples.* Green and semi-black olives (variety *Picholine Marocaine*) were hand-picked from the three main olive-producing areas in Morocco. Within each area and in a radius of about 37 miles, green olives (age about 6 mon) were sampled from three different locations. Semi-black olives (age about 7 mon) were sampled from the same locations and designated with the same sample numbers (S1,S2,S3).

Virgin olive oils were press-extracted with a hydraulic press (Libiu Druge, Brunaix, France) at a gauge pressure of 200 kg/cm$^2$. The chlorophyll and β-carotene analyses were carried out within three months after extraction on oils that had been stored under nitrogen, in the dark, at 2°C.

*Extraction of chlorophyll pigments from olive paste.* The procedure described by Holden (9) was used for the extraction of chlorophyll pigments from olive paste. Ten grams of pulp from green olives were blended for 2 min with 100 mL of the solvent mixture of acetone/hexane (90:10, v/v) in the presence of 2 g $CaCO_3$ to prevent pheophytin formation during pulp reduction (10). The resulting paste was shaken with the solvent mixture for 24 hr in darkness at 2°C then filtered over anhydrous sodium sulfate. The filtrate was transferred to a 250-mL volumetric flask, and the solvent mixture was evaporated under a stream of nitrogen at room temperature. The residue then was made up to 250 mL with hexane/isopropyl alcohol (98.5:1.5, v/v), and aliquots were injected onto the HPLC column. The visible spectrum of the residue was measured in the mobile phase by HPLC with a Varian model 165 spectrophotometer (Varian Analytical Instruments, Walnut Creek, CA).

*Chlorophyll and β-carotene standards.* Chlorophyll A and B (CHL A and B), and β-carotene standards were purchased from Sigma Chemical Co., St. Louis, MO. Pheophytins A and B were not available commercially and were prepared from the corresponding chlorophylls by the method of Schwartz and Von Elbe (11). In this method, ether solutions of CHL A and B were acidified with 13% HCl. The acid was removed by washing the ether layer twice with an equal volume of 5% $Na_2SO_4$. The pheophytin/ether mixture was dried over anhydrous $Na_2SP_4$ and evaporated under a stream of nitrogen. The prepared pheophytins were shown by HPLC to be chromatographically pure, and their purity was further determined by comparing their visible spectra and molar extinction coefficients to those of pure pheophytins reported in the literature (12,13).

*HPLC analysis.* The system consisted of an Altex isocratic pump, model 110 A and Altex model 210 injector equipped with a 20 μL loop (Beckman Instruments, Berkeley, CA). The detector was an ISCO V4 ultraviolet (UV)-visible variable wavelength detector (ISCO Inc., Lincoln, NE). Detector sensitivity was set at 0.05 absorbance unit full scale. The chlorophyll and β-carotene pigments were separated on a 3.9 mm × 30 cm μ-Porasil 10μ column (Waters Associates, Milford, MA) with a mobile phase of 1.5% isopropyl alcohol in hexane at 0.8 mL/min according to the published method of Rahmani and Csallany (14). The hexane and isopropyl alcohol were HPLC-grade solvents (Riedel, Salze, Germany). All samples were injected via a fully loaded 20-μL loop.

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TABLE 1

**Free Acidity, Saponification Value, Iodine Value, Refractive Index, and Concentrations of Chlorophylls, Pheophytins, and β-Carotene in Virgin Olive Oils Extracted from Green Olives[a]**

| Samples | Free acidity (%) | Saponification value | Iodine value | Refractive index ($n_D^{20}$) | Chlorophylls (μg/g) A | Chlorophylls (μg/g) B | Pheophytins (μg/g) A | Pheophytins (μg/g) B | β-carotene μg/g |
|---|---|---|---|---|---|---|---|---|---|
| S1 | 0.19 | 196.4 | 63.4 | 1.467 | ND[b] | ND | 26.28 | ND | 4.06 |
| S2 | 0.22 | 197.8 | 76.1 | 1.466 | ND | ND | 37.17 | 1.84 | 7.70 |
| S3 | 0.19 | 196.4 | 87.3 | 1.466 | 2.78 | ND | 45.45 | 5.91 | 6.69 |
| S1 | 0.17 | 187.6 | 77.4 | 1.466 | 2.93 | ND | 47.88 | 1.72 | 5.36 |
| S2 | 0.19 | 188.0 | 70.2 | 1.466 | ND | ND | 46.12 | ND | 5.16 |
| S3 | 0.19 | 191.0 | 80.4 | 1.467 | traces | ND | 34.01 | ND | 4.64 |
| S1 | 0.13 | 195.0 | 74.4 | 1.470 | ND | ND | 7.43[c] | ND | 1.41[c] |
| S2 | 0.16 | 196.6 | 84.6 | 1.469 | ND | ND | 21.60 | traces | 3.92 |
| S3 | 0.11 | 186.5 | 81.6 | 1.467 | ND | ND | 27.33 | ND | 4.08 |
| Average ± S.D.[d] | 0.17 ± 0.03 | 192.8 ± 4.5 | 77.3 ± 7.4 | 1.467 ± 0.001 | ND—2.86 ± 0.11 | ND | 35.73 ± 10.10 | ND—3.16 ± 2.39 | 5.20 ± 1.36 |

[a]Averages of duplicate determinations.
[b]ND = not detected, minimum detection limit per 20 μL injections = 0.5 ng.
[c]Values excluded from average calculations.
[d]S.D. = standard deviation.

TABLE 2

**Free Acidity, Saponification Value, Iodine Value, Refractive Index, and Concentrations of Chlorophylls, Pheophytins, and β-Carotene in Virgin Olive Oils Extracted from Semi-Black Olives[a]**

| Samples | Free acidity (%) | Saponification value | Iodine value | Refractive index ($n_D^{20}$) | Chlorophylls (μg/g) A | Chlorophylls (μg/g) B | Pheophytins (μg/g) A | Pheophytins (μg/g) B | β-carotene μg/g |
|---|---|---|---|---|---|---|---|---|---|
| S1 | 0.12 | 196.4 | 76.1 | 1.470 | ND[b] | ND | 6.73 | ND | 1.08 |
| S2 | 0.19 | 188.0 | 84.7 | 1.468 | ND | ND | 3.26 | ND | 0.37 |
| S3 | 0.63 | 191.0 | 88.4 | 1.469 | ND | ND | 12.86 | ND | 0.85 |
| S1 | 0.11 | 186.5 | 83.7 | 1.470 | ND | ND | 7.67 | ND | 0.97 |
| S2 | 0.18 | 186.5 | 85.8 | 1.468 | ND | ND | 9.41 | ND | 1.84 |
| S3 | 0.12 | 188.0 | 88.9 | 1.468 | ND | ND | 5.71 | ND | 1.27 |
| S1 | 0.15 | 189.4 | 95.2 | 1.475 | ND | ND | 3.98 | ND | 0.75 |
| S2 | 0.20 | 192.2 | 85.4 | 1.470 | ND | ND | 4.02 | ND | 0.94 |
| S3 | 0.11 | 183.7 | 84.4 | 1.469 | ND | ND | 6.28 | ND | 0.91 |
| Average ± S.D.[c] | 0.20 ± 0.16 | 189.1 ± 3.7 | 85.8 ± 5.1 | 1.470 ± 0.002 | ND | ND | 6.66 ± 3.04 | ND | 1.00 ± 0.40 |

[a]Averages of duplicate determinations.
[b]ND = not detected, minimum detection limit per 20 μL injections = 0.5 ng.
[c]S.D. = standard deviation.

Quantitative measurements were made at 430 nm for CHL A, 452 nm for CHL B and β-carotene, 409 nm for PHY A, and 433 nm for PHY B. These wavelengths correspond with absorption maxima of each pigment in the mobile phase.

*Physico-chemical properties of oils.* Refractive indexes ($n_D^{20}$) of oil samples were measured at 20°C in an Abbe refractometer, model A (Carl Zeiss, Oberkochen, Germany). Before analysis, the refractometer was standardized with distilled water ($n_D^{20}$ = 1.3330) and benzene ($n_D^{20}$ = 1.5014). The iodine value (IV) (15), the saponification value (SV) (16), and the percent of free acidity (PFA) (17) were determined by the official AOCS methods and expressed in terms of oleic acid. Determinations of pH were made on olive paste from green and semi-black olives by direct immersion with a Metrohm Herisau (Herisau, Switzerland) pH meter model E603.

## RESULTS AND DISCUSSION

Concentrations of chlorophylls A and B, pheophytins A and B, β-carotene pigments, free acidity, saponification and iodine values, and refractive index measurements of oils from green and semi-black olives are compiled in Tables 1 and 2. The measured characteristics of Moroccan virgin olive oils are similar to the information reported

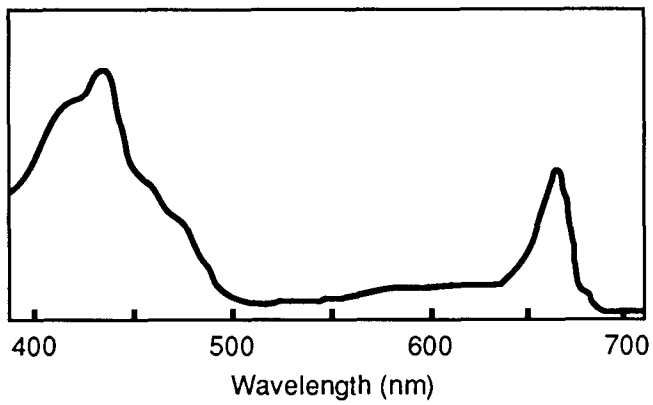


**FIG. 1. Visible spectrum of acetone/hexane (90:10, v/v)-extracted "green olive paste pigments" in acetone.**

in the literature for virgin olive oils from other countries (18). According to the analysis of variance, the only strong positive correlation coefficients ($p < 0.05$) observed were for iodine values and saponification values versus the degree of ripening of the olives.

Pheophytin A was the predominant chlorophyll pigment in all analyzed oil samples (Tables 1 and 2), but the concentrations of PHY A varied greatly from 7.43 to 47.88 μg/g in oils extracted from green olives and from 0.75 to 13.06 μg/g in oils extracted from semi-black olives. Pheophytin A has been reported previously as the major chlorophyll isomer in olive oils (4,6) and in refined edible oils (7,8). Pheophytin B and CHL A were detected only in oils extracted from green olives at amounts up to 5.91 μg/g and 2.93 μg/g, respectively. Chlorophyll B was not found in any of the analyzed oil samples (Tables 1 and 2). The lower limit of adequate detection under the HPLC settings described was 0.5 ng per injection.

The visible spectrum of chlorophyll pigments extracted from the green olive paste was examined in acetone and is shown in Figure 1. The characteristic visible absorption bands were at 410, 430 and 663 nm; these absorption bands correspond to CHL A, the major component of the pigment mixture.

Chlorophyll A, PHY A, and CHL B were measured in the paste from green olives by HPLC. Pheophytin B was not detected in the olive paste samples. The mean relative percent compositions (w/w) of the three pigments were: 64.3% for CHL A, 15.1% for PHY A, and 20.6% for CHL B. These results suggest that PHY A in virgin olive oil is possibly not an effect of oxidation but does preexist *in vivo* where it could be produced from CHL A enzymatically or non-enzymatically. The relatively low pH measured in the olive paste (4.95 for green olives and 5.01 for black olives) might favor a partial "pheophytinization" *in vivo*.

According to the HPLC analysis of green olive paste, at this ripening stage of the olive, CHL A has a relatively high concentration followed by a much lower level of CHL B. The fact that oils extracted in the laboratory from green olives had practically no CHL A and B, but had PHY A and some PHY B shows the instability of chlorophylls during oil extraction. Pheophytin A was found *in vivo* in the green olive paste by HPLC, and this pigment was found in both oils from green and semi-black olives. The concentration of PHY A was five to six times higher in oils from green olives than in oils from semi-black olives (Tables 1 and 2). This difference indicates a possible time-related destruction of the pigment during olive ripening. It seems possible that part of CHL A *in vivo* converts to PHY A, and part of CHL A also degrades to PHY A during oil extraction. The same possibility is true for CHL B. It can also be concluded that during ripening less CHL A is produced; therefore, PHY A production is lowered.

The concentrations of β-carotene ranged from 1.41 to 7.70 μg/g in oils extracted from green olives and from 0.37 to 2.34 μg/g in oils extracted from semi-black olives. A significant difference ($p < 0.05$) was found between the β-carotene content of the oils and the degree of olive ripening. The oil samples extracted from green olives had higher β-carotene content than oil samples from semi-black olives. Similar results were reported in the literature by Vitagliano (19).

In conclusion, the relative concentration of chlorophyll pigments seems to relate to the stage of ripening of the olive. The concentrations of these pigments in the oil, however, are lowered depending on the extraction of the oil.

Since total chlorophyll content has been found responsible for the oxidative stability of edible oils (7,8,20), producing virgin olive oils from mature olives is suggested to minimize the total chlorophyll content and to ensure a greater oxidative stability of the oil.

## ACKNOWLEDGMENTS

Published as paper No. 18,161 of the contribution series of the Minnesota Agricultural Experiment Station on research conducted under Project No. 18-084, supported by Hatch funds, and by the U.S. Agency for International Development, Contract No. 608-0160, IAV, Morocco Project and a grant (E/1010-1) from the International Foundation for Science.

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[Received June 14, 1990; accepted June 5, 1991]