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# Determination of chlorophylls and carotenoids by high-performance liquid chromatography during olive lactic fermentation

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ABSTRACT

Eighteen pigments, including chlorophylls, carotenoids and their degradation products, were separated by reversed-phase ion-pair high-performance liquid chromatography during the lactic fermentation and later preservation phase of green table olives. The method consists of an elution gradient using two solvents: water–ion-pair reagent–methanol (1:1:8, v/v/v) and methanol–acetone (1:1, v/v). Absorbance detection of all the pigments is carried out spectrophotometrically at 430 nm. Pigment concentrations are calculated from an extension of Beer's law. This procedure is compared with the external standard method. The analysis of variance showed no significant differences between the results given by the two methods.

## INTRODUCTION

The use of olive fruit as table olives dates back to the first century A.D. or earlier [1]. In 1930 Cruess was the first to do some research in their elaboration [2] and his work was continued by Vaughn (1943) [1]. In 1947, De la Borbolla *et al.* [2] began research into the fermentation of green olives, succeeding in 1956 in converting the olive elaboration into a truly technological process, subject to physico-chemical and microbiological control standards.

The traditional process of preparation for this type of olives involves treatment with 2% sodium hydroxide solution for 6 h, washing with water for 8 h, and subsequent conditioning of the fruit in brine (10% sodium chloride solution). The sugars, vitamins and amino acids of the fruit pass to the brine by osmotic process, converting it gradually into a suitable medium for microorganism growth, where the fruits undergo a total lactic fermentation. The complete process of fermentation and curing lasts *ca.* 6–7 months, at the end of which period the fruit should have certain organoleptic characteristics [1]. Recent innovations in the traditional process of elaboration of green table olives, to minimize the volume of waste water, have affected the physico-chemical and organoleptic characteristics of the finished product, although this has still not been fully studied [3]. One of the more seriously affected characteristics is that of the colour of the fruit. As colour is an important attribute of quality, special attention has been given for some years to research into the components responsible by means of the qualitative and quantitative evaluation of chlorophylls and carotenoids during the traditional fermentation process of the olive, with the aim of establishing which variables take part in pigment transformation, and attempting to direct their action without giving up the necessary modifications of the process.

Analysis of pigments in olives has proved more

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TABLE I

CHROMATOGRAPHIC AND SPECTROSCOPIC CHARACTERISTICS OF STANDARDS

The pigments were purified by TLC on silica gel 60 $GF_{254}$ using the following eluents: A = ligh petroleum (b.p. 40–60°C); B = dichloromethane–ethyl acetate (4:1) [28]; C = benzene–ethanol (22:1) [25]; D = benzene–acetone (4:1) [28]; E = light petroleum (b.p. 65–95°C)–acetone–diethylamine (10:4:1) [6]; F = light petroleum (65–95°C)–acetone–pyridine (10:4:2.5) [22]; G = hexane–pyridine–diethylamine (10:1:0.5). Peak ratio is % III/II for carotenoids [27] and Soret band absorbance divided by the maximum absorbance in the red region for chlorophylls and derivatives [8].

| Pigment | Purification by TLC | | Spectral data | | | | | | | | IR | | Epoxide test (HCl treatment) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Eluent | $R_F$ values | Light petroleum | | | | Chloroform | | | | OH | C=O | Colour on TLC | Hypsochromic shift ($C_2H_5OH$) (nm) |
| | | | Maxima (nm) | | | Peak ratio | Maxima (nm) | | | Peak ratio | | | | |
| | | | I | II | III | | I | II | III | | | | | |
| *Carotenoids* | | | | | | | | | | | | | | |
| β-Carotene | A | 0.24 | (426) | 444 | 470 | 30 | (434) | 458 | 482 | 7 | – | – | Yellow | 0 |
| Phytofluene | A | 0.12 | 330 | 348 | 367 | 81 | | | | | – | – | Yellow | 0 |
| ζ-Carotene | A | 0.01 | 378 | 400 | 422 | 80 | 384 | 405 | 430 | | – | – | Yellow | 0 |
| Lutein | B | 0.35 | 418 | 442 | 470 | 79 | 430 | 452 | 482 | 71 | + | – | Brown and green | 0 |
| Lutein isomer | B | 0.32 | 416 | 438 | 466 | 58 | | 452 | 480 | | + | – | Brown and green | 0 |
| Mutatoxanthin | B | 0.25 | (402) | 424 | 448 | 31 | 410 | 434 | 460 | 10 | + | – | Blue green | 0 |
| Auroxanthin | B | 0.12 | (378) | 400 | 424 | 109 | 388 | 410 | 436 | 103 | + | – | Blue | 0 |
| Luteoxanthin | C | 0.05 | 402 | 426 | 448 | 107 | 404 | 428 | 456 | 101 | + | – | Blue | 20 |
| Violaxanthin | D | 0.31 | 414 | 436 | 466 | 96 | 422 | 446 | 476 | 93 | + | – | Blue | 40 |
| Neochrome | D | 0.18 | 399 | 418 | 446 | 76 | 402 | 426 | 454 | 65 | + | – | Blue green | 0 |
| Neoxanthin | D | 0.13 | 412 | 436 | 466 | 86 | 420 | 444 | 474 | 82 | + | – | Blue green | 14 |
| | | | Acetone | | | | | | | | | | Colour on TLC (white light) | |
| | | | Maxima (nm) | | | Peak ratio | | | | | | | | |
| | | | I | II | III | | | | | | | | | |
| *Chlorophylls and derivatives* | | | | | | | | | | | | | | |
| Chlorophyll *a* | E | 0.51 | 428 | 616 | 662 | 1.4 | | | | | | | Blue-green | |
| Chlorophyll *b* | E | 0.44 | 454 | 596 | 644 | 3.0 | | | | | | | Yellow-green | |
| Pheophytin *a* | E | 0.57 | 410 | 468 | 668 | 2.4 | | | | | | | Grey | |
| Pheophytin *b* | E | 0.53 | 432 | 522 | 656 | 5.1 | | | | | | | Brown | |
| Chlorophyllide *a* | E | 0.00 | 428 | 616 | 652 | 1.5 | | | | | | | Blue-green | |
| Chlorophyllide *b* | E | 0.00 | 454 | 596 | 644 | 3.1 | | | | | | | Yellow-green | |
| Pheophorbide *a* | F | 0.45 | 410 | 468 | 668 | 3.1 | | | | | | | Grey | |
| Pheophorbide *b* | F | 0.34 | 432 | 522 | 656 | 8.0 | | | | | | | Brown | |
| Pyropheophytin *a* | G | 0.45 | 410 | 468 | 668 | 2.4 | | | | | | | Grey | |
| Pyropheophytin *b* | G | 0.23 | 432 | 522 | 656 | 5.5 | | | | | | | Brown | |

difficult than for other fruits or vegetables. The high lipid content of the olive (15–30%) [4] was a serious obstacle in isolating these liposoluble pigments even by thin-layer chromatography (TLC) and prior attainment of a fat-free pigment extract was necessary [5]. Mínguez-Mosquera *et al.* [6] found that the process of lactic fermentation in olives involves only the transformation of pigments, without their loss or destruction. Recently, they also found that the total quantification of carotenoids, chlorophylls and chlorophyll derivatives from the absorption spectrum of the crude extracts of pigments was correlated with a subjective classification of the fruit by colour, the classification decreasing as the pigment concentration increases [7]. However, for monitoring the individual pigments changes during the fermentation process, the use of high-performance liquid chromatography (HPLC) might be more appropriate, offering significant advantages over TLC, including speed, automatic detection and lower detection limits [8]. Since 1975, numerous studies have been made on the application of HPLC to the determination of chlorophyllic and carotenoid pigments in vegetable tissues. It seems that reversed-phase columns, mainly $C_{18}$ [8–14], offer more advantages than normal-phase columns [15–17]. Thus, pigment degradation and long conditioning times are two drawbacks which have been cited when using silica as HPLC packing material [8,13].

In previous work, using the fat-free pigment extract, reversed-phase HPLC was used successfully for the separation of chlorophylls and carotenoids although only in fresh green olives [18]. In this work, reversed-phase ion-pair HPLC was used for the qualitative and quantitative control of the individual pigment changes throughout the fermentative process and later conservation of green table olives, Spanish style, achieving a satisfactory separation even for the acidic pigment (chlorophyllides and pheophorbides).

## EXPERIMENTAL

### *Samples*

The study was carried out on olives of the Hojiblanca variety, *Olea europaea arolensis*. The fruits were picked from the tree when green-yellowish, and processed using the traditional method of Spanish-style fermentation in brine [1]. The pigments were monitored at the following stages: (a) fresh fruit, (b) at the beginning of fermentation phase, (c) at the end of fermentation phase and (d) in the subsequent brine conservation of the fruit prior to packing.

### *Preparation of extract free of fatty material*

Samples were made from a triturate homogenized from 100 de-stoned fruits, (*ca.* 500 g), by accurately weighing 5–15-g duplicates for each analysis according to the number of days of fermentation. The pigment extraction was made with N,N-dimethylformamide. The filtrates were next treated with hexane in a decantation funnel in order to extract and separate the characteristic fatty olive matter from the previous solution. The hexane phase in turn carried over the carotene fraction, while that corresponding to N,N-dimethylformamide retained chlorophylls, chlorophyll derivatives and the remaining carotenoids. The extraction processes have been described previously [5].

### *Standards*

The reference samples of chlorophyll *a* and *b* were supplied by Sigma (St. Louis, MO, USA). Pheophytin *a* and *b* were obtained by acidification with 13% (v/v) hydrochloric acid of the respective chlorophyll solutions [19]. Chlorophyllide *a* and *b* were prepared by enzymic de-esterification of the respective chlorophylls following the method proposed by Jones *et al.* [20]. Pheophorbides *a* and *b* were then obtained from their respective chlorophyllides by acidification [21]. Pyropheophytins *a* and *b* were prepared by refluxing pheophytins in collidine [10]. Chlorophyll and pheophytin C-10 epimers were prepared by treatment with chloroform according to Watanabe *et al.* [21]. All standards were purified by thin-layer rechromatography on silica gel $GF_{254}$ (20 × 20 cm plates, thickness 0.7 mm) (Merck, Darmstadt, Germany) using different eluents (Table I).

The carotenoid standards were obtained from fresh and elaborated olives whose pigment composition has been studied in detail and identified previously [5,22–24]. The starting point was a pigment extract in acetone obtained by the traditional method of Smith and Benítez [26]. This extract was saponified with methanolic potassium hydroxide solution (20%) for 1 h at room temperature to purify

it of lipids and chlorophylls [24]. Although some carotenoid are sensitive to alkaline media [27], none has been identified in the olive. Hence saponification is a sound method for carotenoid purification. The reference samples were accumulated from separations by TLC as described above. The first separation was carried out using light petroleum (b.p. 65–95°C)–acetone–diethylamine (10:4:1) as eluent. Once a sufficient amount of each of the carotenoids has been obtained it was purified by TLC, using different eluents (specified in Table I). To confirm the identification of all the pigments, the absorption spectra in acetone for the chlorophyllic pigments and in light petroleum and chloroform for the carotenoids were compared with those published in the literature [26–28]. The colour in TLC after spraying with hydrochloric acid and the hypsochromic change in the absorption spectrum in ethanol after acidifying with hydrochloric acid were used to identify the 5,6-epoxycarotenoids [29]. The presence of hydroxyl groups was confirmed by rechromatography after acetylation [30] and by the IR spectrum [28].

*Column liquid chromatography*

The pigment extract (20 $\mu$l), previously filtered through a nylon membrane of 0.45 $\mu$m (Micron Separations, Westboro, MA, USA) was injected into a liquid chromatograph (Perkin-Elmer, Series 4) equipped with a Rheodyne Model 7125 injector valve. Separations were carried out on a 25 cm × 4 mm I.D. column filled with Spherisorb ODS-2, 5-$\mu$m particle size (Supelco, Bellefonte, PA, USA). A short column (5 cm × 4 mm I.D.) of Pelliguard LC-18, 40 $\mu$m (Supelco), was placed immediately before the main column.

The solvents used as the mobile phase were proposed by Mantoura and Llewellyn [13], but in this work their proportions and the type of gradient were modified to obtain an adequate detection (sharper peaks) of pyropheophytins and to improve the separation between carotenoids and pheophorbides. The eluents used were the following: eluent A, water–solution P–methanol (1:1:8, v/v/v) and eluent B, acetone–methanol (1:1, v/v). Solution P (ion-pair reagent) is tetrabutylammonium acetate (0.05 *M*)–ammonium acetate (1 *M*) in water. To avoid any deterioration of silica particles by the ion-pair reagent [13], the column was stored in methanol–water (1:1, v/v).

The pigments were eluted using the gradient scheme outline in Table II, at a flow-rate of 2 ml/min, and detected using an absorbance detector (Perkin-Elmer LC-85B) set at 430 nm. A recording integrator (Hewlet-Packard Model 3396A) was used. Identification was made by comparing the retention times with those of authentic standards. In addition, a programmable photodiode-array detector (Waters Assoc., Model 994) allowed pigment spectra to be obtained without the need to stop the solvent flow. The absorption spectra were measured between 350 and 700 nm and recorded on a Waters Assoc. Model 5200 printer–plotter.

*Quantification*

The weight ($W$, $\mu$g) of pigments were calculated from an extension of Beer's law [13]:

$$W = \frac{aF}{A^{1\%}_{430\ \mathrm{nm}}}$$

where $a$ is the area of the peak expressed as counts supplied by the integrator, $A^{1\%}_{430\ \mathrm{nm}}$ the absorptivity of the pigment at 430 nm and $F$ a calibration factor to transform the units of the integrator into units of absorption. To calculate this factor, for the carotenoid group different solutions of $\beta$-carotene (Sigma) of known concentration under the chromatographic conditions used were analysed for an atten-

TABLE II

GRADIENT SCHEME USED FOR THE SEPARATION OF PIGMENTS

Flow-rate = 2 ml/min. The numbers in parentheses correspond to the curve type included in the programmer of a Perkin-Elmer Series 4 chromatograph.

| Time (min) | Mobile phase | | Curve |
|---|---|---|---|
| | A (%) | B (%) | |
| Initial | 75 | 25 | |
| 8 | 25 | 75 | Linear (1) |
| 10 | 25 | 75 | Isocratic |
| 18 | 10 | 90 | Convex (0.3) |
| 23 | 0 | 100 | Concave (5) |
| 30 | 75 | 25 | Concave (5) |

uation of the detector of 0.04 a.u.f.s. For chlorophyllic derivatives group the same procedure was applied with chlorphyll *a* (Sigma). The values obtained for $F$ were $1.70 \cdot 10^4$ for β-carotene and $2.19 \cdot 10^4$ for chlorophyll *a*. The values of $A^{1\%}_{430\ nm}$ were calculated from the absorption spectrum obtained with the photodiode-array detector of each pigment and from the values of $A^{1\%}_{\lambda max}$ given in the literature [7,27]. For routine determinations, it was necessary to analyse standard samples of β-carotene and chlorophyll *a* periodically, and those factors calculated to take into account the variations in instrumental sensitivity.

The calibration lines for each pigment were calculated from plots of the peak areas against concentration of pure pigment. The approximate detection limit was calculated from the calibration lines as a function of the peak height, taking as the lower limit the peak height equal to twice the noise signal.

*Reagents*

All reagents were of analytical-reagent grade, except acetone and methanol, which were of HPLC grade. The water was deionized and filtered through a 0.45-μm nylon membrane (Supelco, Bellefonte, PA, USA).

## RESULTS AND DISCUSSION

*Separation and identification of the pigments*

Fig. 1 shows the HPLC, using an absorbance detector, of pigment extracts of olives in distinct phases of the fermentation process (Spanish or Sevillian style): (a) fresh fruit, (b) at the beginning of the fermentation phase, (c) at the end of the fermentation phase and (d) in the conservation phase. Table III shows all the pigments identified, with their chromatographic and spectroscopic characteristics. The proposed technique allowed the detection and identification of carotenoids not previously detected in this product by TLC [6]. HPLC of the pigment extract from fresh fruit using the absorption detector (Fig. 1a) showed the following pigments in order of elution: neoxanthin, violaxanthin, luteoxanthin, antheraxanthin, lutein, chlorophyll *b*, chlorophyll *a* and β-carotene. Neoxanthin was shown by HPLC to consist of two isomers (peaks 3 and 3′). The same occurred for anteraxanthin (peaks 8 and 8′). Lutein (peak 10) was accompanied by two isomers (10′ and 10″). The epimers of chlorophylls on C-10 were also separated (peaks 13′ and 14′).

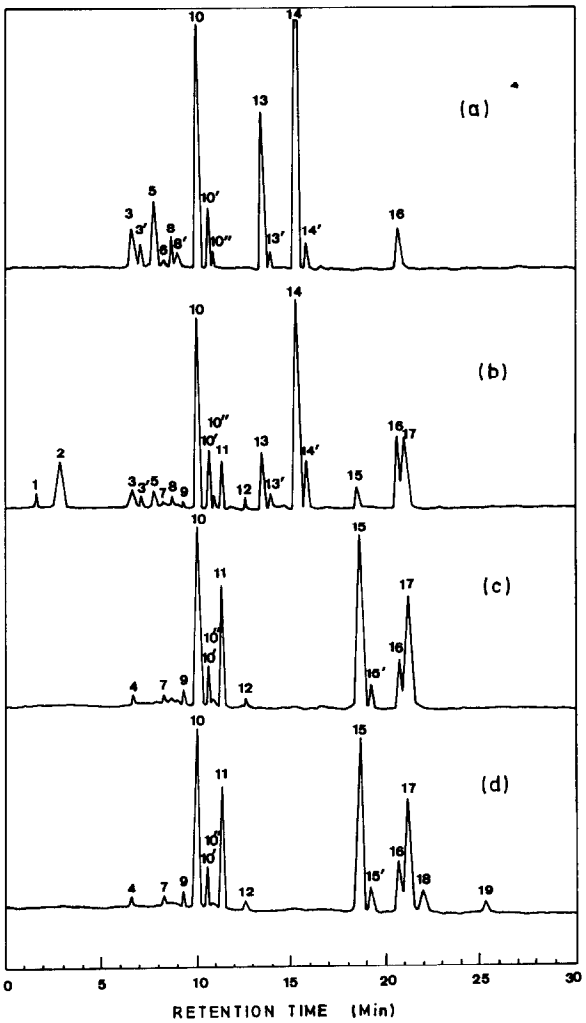


Fig. 1. HPLC using absorbance detector (430 nm) of pigment extracts of olives: (a) fresh fruit; (b) at the beginning of the fermentation phase; (c) at the end of the fermentation phase; (d) in the preservation phase. Peaks: 1 = chlorophyllide *b*; 2 = chlorophyllide *a*; 3 = neoxanthin; 3′ = neoxanthin isomer; 4 = neochrome; 5 = violaxanthin; 6 = luteoxanthin; 7 = auroxanthin; 8 = anteraxanthin; 8′ = anteraxanthin isomer, 9 = mutatoxanthin; 10 = lutein; 10′ = lutein isomer; 10″ = lutein isomer; 11 = pheophorbide *b*; 12 = pheophorbide *a*; 13 = chlorophyll *b*; 13′ = chlorophyll *b*′; 14 = chlorophyll *a*; 14′ = chlorophyll *a*′; 15 = pheophytin *b*; 15′ = pheophytin *b*′; 16 = β-carotene; 17′ = pheophytin *a*; 17 = pheophytin *a*; 18 = pyropheophytin *b*; 19 = pyropheophytin *a*.

The chromatogram from a pigment extract of

TABLE III

PIGMENTS SEPARATED BY HPLC: IDENTITIES, CAPACITY FACTORS AND SPECTRA DATA

$K'_c = (t_r - t_m)/t_m$, where $t_r$ = retention time of the pigment peak and $t_m$ = retention time of an unretained component. Published spectral data are in diethyl ether for chlorophylls and derivatives and in ethanol for carotenoids.

| Peak No. | $K'_c$ | Pigment | Spectral data in the eluent | | | | Published data | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Maxima (nm) | | | Peak ratio | Maxima (nm) | | | Peak ratio | Ref. |
| | | | I | II | III | | I | II | III | | |
| 1 | 0.17 | Chlorophyllide *b* | 466 | 600 | 650 | 3.3 | | | | | |
| 2 | 0.96 | Chlorophyllide *a* | 432 | 616 | 664 | 1.3 | 428 | | 662 | | 8 |
| 3 | 3.28 | Neoxanthin | 414 | 438 | 466 | 90 | 416 | 440 | 468 | 89 | 8 |
| 3′ | 3.60 | Neoxanthin isomer | 414 | 438 | 466 | 90 | | | | | |
| 4 | 3.69 | Neochrome | 398 | 422 | 448 | 78 | 401 | 424 | 451 | | |
| 5 | 4.11 | Violaxanthin | 416 | 440 | 470 | 94 | 417 | 440 | 470 | 93 | 8 |
| 6 | 4.46 | Luteoxanthin | 400 | 424 | 450 | 107 | 396 | 420 | 446 | | 31 |
| 7 | 4.56 | Auroxanthin | 380 | 400 | 424 | 103 | 380 | 402 | 428 | 103 | 8 |
| 8 | 4.75 | Anteraxanthin | (420) | 444 | 474 | 22 | 422 | 444 | 472 | 54 | 8 |
| 8′ | 4.96 | Anteraxanthin isomer | (420) | 444 | 474 | 22 | | | | | |
| 9 | 5.22 | Mutatoxanthin | (404) | 426 | 452 | 39 | | 427 | 457 | | |
| 10 | 5.52 | Lutein | 424 | 446 | 474 | 60 | 422 | 445 | 474 | 62 | 8 |
| 10′ | 6.05 | Lutein isomer | 418 | 440 | 468 | 42 | | | | | |
| 10″ | 6.26 | Lutein isomer | 416 | 438 | 466 | 27 | | | | | |
| 11 | 6.42 | Pheophorbide *b* | 426 | | 650 | 8.3 | 433 | 525 | 655 | 4.56 | 32 |
| 12 | 7.29 | Pheophorbide *a* | 400 | 504 | 662 | 3.3 | 408 | 504 | 667 | 2.07 | 32 |
| 13 | 7.96 | Chlorophyll *b* | 466 | 600 | 650 | 3.3 | 453 | 593 | 642 | 2.89 | 33 |
| 13′ | 8.24 | Chlorophyll *b′* | 466 | 600 | 650 | 2.8 | 453 | 592 | 642 | 2.86 | 34 |
| 14 | 9.18 | Chlorophyll *a* | 432 | 616 | 664 | 1.3 | 430 | 615 | 661 | 1.32 | 33 |
| 14′ | 9.57 | Chlorophyll *a′* | 432 | 616 | 664 | 1.1 | 428 | 614 | 661 | 1.24 | 34 |
| 15 | 11.21 | Pheophytin *b* | 436 | 599 | 654 | 5.1 | 433 | 599 | 654 | 4.81 | 33 |
| 15′ | 11.91 | Pheophytin *b′* | 436 | 598 | 654 | 5.1 | | | | | |
| 16 | 12.84 | β-Carotene | | 452 | 478 | 23 | | 450 | 477 | 20 | 8 |
| 17 | 13.22 | Pheophytin *a* | 410 | 506 | 666 | 1.8 | 408 | 503 | 667 | 2.14 | 33 |
| 18 | 13.79 | Pyropheophytin *b* | 436 | 524 | 654 | 5.4 | 436 | | 655 | | 35 |
| 19 | 16.05 | Pyropheophytin *a* | 410 | 506 | 666 | 2.4 | 409 | | 666 | | 35 |

fruits at the beginning of the fermentation phase (Fig. 1b) shows new peaks, which were identified as degradation products of the pigments present in the fresh fruit. With respect to the chlorophyllic fraction, chlorophyllide *a* and *b*, pheophorbide *a* and *b* and pheophytin *a* and *b* were detected. As shown in previous work [6], the chlorophyllides appear in the few first days of fermentation, when the olives still have an alkaline pH from the sodium hydroxide treatment, which favours the action of the enzyme chlorophyllase, present in most green tissues. The presence of pheophytins and pheophorbides is due to the acidic pH of the medium caused by the products of the lactic fermentation [6]. Because of this, in the carotenoid fraction, the 5,8-epoxides carotenoids neochrome, auroxanthin and mutatoxanthin were also detected.

In the totally fermented fruit (Fig. 1c) the chlorophylls and chlorophyllides have disappeared, being transformed into pheophytins and pheophorbides. In the carotenoid fraction, neoxanthin, violaxanthin and anteraxanthin have been totally transformed into their corresponding furanoid derivatives neochrome, auroxanthin and mutatoxanthin, respectively. Lutein and β-carotene remain the main pigments in this fraction. Finally, in the brine conservation phase, additional degradation reactions may take place. Thus, pyropheophytin *a* and *b* are detected, formed from the corresponding pheophytins by the loss of the C-10 carbomethoxy

group. The last pigment to elute is pyropheophytin *a*, which has a retention time of about 25 min (Fig. 1d). The absorbance chromatograms discussed previously show that during fermentation and subsequent preservation of the fruits it is the chlorophyllic fraction of pigments which undergoes the greatest transformation. The main carotenoids, lutein and β-carotene, remain virtually unaltered.

*Quantification of the pigments*

The slow and labour-consuming operation to obtain carotenoids and chlorophyllic derivatives, and the difficult conservation of the standard mixtures due to the sensibility of these pigments to high temperature, etc., limit the possibilities of using the traditional methods with internal or external standards. These problems extend substantially the time required for the analysis and makes it inappropriate for the control of the pigment changes during the lactic fermentation of olives (6–7 months).

To compare the external standard method with the proposed method, four analyses of two pigment extracts from fresh and elaborated olives were carried out. All the pigment concentrations were calculated from the calibration lines and from the empirical factor for carotenoids and chlorophyllic derivatives. Table IV shows the average values obtained by both procedures and the corresponding absorptivities at 430 nm. Anteraxanthin and mutatoxanthin were not included because insufficient amounts to estimate the calibration line were obtained. The errors of the concentration calculated from the empirical factors and the $A^{1\%}_{430\ nm}$ values ranges from

TABLE IV

QUANTIFICATION BY HPLC OF PIGMENTS USING THE EXTERNAL STANDARD METHOD (METHOD 1) AND BEER'S LAW (METHOD 2)

Means of four injections from fresh and fermented olive extracts (mg/kg). Values of $A^{1\%}_{430\ nm}$ calculated from the absorption spectrum obtained with the photodiode array detector and from the values of $A^{1\%}_{\lambda max}$. Error of the concentrations calculated from method 2.

| Pigment | Method 1 | Method 2 | $A^{1\%}_{430\ nm}$ | Error (%) |
|---|---|---|---|---|
| Neoxanthin | 1.0275 | 1.0175 | 1353 | 1.0 |
| Neochrome | 0.5125 | 0.5000 | 1751 | 2.4 |
| Violaxanthin | 0.5750 | 0.5650 | 1909 | 1.7 |
| Auroxanthin | 0.7725 | 0.7625 | 1709 | 1.0 |
| Lutein | 3.3825 | 3.3625 | 1777 | 0.6 |
| Pheophorbide *b* | 2.0650 | 2.0550 | 1545 | 0.5 |
| Pheophorbide *a* | 0.7750 | 0.7680 | 268 | 0.2 |
| Chlorophyll *b* | 11.6800 | 11.6100 | 356 | 0.6 |
| Chlorophyll *a* | 21.1275 | 21.1050 | 840 | 0.0 |
| Pheophytin *b* | 6.2650 | 6.2350 | 1545 | 0.5 |
| β-Carotene | 2.3250 | 2.3350 | 1844 | 0.0 |
| Pheophytin *a* | 23.9725 | 23.9225 | 268 | 0.2 |

TABLE V

QUANTIFICATION BY HPLC OF PIGMENTS IN PICKLED GREEN OLIVES FROM BEER'S LAW AND ESTIMATION OF PRECISION OF HPLC METHOD AND THE DETECTION LIMITS

Mean ± S.D. of triplicate injections from pitted fresh olive extract.

| Pigment | Extract 1 | | Extract 2 | | Detection limit (ng) |
|---|---|---|---|---|---|
| | mg/kg | R.S.D. (%) | mg/kg | R.S.D. (%) | |
| Neochrome | 0.320 ± 0.014 | 4.37 | 0.340 ± 0.014 | 4.12 | 5.9 |
| Auroxanthin | 0.465 ± 0.021 | 4.52 | 0.530 ± 0.028 | 5.28 | 3.2 |
| Mutatoxanthin | 0.285 ± 0.010 | 0.35 | 0.340 ± 0.014 | 4.12 | – |
| Lutein | 2.995 ± 0.021 | 0.70 | 3.205 ± 0.064 | 2.00 | 2.5 |
| Lutein isomer | 0.500 ± 0.014 | 2.80 | 0.560 ± 0.014 | 2.50 | 2.5 |
| Pheophorbide *b* | 1.745 ± 0.064 | 3.67 | 1.890 ± 0.042 | 2.22 | 8.0 |
| Pheophorbide *a* | 0.845 ± 0.049 | 5.80 | 1.155 ± 0.035 | 3.03 | 44.0 |
| Pheophytin *b* | 2.220 ± 0.042 | 1.90 | 2.735 ± 0.035 | 1.28 | 8.0 |
| β-Carotene | 1.195 ± 0.010 | 0.08 | 1.175 ± 0.021 | 1.79 | 19.0 |
| Pheophytin *a* | 14.980 ± 0.028 | 0.19 | 16.175 ± 0.431 | 2.66 | 44.0 |
| Pheophytin *a'* | 5.040 ± 0.085 | 1.69 | 5.475 ± 0.120 | 2.19 | 44.0 |
| Pyropheophytin *a* | 3.475 ± 0.035 | 1.01 | 3.615 ± 0.078 | 2.16 | 44.0 |

0.2 to 2.4%. The analysis of variance of such data showed insignificant differences between the results given by the two methods. Table V gives the precision of the method (0.1–5.8%) and the detection limits, which varied between 44 ng for pheophytin *a* and 2.5 ng for lutein.

## CONCLUSION

The method developed in this study for rapid control of qualitative and quantitative assessments of individual pigments is of great interest for establishing the presence of appropriate pigments during each fermentation phase. This information permits the correction during the fermentation process of any deviation from the normal pattern that could affect the parameters that influence the pigments. Also, a correlation between the subjective colour and the type and amount of each pigment could be obtained. In general, the proposed method could also be used with other products that contain chlorophylls and carotenoids, if the sum of the pigments is high and the preparation of standards might be too tedious owing to the instability of these compounds.

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## REFERENCES

1 M. J. Fernández, R. de Castro, A. Garrido, F. G. Cancho, F. G. Pellissó, M. Nosti, A. Heredia, M. I. Mínguez, L. Rejano, M. C. Durán, F. Sánchez, P. Garcia and A. de Castro, in *Biotecnología de la Aceituna de Mesa, Madrid 1985*, CSIC, Madrid, 1985, Ch. 1, p. 5 and Ch. 4, p. 51.
2 J. M. R. de la Borbolla, C. Gómez, F. G. Cancho, M. J. Fernández-Díez, R. Gutiérrez, A. Izquierdo, F. G. Pellissó, R. Vázquez and R. Guzmán, *El Aderezo de Aceitunas Verdes*, CSIC, Madrid, 1956, Ch. 6, p. 105.
3 A. Garrido-Fernández, L. Rejano-Navarro, F. Sánchez-Roldán, A. de Castro-Gómez, P. García-García, A. H. Sánchez-Gómez and M. Brenes-Balbuena, in *First Conference on Food Science and Technology for Mediterranean Countries*, Egyptian Society of Food Science and Technology, Cairo, 1986, p. 88.
4 A. Vázquez-Roncero, *Grasas Aceites*, 14 (1963) 262.
5 M. I. Mínguez-Mosquera and J. Garrido-Fernández, *J. Agric. Food Chem.*, 37 (1989) 1.
6 M. I. Mínguez-Mosquera, J. Garrido-Fernández and B. Gandul-Rojas, *J. Agric. Food Chem.*, 37 (1989) 8.
7 M. I. Mínguez-Mosquera, J. Garrido-Fernández and B. Gandul-Rojas, *J. Agric. Food Chem.*, 38 (1990) 38.
8 S. W. Wright and J. D. Shearer, *J. Chromatogr.*, 294 (1984) 281.
9 T. Brauman and L. H. Crimme, *J. Chromatogr.*, 170 (1979) 264.
10 S. J. Schwartz, S. L. Woc and J. H. Von Elbe, *J. Agric. Food Chem.*, 29 (1981) 533.
11 J. F. Fisher and R. L. Rouseff, *J. Agric. Food Chem.*, 34 (1986) 985.
12 F. Khachik, G. R. Beecher and N. F. Whittaker, *J. Agric. Food Chem.*, 34 (1986) 603.
13 R. F. C. Mantoura and C. A. Llewellyn, *Anal. Chim. Acta*, 151 (1983) 297.
14 C. Fisher and J. A. Kocis, *J. Agric. Food Chem.*, 35 (1987) 55.
15 K. Iriyama, M. Yoshiura and M. Shiraki, *J. Chromatogr.*, 154 (1978) 302.
16 J. K. Abaychi and J. P. Riley, *Anal. Chim. Acta*, 107 (1979) 1.
17 M. Rahmani and A. S. Csallany, *Rev. Fr. Corps Gras*, 32 (1985) 257.
18 M. I. Mínguez-Mosquera, A. Montaño-Asquerino, J. Garrido-Fernández and B. Gandul-Rojas, *Grasas Aceites*, 39 (1988) 363.
19 G. Sievers and P. H. Hynninen, *J. Chromatogr.*, 134 (1977) 359.
20 I. D. Jones, L. S. Butler, E. Gibbs and R. C. White, *J. Chromatogr.*, 70 (1972) 87.
21 T. Watanabe, A. Hongu, K. Hond, M. Nakazato, M. Konno and S. Saitoh, *Anal. Chem.*, 56 (1984) 251.
22 M. I. Mínguez-Mosquera, B. Gandul-Rojas and J. Garrido-Fernández, *Grasas Aceites*, 40 (1989) 114.
23 M. I. Mínguez-Mosquera and J. Garrido-Fernández, *Grasas Aceites*, 36 (1985) 376.
24 M. I. Mínguez-Mosquera, J. Garrido-Fernández and J. Pereda-Marín, *Grasas Aceites*, 37 (1986) 272.
25 M. I. Mínguez-Mosquera, J. Garrido-Fernández and J. Pereda-Marin, *Grasas Aceites*, 37 (1986) 320.
26 J. H. C. Smith and A. Benitez, in K. Paech and M. V. Tracey (Editors), *Modern Methods of Plant Analysis*, Vol. 1. Springer, Berlin, Göttingen, Heidelberg, 1955, p. 142.
27 B. H. Davies, in T. W. Goodwin (Editor), *Chemistry and Biochemistry of Plant Pigments*, Vol. 2, Academic Press, London, 1975, p. 38.
28 F. H. Foppen, *Chromatogr. Rev.*, 14 (1971) 133.
29 D. F. Hildebrang and T. Hymowitz, *J. Agric. Food Chem.*, 30 (1982) 705.
30 D. B. Rodríguez, P. L. Simpson and C. O. Chichester, *Int. J. Biochem.*, 4 (1973) 213.
31 B. H. Davies and H. P. Köst, in H. P. Köst (Editor), *Handbook of Chromatography*, Vol. 1, CRC Press, Boca Raton, FL, 1988, p. 3.
32 P. H. Hynninen, *Acta Chem. Scand.*, 27 (1973) 1771.
33 P. H. Hynninen and N. Ellfolk, *Acta Chem. Scand.*, 27 (1973) 1463.
34 P. H. Hynninen, *Acta Chem. Scand.*, 27 (1973) 1487.
35 M. S. Fraser and G. Fankl, *J. Am. Oil Chem. Soc.*, 62 (1985) 113.