Extraction of Pigment Information from Near-UV Vis Absorption Spectra of Extra Virgin Olive Oils

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Supporting Information

ABSTRACT: This work reports a new approach to extract the maximum chemical information from the absorption spectrum of extra virgin olive oils (EVOOs) in the 390–720 nm spectral range, where “oil pigments” dominate the light absorption. Four most important pigments, i.e., two carotenoids (lutein and β-carotene) and two chlorophylls (pheophytin-a and pheophytin-b), are chosen as reference oil pigments, being present in all the reported analytical data regarding pigments of EVOOs. The method allows the quantification of the concentration values of these four pigments directly from the deconvolution of the measured absorption spectrum of EVOOs. Advantages and limits of the method and the reliability of the pigment family quantification are discussed. The main point of this work is the description of a fast and simple method to extract such information in less than a minute, through the mathematical analysis of the UV–vis spectrum of untreated samples of oil.

KEYWORDS: UV–vis spectra deconvolution, olive oil spectra, olive oil pigments, carotenoids, chlorophylls

INTRODUCTION

In the last years, new methods to better characterize extra virgin olive oils (EVOOs) in terms of their minor components were developed by several research groups.1–3 The increasing interest in this field can be attributed to health claims of EVOO related to its minor components, such as polyphenols, vitamins, and pigments.4,5 Moreover, local oil producers are more and more interested in attesting the specificity of the geographical area of olive production, also referring to the particular product.6

Much work has been dedicated to the study of the pigments present in EVOO, of their properties in protecting oil from oxidation processes11,12 or of the way to favor them especially under visible light13 and finally of their analytical determination.14–16 Generally, extraction of pigments from the fat matrix precedes HPLC analyses.17 High sensibility is based on the high extinction coefficient of these pigments in well-defined bands of the visible and near-UV spectral region. In addition, for chlorophyll pigments, fluorescent emission could improve sensibility and selectivity in analytical determinations.17,18

The manipulation and mathematical treatment of the absorption spectra in the vis–near UV spectral region of EVOO was first developed by Ayuso et al.19 According to this method, the measured spectrum is analyzed as a linear combination of two basic spectra: the first one typical of chlorophyll pigments and the second one typical of carotenoids. These two basic spectra are determined by photolizing oil samples in quartz cuvettes with the UV–vis emission of a medium-pressure Hg lamp. This method is based on the evidence that the photodegradation of chlorophyll pigments is much faster than that of carotenoids. This is partially due to the overlapping of chlorophyll pigment absorption with the strong 366 nm lamp emission line, where oil carotenoids have a minimum of absorption. On the basis of this, it is possible to attribute that part of the oil absorbance rapidly lost during the photolysis to the absorbance of chlorophyll pigments and the residual absorbance to the unphotolized carotenoids pigments. According to Ayuso et al.,19 the linear combination of these two spectra can fully determine the spectral profile of each oil. Clearly, this implies that the composition and relative proportions of carotenoids and of chlorophyll pigments are approximately constant in EVOO. However, looking at published data15,17,20–22 this assertion seems too drastic, as both carotenoids and chlorophylls present in oils can show neatly different absorption spectra, and they are present in quite different ratios in each of the two pigment families. For chlorophylls, the pheophytin-a/pheophytin-b relative proportion can vary substantially from one oil to the other. For instance, the ratio between concentrations of pheophytin-a and pheophytin-b spans from 6.4 to about 130,17,21 while, for carotenoids, the ratio of β-carotene/lutein significantly changes in EVOOs from about 0.08 up to 5.9.21–23 Nevertheless, the results reported by Ayuso et al.19 strongly suggest that in olive oil the light absorption in the 390–720 nm spectral region is essentially due to pigments, i.e., substances in low concentration and with high extinction coefficients dissolved in a quasi-transparent medium and pertaining to two classes: carotenoids and chlorophylls.

Following these considerations, a new method to obtain the pigments concentration in EVOOs by analyzing their near
UV–vis absorption spectra is discussed in the present paper. This method requires only a mathematical treatment of the measured oil absorption spectrum avoiding any chemical manipulation of the sample. Within this approach, the spectra of EVOOs are analyzed in terms of the molar absorption spectra of four main pigments dissolved in purified triolein, namely, lutein (a xanthophyll), β-carotene (a carotenoid), pheophytin-a and pheophytin-b (two chlorophylls derivatives). The choice of this set of “main pigments”, suggested by experimental analytical data, is discussed, as well as the use of triolein as the best solvent. Several examples of the application of this method to real experimental cases are reported, underlining its possible uses for the assessment of quality, freshness, and aging effects in EVOOs.

**MATERIALS AND METHODS**

**Oil Samples.** The oil samples from Spain (Coprovir, Poniente de Granada, Fuentes de Dios, Loja) were a gift from San Isidro Deifontes S.C.A. (Granada), and they are of the same vintage year. The Italian oils (mixed cultivar of different origin) were provided by Salov S.P.A. (Massarosa, Lucca), and they were from the 2012 and 2013 harvests. Tuscan oils of Frantoio cultivar were provided from Carapelli Firenze S.p.A, and the harvest year is indicated case by case. The rest of oils Tuscan oils of Frantoio cultivar were provided from Carapelli Firenze S.p.A, and they were from the 2012 and 2013 harvests. S.C.A. (Granada), and they are of the same vintage year. The Italian Granada, Fuentes de Dios, Loja) were a gift from San Isidro Deifontes S.C.A. (Granada), and they are of the same vintage year (2012). For each measurement reported in this work, a minimum of three replicates were performed. All oil samples were maintained in dark conditions, in 25 mL dark glass bottles, at a temperature between 1 and 5 °C.

**Solvents and Purification Methods.** Methanol, n-hexane, and acetone were Carlo Erba HPLC solvents (Milan, Italy).

β-Carotene (>97% purity) and triolein were purchased by Fluka (Milan, Italy). β-Carotene was used as such, while triolein was purified by washing cycles with pure MeOH and freezing/partial crystallization of the mixture at T < −20 °C, followed by a phase separation, until the measured absorbance of triolein was equal to that of the washing cycle (minimum four cycles). Lutein and zeaxanthin were obtained through HPLC semi-preparative isolation/purification of methanol extracts of maize flour. The mobile phase was methanol. The column was a Waters Spherisorb SS-ODS2 10 × 250 mm RP-C18, the flux was 2.4 mL/min at T = 25 °C, and the spectrophotometric detector was at 450 nm. Retention times (Rt) were 15 and 16 min, respectively, for lutein and zeaxanthin.

Pheophytin-a and pheophytin-b were obtained through HPLC semi-preparative isolation, by Waters Spherisorb SS-ODS2 10 × 250 mm RP-C18 column, detector at 415 nm, of acetone extracts of leaves of Acer Negundo L. (Aceraceae) acidified with 1 N HCl, after complete pheophytinization of chlorophylls present in these extracts. Pheophytinization was controlled through UV spectrophotometry by observing the sharp diminution of absorbance at 434 nm, where the maximum of chlorophyll-a is located, and the corresponding increase at 410 nm near the absorbance maximum of pheophytin-a (65% of spectral change in 12 min). Retention times were Rt (pheo-b) = 25.9 min; Rt (pheo-a) = 32.5 min; flux 4.5 mL/min; T = 45 °C; mobile phase: methanol.

**Instruments.** Absorbance spectra were measured with a Jasco V-550 spectrophotometer using quartz cells with 0.1–1 cm optical path length in order to measure absorbances smaller than 2 absorbance units.

A HPLC Jasco 880-Pu pump equipped with a Shimadzu SP-10A spectrophotometric detector was used isocratically for analytic and semipreparative experiments.

**Spectrophotometric Measurements.** Before absorbance measurements, the oil samples were centrifuged for 30 min at 5000 rpm in order to minimize the light absorption due to suspended materials. The absorbances used in the present analyses of EVOOs spectra are referred to 1 cm optical path. Clearly, in the actual absorbance measurements, cells whose optical path was compatible with the requirement of precise measurements, in particular to avoid any sensible stray light effect, were used. Spectroscopic parameters included spectral bandwidth = 1 nm and measurement step Δλ = 1 nm.

**Extinction Coefficients of the Pigments.** Because of the lack of relevant literature, the molar extinction spectra of lutein, pheophytin-a, and pheophytin-b in triolein (see Figure 1) were obtained through the experimental procedure hereafter described. The methanol solutions of purified pigments from semipreparative HPLC were about 2 × 10⁻⁵ M of pure pigments in volumes of 5–10 mL, i.e., sub-milligram masses of pigments. Their absorbance in methanol was measured, and the concentration of the pure analytes was calculated by using the values of their extinction coefficients found in the literature.25–27 The volatile solvent in a precise volume (1 mL) of methanol solution was finally evaporated and a measured volume (1 mL) of purified triolein was added to the dry pigment.

For β-carotene a weighted mass (4.26 mg) was dissolved in a measured volume (25 mL) of n-hexane to obtain a relatively concentrated solution. A measured aliquot (40 µL) was conveniently diluted in 2 mL of solvent and its absorbance was measured. The molar extinction of β-carotene in hexane found in the literature was used to calculate its concentration. The volatile solvent was evaporated, and a measured volume of pure triolein was added. In this case, it was also possible to verify that the procedure was correct. In fact, the initial mass of β-carotene and the used volumes of solutions and solvents were measured, and the difference between the concentrations found by the two procedures is less than 2%.

The spectra were measured in pure triolein, since this quasi-transparent solvent is the most appropriate to represent the interactions of the olive oil matrix with the pigments. The spectra obtained was neutally different from the spectra in the more usual solvents (methanol, ethanol, acetone, diethyl ether, or hexane) reported in the literature.28–29

A sample of HPLC-pure zeaxanthin, a xanthophyll isomer of lutein, very common in nature, was also prepared. In two parallel experiments, known amounts of lutein and zeaxanthin were added to samples of a Tuscan EVOO. The measured increase of absorbance of the oil due to the added lutein, or zeaxanthin, was identical to the absorbance of an equal quantity of lutein, or zeaxanthin, dissolved in a volume of triolein equal to that of the EVOO used in the experiment. The two major peaks in the absorption spectrum of zeaxanthin in triolein were 4 and 7 nm red-shifted with respect to the corresponding lutein spectrum. The zeaxanthin spectrum peaks, in addition, seem to be less resolved than those of lutein. The above findings demonstrate the good choice of triolein as a solvent: no particular interactions of xanthophylls with the components of EVOOs different from triolein, should be present. Differences in the shapes and shifts of the order of
5–10 nm are expected for the absorption spectra of the other carotenoids present in EVOOs in concentration usually much smaller than lutein and β-carotene.

With a procedure similar to that used for lutein, triolein has been proven to well represent the medium in which pheophytin-a and pheophytin-b are present in olive oils.

Mathematical Treatment of UV–vis Absorption Spectra. In this section, the mathematical treatment of the EVOO absorption spectrum adopted in order to extract the concentration of the aforementioned four main pigments is reported.

As shown in Figure 1 and summarized in Table 1, while the two pheophytins spectra show sensible differences in their shapes and absorption intensities, lutein and β-carotene show spectra similar in shape and intensity. In fact, the spectrum β-carotene is red-shifted and has a lower resolution of the peaks with respect to the xanthophyll one. However, there is no doubt about the linear independence of the four spectra, i.e., not one of them can be expressed as a linear combination of the other three spectra.

The UV–vis absorption spectrum of any EVOO can be conveniently described by four orthogonal functions (eigenvectors) by using a treatment similar to that adopted by Hennessey and Johnson\textsuperscript{10} to calculate the contributions of various proteins basic structures (α-helix, β-sheet, etc.) to the measured circular dichroism (CD) spectra of proteins in solution.

First, the symmetric $4 \times 4$ overlap matrix, $S$, of the four molar extinction spectra $\varepsilon(\lambda)$ of the four pigments in triolein, is obtained. The elements $S_{ji}$ are defined as

$$S_{ji} = \int \varepsilon_j(\lambda) \varepsilon_i(\lambda) \, d\lambda$$

the integration being performed from 390 to 720 nm, the spectral range where light absorbance in EVOOs can be ascribed to the chosen pigments.

Then, four eigenvectors and the correspondent four eigenvalues are obtained by diagonalization of the $S$ matrix. The eigenvectors, $\Phi_j(\lambda)$, with $j = 1, 2, 3, \text{ and } 4$, are linear combinations of the four molar extinction coefficient of pigments, $\varepsilon_i(\lambda)$ according to eq 2, and they are reported in Figure 2.

$$\Phi_j(\lambda) = \sum_{i=1}^{4} P_{ji} \varepsilon_i(\lambda)$$

where $P_{ji}$, a fixed parameter, is the contribution to the eigenvector $\Phi_j(\lambda)$ of the molar extinction spectrum of pigment $r$. It must be observed that the diagonalization of the $S$ matrix is possible only when the four spectra are linearly independent, as it is in this case.

The eigenvalues, $\sigma_r$ ($\times 10^{-4}$), are 0.022298, 0.02191, 0.403061, and 2.337122 M$^{-1}$ cm$^{-1}$ in correspondence to the eigenvectors $\Phi_j(\lambda)$. Only the eigenvector corresponding to the highest eigenvalue is positive in all the spectral range, while the other curves also have negative values in a number of spectral intervals separated by crossing points with the $x$-axis. These progressively increase from 0 up to 7 (0, 2, 6, 7 with decreasing eigenvalues).

If the absorbance of an EVOO in the 390–720 nm range is due to the four pigments only, it can be expressed exactly as a linear combination of the above four functions $\Phi_j(\lambda)$. In any case, it is simple to calculate (eq 3) the four coefficients $\gamma_r$ of the linear combination that best represents the oil spectrum in terms of the spectra of the reference pigments:

$$\gamma_r = \int \Phi_r(\lambda) \text{ABS}(\lambda) \, d\lambda / \sigma_r$$

where $\text{ABS}(\lambda)$ is the measured absorbance of the oil and $\sigma_r$ is the eigenvalue corresponding to $\Phi_r(\lambda)$.

Finally, the molar concentration of the pigment $r$, $C_r$, in the oil is determined through eq 4:

$$C_r = \sum_{j=1}^{4} P_{ji} \gamma_j$$

With the values of the molar concentrations for the pigments, it is possible to calculate (eq 5) the absorption spectrum $\text{ABS}_{\text{calc}}(\lambda)$ of the EVOO, to be compared with the experimental one, $\text{ABS}(\lambda)$.

$$\text{ABS}_{\text{calc}}(\lambda) = \sum_r C_r \varepsilon_r(\lambda)$$

From the discrepancies between these spectra, it is possible to evaluate the quality of the concentration values $C_{r,\text{calc}}$ extracted from the experimental absorption spectrum, as well as the goodness of the procedure.

Spectral and Data Analysis. The measured spectra were mathematically treated by using properly adapted Excel sheets. The diagonalization of the matrix $S$ was performed by using a package of MatLab 6. The evaluation of relevant statistic parameters was also performed by using Excel software.

Validation of the Method. In order to validate the method, the error due to both the experimental procedure and the mathematical treatment was evaluated. For the experimental contribution to the error, all UV–vis spectra were acquired by using rectangular quartz cuvettes with different path lengths of 0.1, 0.2, 0.5, and 1 cm and two cuvettes with the same path length of 0.1 cm, but with different geometry and shape were used: five spectra were acquired for each oil. After normalization for the path length, the five experimental spectra were scaled in order to have the absorbance values at $\lambda > 720$ nm equal to zero. These five scaled spectra were used to calculate the average spectrum and the standard deviation, for each oil sample. From the statistical point of view, the method has been applied to more than 80 oils from Tuscany and Apulia\textsuperscript{31} and the average value of the standard deviation, $\sigma_{\text{std}}$, calculated for each pigment, is reported in Table 2. It should be noticed, that the experimental error also contains a systematic error, which is not easy to evaluate, related to the intrinsic differences among the different cuvettes. In fact, by decreasing the path length, the effect of the stray light decreases, and it is not constant within the observed spectral window. However, the values reported in Table 2 can be taken as representative for the general case.

The analysis of the UV–vis spectra by using the mathematical approach presented above introduces an error, too. This error can be evaluated through the residues (see the Supporting Information for details). Moreover, the goodness of the mathematical treatment was...
Table 2. Values of Standard Deviation of Concentration (M) for Investigated Pigments Due to Experimental (σexp) and Deconvolution (σcalc) Contributions

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<td>σexp</td>
<td>0.3</td>
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<td>5 × 10⁻²</td>
<td>1 × 10⁻⁷</td>
<td>2 × 10⁻⁴</td>
</tr>
<tr>
<td>σcalc</td>
<td>0.3</td>
<td>5 × 10⁻²</td>
<td>1 × 10⁻⁷</td>
<td>3 × 10⁻⁷</td>
<td>2 × 10⁻⁷</td>
</tr>
<tr>
<td>LOD</td>
<td>1 × 10⁻⁸ ± 6 × 10⁻⁷</td>
<td>7 × 10⁻⁸ ± 3 × 10⁻⁴</td>
<td>1 × 10⁻⁷ ± 6 × 10⁻⁸</td>
<td>8 × 10⁻⁷ ± 2 × 10⁻⁸</td>
<td></td>
</tr>
<tr>
<td>LOQ</td>
<td>1 × 10⁻⁸ ± 6 × 10⁻⁷</td>
<td>7 × 10⁻⁸ ± 3 × 10⁻⁴</td>
<td>1 × 10⁻⁷ ± 6 × 10⁻⁸</td>
<td>1 × 10⁻¹³ ± 6 × 10⁻⁸</td>
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</table>

*LOD and LOQ values calculated as reported in the text.

verified by the "R-square" test, which estimates the correlation between the experimental values and the values predicted by the deconvolution procedure:

\[
R\text{-square} = 1 - \frac{\sum_{i=1}^{n} (y_i - f_i)^2}{\sum_{i=1}^{n} (y_i - \bar{y})^2}
\]

(6)

where \( f_i \) is the value predicted by the fitting, \( \bar{y} \) is the mean of the observed data, and \( y_i \) is the observed data value. In particular, two tests were done by choosing two EVOOs representative of two opposite situations: a good and a bad fit, due, for example, to the turbidity of the oil, as reported in the Supporting Information.

The values of \( R \) are 0.85 for the bad case and 0.96 for the good one. The range 0.85 ± 0.96 can be taken as representative for all other intermediate cases.

The standard deviation due to the deconvolution procedure, \( \sigma_{\text{calc}} \), reported in Table 2, can be determined according to the following equation:

\[
\sigma_{\text{calc}} = \sqrt{\frac{\sum_{i=1}^{n} (x_i - \bar{x})^2}{n-1}}
\]

(7)

where \( x_i \) is the pigment concentration obtained after fitting process for each path lengths cuvette, \( \bar{x} \) is the mean value of the pigment concentration, and \( n \) is the number of the values considered. In this case, it is equal to five (number of cuvettes used). It can be noted that triolein concentration is visibly higher than the other values, because it represents the matrix of the oil without any pigment.

A sample of refined olive oil was used for the measurement of the limit of detection (LOD) and limit of quantitation (LOQ). According to IUPAC definition, LOD and LOQ are mathematically defined equal to 3 and 10 times, respectively, the standard deviation of the results for a series of replicates (five in our case) used to determine a reliable limit of detection. The respective values of LOD and LOQ are reported in Table 2.

## RESULTS AND DISCUSSION

**Discussion about the Mathematic Treatment.** In this work on the extraction of pigment information, four pigments were chosen. In principle, other pigments could be added to these four ones, provided that their molar spectra are linearly independent from each other and from the previous ones. The maximum number of basis spectra is actually defined by the dimensionality of the functional space of the absorption spectra of all extra virgin olive oils. Physical restrictions have to be taken into account. For example, the precision of the absorbance measurements both of the oil samples and of the reference spectra, ca. 1%, has to be considered. Two spectra differing only within the experimental error cannot be considered linearly independent, and pigment concentrations identical, within the experimental error, have to result using the above treatment of their absorption spectra, i.e., by using eqs 3–5, in the previous section.

Two independent spectra cannot be proportional to each other and they must be different in their shape. As shown in Figure 1, even if the differences in the shape of the molar extinction of lutein and β-carotene are much smaller than that of any other couple of reference pigments, they are sufficient to represent EVOOs carotenoids. From a quantitative point of view, the difference in shape between spectra can be evaluated by the values of the overlaps of normalized extinction spectra (see the Supporting Information).

In this work, the minimum number of pigments, normally present in EVOOs, whose absorption spectra are significantly different from each other, was chosen in order to obtain calculated absorption spectra nearest to the measured ones. The possible presence of other pigments not included among the above reference components has to be addressed as well. If the outsider pigment has a spectrum equal to any of the reference components, its contribution to the absorbance of the oil is simply added up to the absorbance of this component, through eq 3, thereby increasing its calculated concentration, through eq 4. This situation actually occurs for some isomers of lutein, for lutein esters,17,18 and for some pheophytins.32 It must be noticed that, while there are large differences in the CD spectra of pheophytin stereoisomers, differences in their absorption spectra are undetectable.30 When the spectrum of the outsider component pertains to the functional space of two of the basic four pigments, its contribution to the whole spectrum is included in the calculated amounts of these two pigments. This case is here briefly described.

A known amount of zeaxanthin, i.e., a carotenoid isomer of lutein that, as far as we know, has never been detected in EVOOs, was added to a multicultivar Italian EVOO. The absorption spectra of the EVOO before (curve a) and after the addition (curve b) are reported in Figure 3A. The spectrum (curve c) is the difference between spectra (b) and (a) and represents the measured increase of the absorbance of the EVOO due to the added zeaxanthin. The spectra (a), (b), and (c) were analyzed by means of the mathematical method described above, and the calculated spectra are shown in dashed lines. The spectrum calculated from experimental (c) overlaps perfectly with the difference between the two absorption spectra (a) and (b), even if zeaxanthin is a carotenoid not likely to be found in EVOOs. Moreover, Figure 3B shows the contributions to absorbance proceeding from the two reference pigments lutein and β-carotene. It is evident the prevalent contribution of β-carotene to the absorbance of this "oil", as expected from the spectral characteristics of zeaxanthin. An increase of the calculated concentrations of lutein and of β-carotene corresponds to the increased absorbance of the oil caused by the added zeaxanthin. These concentration increments are a mathematical consequence of the linear dependence of the zeaxanthin spectrum on the spectra of lutein and β-carotene.

From these findings several considerations can be drawn:

1. The presence of a carotenoid (here zeaxanthin) different from lutein and β-carotene leads to concentrations different from their analytical (e.g., determined by HPLC) values.
The introduction of zeaxanthin as a third carotenoid in the set of reference spectra would result in the impossibility to diagonalize the overlap matrix $S$ (eq 1), given the evident linear dependence of its spectrum on those of lutein and $\beta$-carotene in the 390–720 nm spectral region. Hence, this example demonstrates that it is impossible to extract specific and quantitative information about all carotenoids from the oil absorption spectrum, even when they are present in non-negligible concentrations.

Even if at least 15 carotenoids were found in extracts of different oils, a much smaller number of carotenoids, two or three, is sufficient to describe their functional space. This is supported by two general observations: (1) the spectral range of their absorption spectra is limited to the 390–550 nm range; (2) most of them are typically shaped in a very similar way in this spectral range. For this reason, lutein and $\beta$-carotene have been chosen, since they are probably the most significant from both the analytical and the nutraceutical point of view.

Addition of a Nonpigment Substance Spectrum to the Reference Spectra. In our approach, a fifth spectrum vector has been added in order to reproduce the absorption in the 390–720 nm spectral region of an EVOO deprived of any pigment. In fact, other substances are present in oils, partially due to the degradation processes of the original components, including the same pigments. This absorption curve is well reproduced by the shape of the absorption spectrum of aged triolein or of refined olive oil. This absorption has an absorbance close to zero in the visible and gradually increases moving toward the near-UV spectral region. The absorption of an aged sample of triolein, reported in Figure 4 together with the spectrum of purified triolein, is clearly due to impurities, whose concentration increases with the triolein aging: this absorption is used as that equivalent to an unspecified one-molar substance. It is interesting to observe that its shape is similar to the residual absorbance of EVOOs after complete photodestruction of the pigments. Since the shape of this function is quite different from the absorbance of the pigments, it cannot be represented by the eigenvectors of the true pigment-space. Moreover, the presence of this function is especially interesting for studies of EVOO aging, as described in the following paragraph.

Several Applications of the Method to EVOO Samples. In Figure 5, the measured absorption spectra of four one-year-old EVOOs from Granada (Spain) are reported. Notable differences of their absorbance both in shape and in intensity can be observed. The calculated spectra (eq 5) and the difference between the calculated and the measured absorbances are reported as well. The experimental absorbance has been reproduced with remarkable precision by the absorptions of the four $+1$ light absorbers described: differences of ca. 1% are found only at the 485 nm maximum. The experimental spectra in Figure 5 present well resolved peaks. In correspondence to the differences of absorbance spectra, the four EVOOs have significant differences in pigment composition, as reported in Table 3.

However, not all the EVOOs are reproduced with the above quasi-absolute precision. In Figures 6A,B the spectra of two Italian EVOOs are represented. They show, respectively, medium and intense differences between experimental and

![Figure 3](image_url)

Figure 3. (A) UV−vis absorption spectra of an EVOO (a) and of the same one with the addition of a certain amount of zeaxanthin (b) with the respective calculated spectra (dashed lines) and residues (black line). The spectrum of the added zeaxanthin (c) is also reported. (B) Contributions of lutein and $\beta$-carotene to the same EVOO sample due the addition of zeaxanthin.

![Figure 4](image_url)

Figure 4. UV−vis absorption spectrum of purified (red) and “raw” commercial triolein (brown) curves.
calculated absorbance. In Table 3 the correspondent concentration of the pigments, calculated by means of eqs 3 and 4, is reported.

The spectrum in Figure 6A is typical of many Italian EVOOs. In the example (a one-year old monovarietal oil from fruits of "Frantoio" cultivar), the disagreement between calculated and experimental spectra is appreciable in a single spectral zone, i.e., in the 465–495 nm range (on the average 5.3%), where the calculated absorption is much more resolved than the experimental one. This disagreement regards part of the carotenoid spectral region, for which only lutein and $\beta$-carotene spectra define the spectroscopic functional space, as previously discussed. In Figure 6A, the contribution of these two pigments to the spectrum of "Frantoio" EVOO is also reported: the maxima of both pigments, at 484 and 491 nm, respectively, in the disagreement zone, are red-shifted with respect to the actual maximum (481 nm) of the oil. Thus, there is no possibility to obtain a maximum at 481 nm by combining the extinctions of lutein and $\beta$-carotene with positive coefficients. On the other hand, they are the only pigments, among the four mathematically present, appreciably absorbing light in the 480 nm region.

In a recent work on Italian EVOOs from different cultivars, it has been shown that, on the average, lutein is present in these oils as 39% by weight of the sum of the weights of the other 12 xanthophylls HPLC-determined. In "Frantoio" oils lutein represents the 61%, i.e., the maximum content in the oils there investigated. Thus, minor carotenoids, such as neoxanthin, violaxanthin, luteoxanthin, antheraxanthin, mutatoxanthin etc., are present in concentrations well over the values reported for many Spanish EVOOs in which the above minor xanthophylls are present in neatly lower percentage, and lutein is present from 61 up to 93.1% of total xanthophylls. The above data suggest that the contribute of minor xanthophylls to oil absorbance in the Italian EVOOs of Figures 3 and 6(a) is higher than in the Spanish EVOOs of Figure 5. As a consequence, the functional space of lutein and carotene spectra cannot represent the spectral contributions of some of these "minor" pigments. This is reflected by some inaccuracy in

### Table 3. Molar Concentration (mol/L) of the Four Pigments of the Four Andalusian EVOOs (Figure 5), of a Typical Tuscan "Frantoio" EVOO (Figure 6a), a Young Tuscan EVOO (2008 Harvest; Spectra Recorded in 2009) (Figure 6b) and the Aged Corresponding Tuscan EVOO (the Same Sample, but Spectra Recorded in 2013) (Figure 6c)

<table>
<thead>
<tr>
<th>EVOO samples</th>
<th>Coprovir</th>
<th>Poniente de Granada</th>
<th>Fuentes de Dios</th>
<th>Loja</th>
<th>Typical Tuscan &quot;Frantoio&quot;</th>
<th>Young Tuscan</th>
<th>Aged Tuscan</th>
</tr>
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<tr>
<td>pheophytin-a</td>
<td>$8.74 \times 10^{-08}$</td>
<td>$5.00 \times 10^{-08}$</td>
<td>$4.96 \times 10^{-08}$</td>
<td>$5.13 \times 10^{-08}$</td>
<td>$1.65 \times 10^{-05}$</td>
<td>$5.20 \times 10^{-05}$</td>
<td>$4.23 \times 10^{-05}$</td>
</tr>
<tr>
<td>pheophytin-b</td>
<td>$9.43 \times 10^{-07}$</td>
<td>$6.65 \times 10^{-07}$</td>
<td>$4.42 \times 10^{-07}$</td>
<td>$7.54 \times 10^{-07}$</td>
<td>$1.58 \times 10^{-05}$</td>
<td>$2.00 \times 10^{-05}$</td>
<td>$6.10 \times 10^{-06}$</td>
</tr>
<tr>
<td>lutein</td>
<td>$8.46 \times 10^{-08}$</td>
<td>$7.16 \times 10^{-08}$</td>
<td>$6.65 \times 10^{-08}$</td>
<td>$1.00 \times 10^{-05}$</td>
<td>$1.31 \times 10^{-05}$</td>
<td>$4.60 \times 10^{-05}$</td>
<td>$3.05 \times 10^{-05}$</td>
</tr>
<tr>
<td>$\beta$-carotene</td>
<td>$2.35 \times 10^{-08}$</td>
<td>$2.56 \times 10^{-08}$</td>
<td>$1.82 \times 10^{-08}$</td>
<td>$2.83 \times 10^{-06}$</td>
<td>$2.73 \times 10^{-06}$</td>
<td>$3.01 \times 10^{-07}$</td>
<td>$6.95 \times 10^{-07}$</td>
</tr>
</tbody>
</table>
Another interesting example is reported in Figure 6(a): in this case, the method fails in reproducing the intense experimental absorption spectrum of a young (less than two months) EVOO from southern Tuscany. In fact, there are two spectral intervals, centered at 430 and 665 nm, where the calculated absorption is about 1 absorbance unit lower than the measured one, in exact correspondence with the absorption peaks of chlorophyll-a in extra-virgin oils, as determined by addition of chlorophyll-a to triolein and to an EVOO. The same oil of Figure 6(b), after aging almost three years at ca. 4 °C in dark conditions and complete spontaneous pheophytinization, showed a changed absorption spectrum as shown in Figure 6(c), where the usual trend of calculated vs measured spectrum is observed. In Table 3, the calculated pigment concentrations are reported. In order to fit the spectrum of this particular oil before pheophytinization, it would be necessary to add in our treatment other pigments, i.e., chlorophylls. Since chlorophylls are present usually only in freshly extracted oils, the number of pigments was limited to the four previously described. It is clear that the functional spectral space of chlorophyll pigments is complete only for oils in which pheophytinization processes are finished and pyropheophytinization processes are marginal.

In addition to the successful reproduction of the absorption spectra of nonfreshly extracted EVOOs, the method has been tested by measuring the spectra of some EVOO samples obtained by mixing, in defined proportions, two EVOOs with different spectra and pigments composition. The resulting composition in pigments reproduced the proportion of the constituent oils and their pigment compositions with an average error of 1.1 ± 0.6%.

Use of the Method to Check Eventual Thermal Treatments. An experiment was done in order to reproduce a case of bad storage. A sample of olive oil was forced to a controlled thermal degradation, to induce pigments degradation. Chlorophyll content generally reduces up to 80% during the oil extraction process, using only mechanical and physical processes for extraction. These processes result in a considerably reduced chlorophyll level in the oil compared to the olive fruit itself. Pyropheophytins are also produced by subsequent degradations of chlorophyll pigments, and the presence of pyropheophytins is an indication of probable heating or aging effects. In fact, chlorophyll converts to pheophytin and ultimately to pyropheophytins. The proportion of pyropheophytin-a to the total pheophytins is useful in discriminating fresh oil from oil which has been in long-term storage or which has been heated in the refining process.35–37 Freshly extracted virgin olive oils do not contain pyropheophytins. However, they are generated during storage in different amounts depending on the conditions of exposition of oil. This reaction is boosted by heat treatments during refining processes, especially during deodorizing. This leads to the possibility of using these degradation products of chlorophyll as a marker for quality and traceability of virgin olive oils. For instance, in this way, it can be determined if an olive oil, labeled as virgin, was properly stored or, in contrast, if the virgin olive oil was cut with refined oils. For all investigations, it is essential to consider the exact harvesting date. Generally, the typical analysis according to ISO 29841:2009 is a chromatographic analysis. In this paragraph, we would like just to put the attention on the large spectral changes affecting the UV–vis spectra of EVOOs, when the temperature of storage increases. In Figure 7, a case of thermal degradation at 80 °C, after a different heating time, is shown. The curves obtained after heating at T = 80 °C and after 6, 24, 28, and 46 h show a gradual change. It is possible to see that after few hours from the warming the oil spectrum changes in its shape and relative intensity. After more than 24 h, the changes are drastic. This method could indeed reveal the presence of oil undergone to a refined treatment or simply subjected to a bad storage condition for example during its transport, and further extensions of our method to include other pigments or to study different cases of thermal or illumination effects are expected to provide a useful, fast, and simple tool to check the quality of olive oils.

In this work, a mathematical treatment of UV–vis absorption spectra of olive oil is developed to extract quantitative information about four among the most important pigments in extra-virgin olive oils, namely, β-carotene, lutein, pheophytin-a, and pheophytin-b. To explain the reliability and applicability
of this fast and not destructive method, several examples, underlining positive aspects and limits, have been reported.

The original aim of this research was to extract from the absorption spectrum (390–720 nm) of extra virgin olive oils the most complete information about the concentration of the pigments of interest. Our experiments show that reliable data on the concentration of the chromophores corresponding to these pigments can be extracted. For this reason, a direct comparison with data found by other techniques, such as HPLC, is not appropriate, as shown through an example in the Supporting Information. For instance, because of the typical presence in many EVOOs of fat acids-esterified lutein,\(^2\) the concentration of lutein found by this spectral analysis is slightly higher than that determined by HPLC, since the absorption spectra of this xanthophyll and that of its known esterified-forms coincide within experimental errors. In parallel, the obtained UV-concentration of pheophytin-a represents the sum of the concentrations of pheophytin-a and its epimers, since the molar extinction spectra of these coincide almost exactly with the pheophytin-a one. This is also shown in the Supporting Information. The intrinsic differences between the HPLC method and that proposed in this work confirm that a direct comparison between them is not appropriate. However, as shown by the examples reported in this work, the data obtained from the analysis of UV–vis spectra, analytically oriented to the chromophores, preserve analytical interest for systems where the four pigments, chosen as mathematical spectral references, are dominant, as seems to be the case for most of the EVOOs analyzed so far.

Moreover, among the possible applications of this fast method, its successful applicability to put in evidence eventual thermal degradation or not-appropriate storage conditions of EVOOs is discussed. Despite its not strictly quantitative use, this method appears very useful for a fast screening of EVOOs.

### ASSOCIATED CONTENT

[Supporting Information](#)

Values of overlapped matrix for the four pigments used to perform the mathematical treatment. A detailed description of the validation of the method. A detailed comment about the comparison between data calculated from the method proposed and from HPLC analysis, with an example of HPLC determination of pigments quantity for an EVOO sample. This material is available free of charge via the Internet at http://pubs.acs.org.

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**Notes**

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