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Fluorescence of Vegetable Oils: Olive Oils

MAURIZIO ZANDOMENEGHI,* LAURA CARBONARO, AND CHIARA CAFFARATA

Dipartimento di Chimica e Chimica Industriale, Università di Pisa, Via Risorgimento 35, 56126 Pisa, Italy

Fluorescence spectra of undiluted extra virgin olive oil obtained with the traditional setup (right-angle fluorescence) show considerable artifacts and deformations due to self-absorption phenomena, even when the spectra are corrected for inner filter effects. On the other side, front-face fluorescence spectra are much less affected by self-absorption. Front-face fluorescence of native olive oil reveals the presence of different fluorophores and can provide information about their amount. From the intense emission at ca. 315−330 nm, it is possible to detect fluorescent polyphenols and pherols and to evaluate their overall content. Low-intensity emission bands at 350−600 nm are correlated to vitamins and other important molecules. Among them, the fluorescence of the riboflavin fluorophore can be used to evaluate its concentration. The intense emission of chlorophyll derivatives, measured in the 640−800 nm spectral region, can provide information on their concentration.

KEYWORDS: Front-face fluorescence; olive oil; polyphenols; vitamins; pheophytins

INTRODUCTION

Direct spectroscopic analysis of native oils is clearly more convenient than methods (*1*−*7*) which require procedures such as solubilization in solvents, extraction, and treatment with chemicals. At the present, direct spectroscopic techniques in the UV−vis spectral region have been scarcely employed in the investigation of edible vegetable oils. In fact, UV−vis absorption spectroscopy has been used mainly with 1% solutions of oils in hydrocarbon solvents, where the absorbance (ABS) can be measured until the far-UV region, providing some information on quality, state of preservation, and possible alterations of the oil due to industrial processes. On the other hand, ABS measurements in the middle-UV spectral region and direct fluorescence studies of oils are limited because of the typically very high light absorption in such spectral region, i.e., from 10 up to 150 ABS units (AU), on passing from 325 to 260 nm. Although the ABS of olive oil samples here considered is 2−12 AU in the same spectral region, i.e., much more transparent than, for instance, undiluted peanut, maize, and sunflower oil, such absorbance is still by far too high for traditional vertical beam "right-angle" (RA) fluorescence measurements. The latter require that the absorbance at the excitation wavelength is smaller than 0.1 and close to zero in the emission spectral zone (*8*) to measure a fluorescence intensity which is proportional to the fluorophore concentration and to avoid self-absorption effects. To the best of our knowledge, only two studies on the fluorescence of olive oils are reported. In the first (*3*), diluted solutions of oils in acetone were investigated to quantify the chlorophylls and pheophytins using a general method (*9*, *10*). In the second study (*11*), direct RA fluorescence measurements on undiluted olive and other vegetable oils were undertaken by exciting oil samples with the strong 365 nm emission lines of an Hg lamp. The features of the spectra reported raise doubts about the presence of experimental artifacts related to massive absorption phenomena regarding both the excitation light (*primary inner filter effects*) and the emission light (*secondary inner filter effects*) (*8*). Probably, only the emission band at 670−680 nm, clearly due to chlorophyll-like chromophores, is not strongly affected by such artifacts.

Hence, the fluorescence of olive oil is not known. This is quite surprising as natural fluorophores, such as polyphenols (*7*, *12*−*14*), vitamins (*13*, *15*), and chlorophyll derivatives (*3*, *16*−*19*), are present in olive oil and their emission characteristics could be important in both scientific research and technical and commercial applications.

In this study, we report the fluorescence spectra of native extra virgin olive oils obtained with a front-face (FF) fluorescence technique, which eliminates or strongly reduces the typical artifacts of the ordinary RA measurements of high-absorbance samples. We will show the main classes of fluorescent molecules in virgin olive oil and propose some methods to evaluate their concentration. In addition, we will evidence peculiar spectral characteristics of olive and seeds oils.

MATERIALS AND METHODS

Materials. All solvents, of HPLC grade, were from Carlo Erba, Italy. RBF (98% purity), 7,8-dimethyl-10-D-ribitol-1-yl-10*H*-benzo[*g*]pteridin-2,4-dione; hydrocaffeic acid (HCA) (98%), 3,4-dihydroxyhydrocinnamic acid; gallic acid (97%), 3,4,5-trihydroxybenzoic acid; syringic acid (98%), 4-hydroxy-3,5-dimethoxybenzoic acid; caffeic acid (97%), 3,4-dihydroxycinnamic acid; ferulic acid (99%), *trans*-4-hydroxy-3-methoxycinnamic acid; vanillic acid (97%), 4-hydroxy-3-methoxybenzoic acid; and vitamin E, α-tocopherol (97%) were from Sigma-Aldrich Co. (Milano, Italy).

* Author to whom correspondence should be addressed. Phone: +39-050-2219267. Fax: +39-050-2219260. E-mail: zando@dcci.unipi.it.

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Samples of extra virgin olive oil O1, an intensely green oil of southern Tuscany produced in December 2003, were provided by Oleificio Aldobrandeschi (Campagnatico, Grosseto, Italy); samples of oil O2, a green-yellow oil of northern Tuscany of identical age as O1, were provided by S. Corti (Pisa). Oil O3, a green-yellow oil of Assisi, Umbria, similar in color to O2 but produced one year before O2, and oil O4 "Ligustro", a tuscan oil of the same age as O3, were provided by A. Mattei (Carapelli spa, Florence). Edible maize, peanut, and sunflower oils were purchased in a store. The samples were stored in the dark and at room temperature.

Addition of Antioxidants and of Riboflavin to Olive Oil. Materials to be added were first dissolved in solvents (vitamin E in hexane/isopropyl alcohol (IPA) 2:1, ca. 15 mg/mL; gallic acid and HCA in ethanol, ca. 15 mg/mL; RBF, in DMSO or in IPA, ca. 10 or 5 mg/mL). Progressive additions of negligible volumes (up to 20 μL) of the above solutions to 3 or 5 mL oil samples and slow mixing until uniformity of solutions was achieved led to the oil samples enriched with the added substance.

Extraction of Olive Oils. (a) Polyphenols: 2.5 mL of oil was extracted with 3 mL of MeOH/H_2O, 4:1 v/v, three times. In each extraction, phases were subjected to strong agitation for 1 h. Separation of the phases was carried out after 15 min of centrifugation at 5000 rpm. (b) Carotenoids: 3 mL of methanol was added to 2.5 mL of oil. After 1 h of agitation, the methanol phase was recovered. The extraction was repeated five times.

HPLC and Spectroscopic Measurements. An HPLC Jasco 880 Pu pump and a spectrophotometric Shimadzu SPD10A detector were used, with 4.6 mm × 250 mm a S5 ODS2 Spherisorb column. The mobile phase CH_3CN/H_2O/TFA in the volume ratios 200:400:1 was suitable for the isocratic RP-HPLC separation of HCA, caffeic, vanillic, and ferulic acids in methanol extracts of oil with retention times of about 9.5, 10.7, 11.5, and 16.8 min, respectively, at a flux of 0.5 mL/min at 30 °C. For the RP-HPLC determination of RBF, the mobile phase was MeOH/H_2O 100:50 (v/v). The determination of the content of lutein was made according to an RP-HPLC method (*20*). Standard solutions of pure lutein in methanol were obtained by semipreparative HPLC from methanol extracts of *Zea mais* flour; the lutein concentration in them was determined by measuring the ABS at 445 nm, ϵ (445) $=133\,000\ M^{-1}\ cm^{-1}$.

A Cary 219 spectrophotometer was used for absorbance measurements. The ABS values reported refer to a 1 cm optical path. Suprasil cells, 0.2−10 mm optical path, were used for absorption measurements.

Fluorescence spectra were recorded with an ISA Fluoromax II photon-counting spectrofluorometer. The instrument was equipped for front-face measurements with a cell holder designed to accommodate 1−10 mm optical path spectrophotometric cells that could be oriented at various incidence angles, α, of the excitation light beam, $0° \le \alpha \le 90°$. With $\alpha = 31°$, the light signal due to scattering and stray light from pure liquids (water, methanol, hexane, IPA, and DMSO) was minimized, meanwhile avoiding any reflection of the excitation beam by the cell window and by the underlying liquid surface of the sample into the emission monochromator. The emission was observed at 90° to the incident beam, i.e., 31° with respect to the illuminated cell surface. This surface was in the optical focus of the exciting beam. All the surfaces of the cell holder were black.

A 5 mm × 10 mm optical path suprasil cell was used in FF fluorometry; a 10 mm × 10 mm suprasil cell was used for RA fluorometry. Spectral bandwidths of 0.25 (or 0.5) and 6 nm for the excitation and the emission slits, respectively, were employed for the emissions excited at $\lambda_{exc} \ge 400$ nm, in particular, for the fluorescence of chlorophyll pigments. The excitation and the emission slits were 1.5 and 3 nm, respectively, for $\lambda_{exc} < 400$ nm, mainly for the emission of other fluorophores. The integration time was 0.5 s, and the wavelength increment during spectrum scanning was 2 nm. In these experimental conditions, spectra with satisfactory intensity, resolution, and signal-to-noise ratios were recorded, while the substrate photolysis was negligible.

The intensity of the spectra was determined as the ratio of the emission signal, *S* (counts per second), to the intensity of light from the excitation monochromator, *R* (mV), measured by means of a photomultiplier and a photodiode, respectively. When necessary, a correction of emission spectra with $\lambda_{exc} < 400$ nm to eliminate gratings "second order" effects has been introduced (next section).

Calculation of the Phantom Band in the Red Spectral Region. We recorded the FF emission spectra of a methanol solution of HCA (λ_{exc}= 275, 280, and 290 nm; emission maxima at 315 and 630 nm) and vanillic acid, (λ_{exc}= 280, 285, and 290 nm; emission maxima at 341 and at 680 nm) up to 800 nm. The bands at 630 and 680 nm were phantom bands due to the second-order transmission of the 315 and 341 nm fluorescence bands, respectively, by the emission monochromator. Then, we calculated the ratio $I(2\lambda)/I(\lambda)$ of the emission intensity, *I*, for each λ of the six spectra above, with λ varying from 290 to 400 nm. We used the mean value (standard deviation 3.2%, on the average) of the six ratios, at each single λ, as second-order transmission factors of the UV fluorescence to the red spectral region.

The second-order effect of the UV fluorescence in any oil emission spectrum at, for example, 630 nm is calculated by multiplying the measured intensity at 315 nm for 0.384, i.e., the transmission factor corresponding to 630 nm. Then, this value is subtracted from the measured emission at 630 nm, obtaining the fluorescence at 630 nm, free of the second-order reflections of the emission monochromator. The transmission factor values are in the range between 0.550 (for emissions at 580 nm) and 0.102 (at 750 nm). Very similar transmission factors were obtained by using RA spectra.

Scattering and Fluorescence in Emission Spectra. When we record the emission spectrum of a pure liquid sample completely transparent in the spectral region of interest, some light is always collected. This effect is due both to stray light of the excitation monochromator and to the incomplete rejection of the excitation photons by the emission monochromator. The presence of insoluble material aggregates further enhances this scattering effect. Since aggregates are generally present in olive oils, our samples were subjected to centrifugation (5000 rpm for 20 min) in order to reduce turbidity to a minimum. This procedure revealed itself to be sufficiently effective and further procedures to reduce the scattering throughout optical means, for example, by the use of polarizers, were not undertaken.

RESULTS AND DISCUSSION

Physics in Right-Angle and in Front-Face Fluorescence Measurements. In the traditional RA technique, the excitation beam propagates perpendicularly to the input window. The emission beam coming out from the center of the sample is analyzed at 90°. The measured emission at the excitation and emission wavelengths, E_m (λ_{exc}; λ_{em}), depends on the light absorbed in both the excitation and emission paths through the sample according to Lambert and Beer's law, as described by the equation:

$$E_m(\lambda_{exc};\lambda_{em}) = KF(\lambda_{exc};\lambda_{em})\ 10^{-(ABS(\lambda_{exc})+ABS(\lambda_{em}))0.5} \quad (1)$$

Where 0.5 cm is the typical light path, $F(\lambda_{exc};\lambda_{em})$ is the fluorescence measured at a very low ABS of the solution, and *K* is a normalization constant. Here, we define the $10^{(ABS(\lambda_{exc})+ABS(\lambda_{em}))0.5}$ factor as the "ABS correction factor". Equation 1, which is valid approximately up to 0.5−1.0 AU, depending on the optics of the fluorometer (*21*), allows the determination of $F(\lambda_{exc};\lambda_{em})$ through the use of the ABS correction factor and the experimental $E_m(\lambda_{exc};\lambda_{em})$.

The setup of FF fluorescence measurements needs the same window for input and output lights. With highly absorbing samples, the layers next to the sample surface are, at the same time, the ones that absorb more input light and the ones from which the fluorescence is collected to the emission monochromator with the minimum path inside the sample. Hence, these fluorescence rays are simultaneously the most intensively

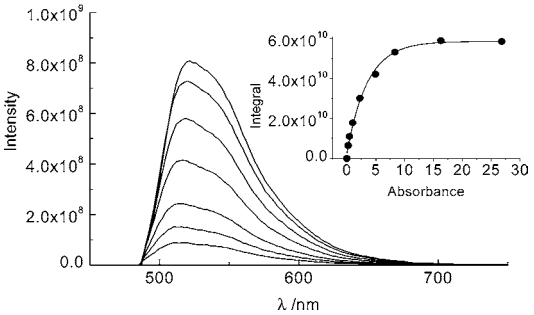

Figure 1. FF Fluorescence spectra of RBF solution in DMSO (0.0159; 0.0318; 0.0630; 0.127; 0.254; 0.382; 0.764 mg/mL) with $\lambda_{exc} = 470$ nm. The curve corresponding to 1.27 mg/mL overlaps the 0.764 one, and it is not shown. Spectra are reported after subtracting the DMSO emission spectrum. In the box inset, the integrals, from 500 to 700 nm, of the above spectra are reported vs the ABS. The solid line fits the experimental data according to eq 2 ($\alpha = 5.85\ 10^{10}$; $\beta = 0{,}28$; $\gamma = 0{,}95$).

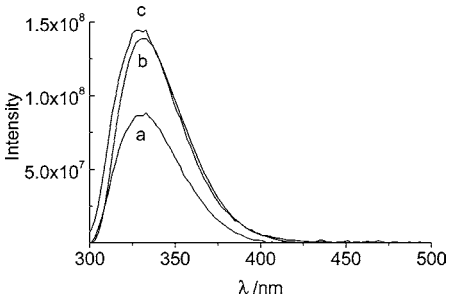

Figure 2. Fluorescence spectra of vitamin E dissolved in MeOH with $\lambda_{exc} = 285$ nm. (a) FF spectrum of a solution with ABS = 0.093 AU; (b) FF spectrum of a concentrated solution with ABS = 9.4 AU. The spectrum intensity is divided by 10 for comparison purposes; (c) RA spectrum of a solution with ABS = 0.078 AU.

produced and the least absorbed. The layers located deeper in the sample, in which the excitation light is weak, are also those whose fluorescence may be extinguished by self-absorption because of the large path inside the sample. From these considerations, we deduce that these absorption events affect the FF spectra less than the traditional RA ones (*21*). However, these suppositions have to be validated experimentally since it does not take into account experimental aspects, such as incidence angle, convergence degree of the excitation beam, geometry of the light-collecting optics, positioning of the sample (*21* and references *54*−*60* cited therein), which can deeply affect the measurements.

RA and FF Emission Spectra of Test Fluorophores at High Concentration. To verify how the intrinsic properties of the fluorometer (optics and measurement devices) affect the measurements, we determined the fluorescence of two pure test fluorophores using the FF technique in conditions of medium-to-high ABS and the RA technique in the low-ABS limit (**Figures 1** and **2**), under otherwise equal spectroscopic conditions, i.e., slits and integration time. We investigated two test fluorophores whose spectra cover the probable spectral range for olive oil emissions, namely vitamin E, with an absorption band in the 250−310 nm spectral region and emission in the 300−450 nm region, and RBF that absorbs light up to 500 nm, with an emission in the visible region from 500 up to 700 nm.

Figure 1 shows the FF fluorescence curves of RBF at different values of ABS(λ_{exc}), obtained at different concentrations of RBF in DMSO. The fluorescence spectra approach a limiting band for ABS(λ_{exc}) larger than 15 AU. The integral, *I*, of the fluorescence intensity in the emission range versus ABS(λ_{exc}) reported in the inset of **Figure 1** can be described by the equation:

$$I = \alpha\,(1 - \gamma e^{-\beta \mathrm{ABS}(\lambda_{exc})}) \qquad (2)$$

where α, β, and γ are parameters depending both on the fluorophore and on the fluorometer features. It is worthwhile to point out that the FF fluorescence spectra of RBF in solutions up to 15 AU are shaped as the reliable fluorescence spectra obtained with a RA setup at low absorbance (ABS(λ_{exc}) < 0.1 AU) in the same solvent. A similar behavior was observed for the solutions of vitamin E in methanol, although the asymptotic behavior was detected at lower absorbances (ABS(λ_{exc}) > 2.5 AU). **Figure 2c** shows the RA fluorescence spectrum of a vitamin E solution with ABS(λ_{exc}) = 0.078 that is in the range of a linear dependence of the fluorescence intensity on the absorbance. The corresponding FF spectrum (**Figure 2a**) showed an intensity about 1.6 times lower than that of the RA spectrum (**Figure 2c**). When the ABS is very high, ca. 9.4 AU, an FF spectrum 10-fold more intense than the RA one, but with a very similar band-shape, was obtained (**Figure 2b**).

From the above experiments, some conclusions can be drawn. (i) The FF spectra, measured with ABS(λ_{exc}) up to 10 AU, are very similar to the "ideal" ones, i.e., the RA spectra measured at very low ABS(λ_{exc}). (ii) The intensity of the FF spectra depends exponentially on the fluorophore concentration, as described by eq 2. (iii) The asymptotic behavior, i.e., the value of ABS(λ_{exc}) at which the fluorescence intensity becomes constant with increasing absorbances, depends on the fluorophore.

In the presence of two or more fluorophores, as in the olive oil samples, we expect that the fluorescence intensity is linear with the concentration of the respective fluorophores only at those excitation wavelengths, where the absorption of all fluorophores is relatively low. When this is not the case, we should observe that the overall spectrum is deformed and that the contribution to the fluorescence becomes independent from the fluorophore concentration.

Direct Fluorescence of Oils: Right Angle Emission. Figure 3 presents the fluorescence spectra of olive oil O2, obtained with the traditional RA technique. The excitation wavelengths are in the region 280−450 nm, where the ABS of the oil is high, and in the visible region, 500−650 nm, where the ABS is much lower (**Figure 4a**). In **Figure 4b**, the ABS spectrum of a maize oil solution in *n*-hexane is reported for comparison with the extra virgin olive oil. The absorption spectra of sunflower and peanut oils are similar in shape and in intensity to the maize spectrum for $\lambda > 250$ nm.

Focusing on the 300−600 nm region, the emission spectra of an olive oil with $\lambda_{exc} = 320$ and 350 nm resemble those previously reported (*11*), with $\lambda_{exc} = 365$ nm, at least for the position of the minima and maxima. For example, the spectrum measured with $\lambda_{exc} = 320$ nm (**Figure 5d**) for wavelengths up to 500 nm, shows peak maxima at 385, 439, and 470 nm and minima at 415, 453, and 480 nm. These minima correspond almost exactly to the absorption peaks centered at 417, 455, and 482 nm of the oil absorption spectrum in **Figure 4a**. This correspondence indicates the presence of fluorescence self-absorption, whose magnitude, however, cannot be quantified unless the "true" fluorescence is measured. The "true" fluorescence can be defined as the fluorescence measured in a sufficiently dilute solution in an ideal solvent, which does not affect the fluorescence properties of oil. Since the ABS of the oil in the excitation and emission regions is quite high, we applied the correction for inner filter effects due to the absorption

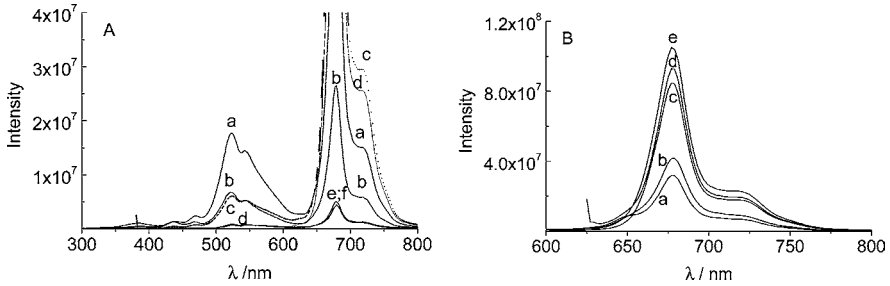

Figure 3. (**A)** RA fluorescence spectra of the extra virgin olive oil O2 where λ_{exc} is (a) 350 nm; (b) 320 nm; (c) 380 nm; (d) 410 nm; (e) 300 nm; (f) 280 nm; **B**) (a) 580 nm; (b) 550 nm; (c) 510 nm; (d) 620 nm; (e) 530 nm. The excitation and emission bandwidths are 1.5 and 3 nm, respectively.

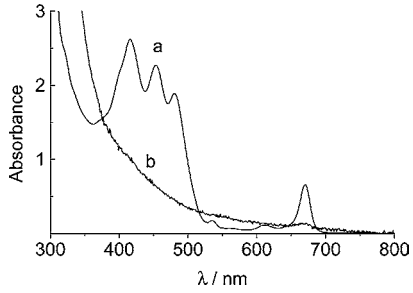

Figure 4. (a) Absorption spectrum of olive oil O2. (b) Absorption spectrum of a solution of maize oil in *n*-hexane (1:100) v/v. The spectrum intensity is multiplied by 100.

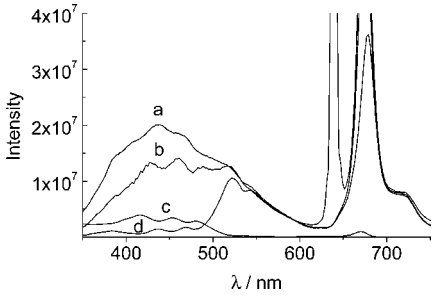

Figure 5. Emission spectra of olive oil O2 with λ_{exc} = 320 nm. (a) FF spectrum; (b) RA spectrum with ABS correction; (c) ABS spectrum of the oil multiplied by 10^6; (d) RA spectrum without ABS correction.

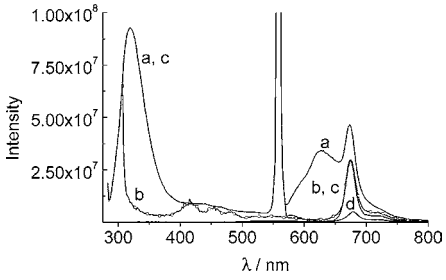

Figure 6. Emission spectra of olive oil O2 with λ_{exc} = 280 nm. (a) FF spectrum; (b) RA spectrum with ABS correction and multiplied by 3.7; (c) FF spectrum second-order corrected; (d) RA spectrum.

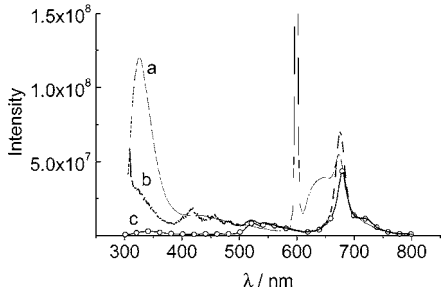

Figure 7. Emission spectra of olive oil O2 with λ_{exc} = 300 nm. (a) FF spectrum; (b) RA spectrum with ABS correction and multiplied by 10; (c) RA spectrum multiplied by 10.

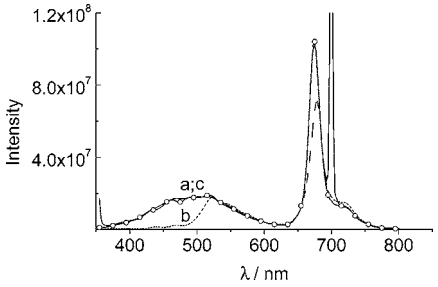

Figure 8. Emission spectra of olive oil O2 with λ_{exc} = 350 nm; (a) (—) FF spectrum; (b) (- - -) RA spectrum and (c) (–○–) RA spectrum obtained by applying the ABS correction.

both of the excitation and the fluorescent radiation, eq 1. The corrected spectrum is shown in **Figure 5b**. We note, a wavelength-dependent intensification of the spectrum, such that the minima in the uncorrected spectrum become maxima in the corrected one and vice versa. This behavior is due to the high value of the ABS correction factor and its rapid variations in the 375−525 nm range where the correction factor acts on the very low values of the emission intensity. In contrast, in the 525−640 nm region, the correction factor is almost constant and about 2 orders of magnitude lower than at shorter wavelengths. The FF emission spectrum of olive oil O2 is reported in **Figure 5a** for a preliminary comparison between RA and FF spectra with regard to their band shape, which is the only meaningful feature to take into account. The curves have been normalized to the same values in the lowest absorption spectral zone, 550−600 nm, where the corrections for inner filter effects are less dramatic.

Figure 6d reports the measured RA emission with λ_{exc} = 280 nm, a wavelength at which the ABS of the oil is about 12 AU. In this case, the intensity of the signal up to 500 nm, curve **d**, is extremely low, $\sim 10^{-3}$ times the FF signal, curve **a**, constant and of the order of magnitude of the noise. Such experimental data generate, after correction for the absorption, a spectrum, curve **b**, which is about proportional to the absorption spectrum but has no meaning. Instead, at the red end of **Figure 6d**, a band due to chlorophyll-type chromophores appears, with a maximum at 679 nm that shifts to 675 nm after ABS correction (**Figure 6b**).

Figures 7 and **8** show the RA emissions, with λ_{exc} = 300 and 350 nm, respectively. These spectra resemble the emission spectra with λ_{exc} = 280 and 320 nm already analyzed in **Figures 6** and **5**, respectively, with the difference being that, with λ_{exc} = 350 nm, the RA-corrected spectrum (**Figure 8c**) is more similar to the one measured with the FF technique both in the

350−500 nm and in the chlorophyll spectral region. In fact, the minimum at 360 nm of the ABS spectrum of olive oil is close to $\lambda_{exc} = 350$ nm, where ABS $\approx$ 1.45 AU. Thus, the information contained in this RA spectrum is sufficient to provide, once the correction for the absorbance has been applied, a band-shape approximately similar to the FF spectrum, which we assume as the correct one (see below).

Since the fluorescence spectra of Kyriakidis and Skarkalis (*11*) have been obtained with 365 nm excitation, i.e., close to the minimum of the absorbance of extra virgin olive oils, we think that spectra much more reliable than those published could be obtained by applying the absorbance correction, eq 1.

In any case, it is clear that the intensification of the fluorescence, observed after oxidative heating of greek olive oils (*11*), is due to the lowering of the self-absorption of emission consequent to the lowering of the absorbance in the region of carotenoids, which are partially destroyed by the oxidative thermal treatment (*22*).

With regard to of chlorophyll chromophores fluorescence, pheophytins are known to emit approximately at 670 nm, depending on the solvent (*9*, *3*). We observed in all the RA and FF spectra, **Figures 3**, **5**, **6**, **7**, and **8**, a strong band in the 640−780 nm region. This emission band differs in the RA and FF spectra both in intensity and in the position of its maximum, at 679 nm in the RA spectra and at 675 nm in the FF and in the ABS corrected RA spectra, thus indicating that 675 nm is the right wavelength for the emission maximum.

Finally, it is worth noticing the agreement, in the measured range, between the FF and the corrected RA spectra, when $\lambda_{exc} = 350$ nm, **Figure 8**.

Direct Fluorescence of Oils: Front-Face emission. Figures 5−8 show the FF emission spectra together with the corresponding RA spectra. Besides the chlorophyll region, significant differences in the number, shape, intensity, and position of the bands in RA and FF spectra are evident. A very intense band centered at about 325 nm, absent in the RA spectra, appears in the FF spectra with $\lambda_{exc} = 280$ and 300 nm, **Figures 6** and **7**, respectively, while the sharply structured emission of the RA spectra in the 400−600 nm range is replaced by a large, almost unstructured, and more intense emission in the FF spectra. After ABS correction, the RA spectrum (**Figure 8c**) with $\lambda_{exc} = 350$ nm approaches the FF one (**Figure 8a**), as previously noticed.

The FF spectra at $\lambda_{exc} = 280$ nm (**Figure 6a**) and 300 nm (**Figure 7a**) display an emission at $2\lambda_{exc}$ i.e., at 560 and 600 nm, respectively. This emission is an instrumental artifact due to the scattered excitation light at 280 or 300 nm that partially passes through the emission monochromator as reticule second-order reflection. In addition, part of the fluorescent light, centered at ca. 325 nm, goes through the emission reticule as a second-order band centered at 650 nm, overlapping with the chlorophyll emission light. It is possible to calculate the intensity of this "phantom" band at 650 nm and subtract it from the FF spectrum (see the section "Calculation of the Phantom Band"). The single case of $\lambda_{exc} = 280$ nm is also reported in **Figure 6a, c** which evidences the correction effects and the consequent emerging of the fluorescence band of pheophytins with maximum at 675 nm.

It is still to be demonstrated that the FF spectra are similar to the true fluorescence spectra of olive oil. This is a difficult task to assess since we are able to compare the FF spectra only with traditional RA ones obtained in sufficiently diluted oil samples. Unfortunately, even the choice of the solvent is critical. For example, we found that the emission spectra of two phenol derivatives, such as vanillic and hydrocaffeic acid in two different solvents, *n*-hexane and methanol, but otherwise with identical experimental conditions and similar ABS(λ_{exc}), show a significant dependence of quantum yield and Stokes shift on the nature of the solvent (**Figure 9A, B**). Passing from methanol to hexane, vanillic acid has an ∼30% higher fluorescence yield and an ∼10 nm blue-shifted emission. In contrast, the fluorescence quantum yield of HCA is 6-fold higher in methanol than in hexane, and the Stokes shift of fluorescence reduces to 3 or 4 nm on passing from hexane to methanol. This solvent dependence of the quantum yield may even be higher for other acid polyphenols. We found that the fluorescence of syringic acid is one fifteenth less intense in methanol than in a 2:1 (v/v) mixture of hexane and IPA. The maximum is at 352 nm, around 7 nm red-shifted with respect to the fluorescence in hexane−IPA. The above three phenol derivatives are known to be typical components of the antioxidant pool of olive oils, and the antithetic behavior of their fluorescence with solvents illustrates well the difficulty to determine the influence of the solvent on the fluorescence of olive oil. In addition, as fluorescence might be very sensitive to interactions between fluorophores, chromophores, and quenchers through a number of mechanisms, the existence of any solvent able to dilute the oil sample without altering its emissive properties is questionable. Thus, on the basis of (i) the agreement between FF spectra and the ABS corrected RA spectra at $\lambda_{exc} = 350$ nm, in relatively low-absorbance condition, (ii) the greater reliability of FF with respect to RA spectra, as discussed in the section "Physics in RA and FF Fluorescence Measurements", and (iii) the strict similarity between RA spectra at low-concentration and FF emission spectra at high concentrations of test fluorophores, we assume that the spectra closest to the "true" fluorescence are those ones obtained by means of the FF technique with corrections for second-order effects.

Near-UV Fluorescence. Addition and Subtraction of Antioxidants to Olive Oils. We attempted to characterize the origin of the FF fluorescence bands of our olive oil samples. The highest-energy band centered at ca. 325 nm is mainly due to polyphenols whose concentration may exceed 1000 ppm (*23*). Most of these compounds are fluorescent substances which absorb in the 260−310 nm spectral region and emit in the near-UV region with bands centered at 310−370 nm, as it is shown in the cases of vanillic acid, **Figure 9A**, and HCA, **Figure 9B**.

Figure 10 reports four FF emission spectra of olive oil O2 (excitation in the range 275−300 nm) and two FF excitation spectra ($\lambda_{em} = 315$ and 325 nm). The latter spectra have overlapping bands with maxima at 290 and 296 nm, respectively. The position of these maxima, as well as of the emission maxima around 325 nm, rules out that the emission observed in the 320−330 nm range is due to trace amounts of aromatic residues of proteins in the O2 oil. Upon addition of known amounts of one of the three phenol derivatives, HCA, vitamin E, and gallic acid, to four extra virgin olive oils, the fluorescence in the above UV spectral zone becomes more intense. The resulting spectra are shown in **Figure 11 A−D** together with the curves corresponding to the difference between the emission of each sample after and before fluorophore additions. These difference spectra are to be considered the fluorescence spectra of HCA, vitamin E, and gallic acid dissolved in the olive oil as a solvent.

Fluorescence increase is *quasi*-proportional to the increase of the concentration of each antioxidant in all the oils studied. Vitamin E emits in the same spectral region of the oil; therefore, we can express the antioxidant's overall content in oil in terms of the vitamin E concentration which is able to reproduce the observed 325 nm fluorescence band of the native oil. For

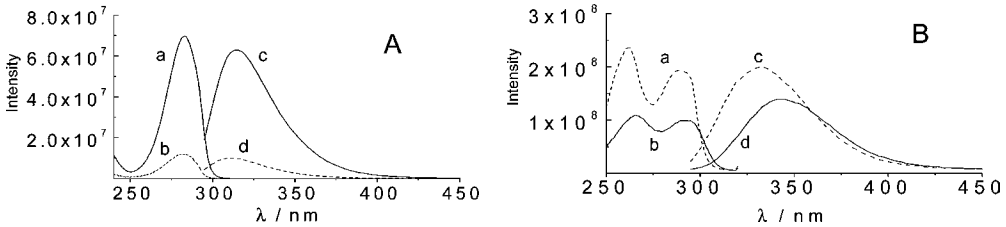

Figure 9. (**A**) RA excitation spectra of HCA (λ_{em} = 315 nm): (a) MeOH, (b) *n*-hexane. RA emission spectra of HCA (λ_{exc} = 285 nm): (c) MeOH, (d) *n*-hexane. (**B**) RA excitation spectra of vanillic acid (λ_{em} = 325 nm): (a) MeOH, (b) *n*-hexane. RA emission spectra of vanillic acid (λ_{exc} = 285 nm): (c) MeOH, (d) *n*-hexane.

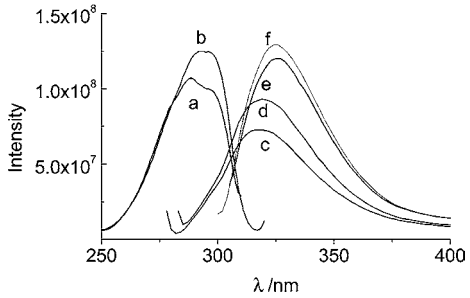

Figure 10. FF Fluorescence excitation and emission spectra of olive oil O2: (a) λ_{em} = 315 nm; (b) λ_{em} = 325 nm; (c) λ_{exc} = 275 nm; (d) λ_{exc} = 280 nm; (e) λ_{exc} = 300 nm; (f) λ_{exc} = 290 nm.

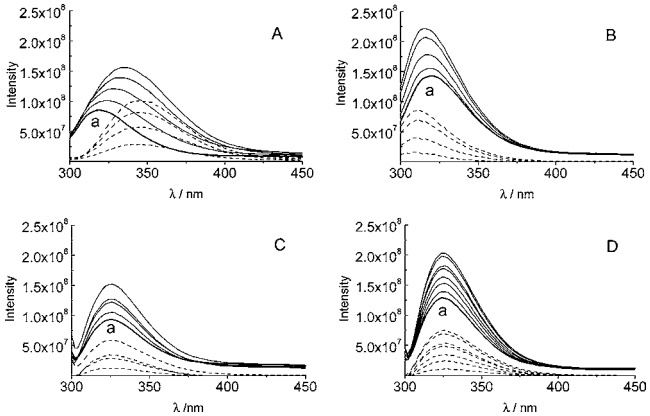

Figure 11. FF Fluorescence emission spectra (**A**) Olive oil: O2; chromophore: gallic acid 0.018–0.073 mg/mL added; λ_{exc} = 280 nm. (**B**) Olive oil: O3; chromophore: HCA 0.095–0.37 mg/mL added; λ_{exc} = 285 nm. (**C**) Olive oil: O4; chromophore: vitamin E 0.088–0.35 mg/mL added; λ_{exc} = 295 nm. (**D**) Olive oil: O1; chromophore: vitamin E 0.079–0.53 mg/mL added; λ_{exc} = 295 nm. 'a' lines: spectra of native oils. Solid lines: from 'b' to top, spectra correspond to increasing amounts of added fluorophore. Dashed lines: differences between added and native oil spectra.

example, we found that the fluorescence, with excitation at 295 nm, corresponds to ca. 670 μg/g of vitamin E in the oil O3, while for oil O1, the concentration was 860 μg/g by applying the method used for RBF determination in cereal flours (*24*).

Finally, a sample of O2 oil was extracted with a methanol−water mixture. The FF emission spectra of the extracted oil, under 270−300 nm excitations, showed a reduction of the intensity of the 325 nm band larger than 50%. The aqueous MeOH phase had a strong emission band in the same spectral zone, and HPLC analysis of this solution revealed the presence of antioxidants. Thus, a subtractive technique confirmed the origin of the 325 nm fluorescence band of oils.

Near-UV−Vis Fluorescence. The Riboflavin Case. As shown in **Figures 5−8**, low-intensity FF fluorescence bands are found in the 350−600 nm emission spectral range for λ_{exc} = 280, 300, 320, and 350 nm. It is difficult to assign these bands because many endogenous molecules fluoresce in the 350−600 nm spectral range. For example, pyridoxine and some of its derivatives have fluorescence maxima in the 385−425 nm region under 280−350 nm excitation, meanwhile vitamins A, K, and D fluoresce in the 480−510 nm range (330−390 nm excitation), NADH and NADPH at 440−464 nm (290−350 nm excitation), and finally, FAD and flavins emit in the 520−535 nm region, with λ_{exc} between 250 and 480 nm (*25*).

In the emission spectra of olive oils, a weak emission centered at 524 nm is detected (**Figure 12a**). The shape of this weak band does not change on varying the excitation wavelength from 400 to 480 nm. This emission likely is due to vitamin B2 or some of the chemically correlated molecules, FAD and FMN. Moreover, on addition of RBF in IPA to the olive oil O2, the 524 nm fluorescence band increases almost proportionally to the added RBF (**Figure 12b−d**).

By evaluating the area of the "native" fluorescence band and taking into account the increases of fluorescence (*24*) due to the added RBF (**Figure 12**), we calculated that the concentration of RBF in the oil O2 is 0.9 μg/g. This quantity agrees well with the reported values of vitamin B2 in olive pulp (*15*). No

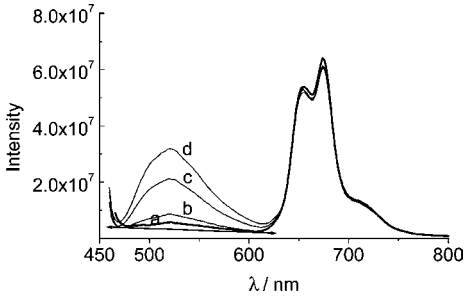

Figure 12. FF fluorescence spectra of oil O2, λ_{exc} = 450 nm. (a) the native oil; (b–d) oil added with amounts of RBF varying from 0.0022 to 0.0087 mg/mL. The baseline, considered to calculate the fluorescence band area, is represented by the two-arrow segment.

data regarding the presence of RBF in olive oils have been reported up to now.

Carotenoids are present in olive oil with a relatively high concentration. For instance, after exhaustive extraction with methanol, the concentration of lutein in our O2 oil was found to be 9.4 μg/g. Carotenoids strongly compete for incident light with other chromophores present in oil due to their concentration and high extinction coefficient, which is of the order of 10^5 M^{-1} cm^{-1} in the violet zone. Consequently, it is probable that carotenoids cause the observed low intensity of the emission of RBF. In fact, RBF is characterized by a low concentration and an extinction coefficient of an order of magnitude smaller than lutein, in the 430−480 nm range.

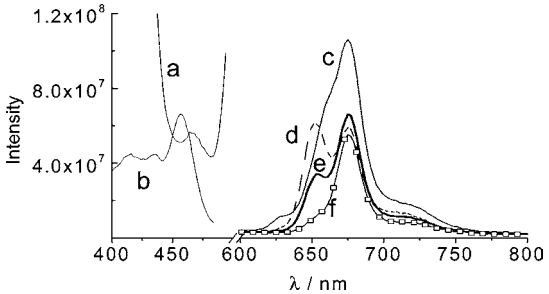

Figure 13. FF Fluorescence excitation spectra of olive oil O2 with (a) $\lambda_{em} = 670$ nm; (b) $\lambda_{em} = 650$ nm. Emission spectra with: (c) $\lambda_{exc} = 440$ nm; (d) $\lambda_{exc} = 460$ nm; (e) $\lambda_{exc} = 470$ nm; (f) $\lambda_{exc} = 480$ nm.

Red to Near-IR Fluorescence. Pheophytins. Pheophytin emission bands, in the 640−800 nm range, absorb light almost at any wavelength shorter than 710 nm. Hence, all the oil emission spectra have some contribution from these fluorophores. The extent of such a contribution depends on the ABS of the pheophytins relative to the ABS, at the same λ, of the other chromophores present. Thus, intense emission is collected under excitation in the near-UV and visible regions, where the absorption of competing-for-light absorbers gradually vanishes on increasing the wavelength.

At least two distinct pheophytin chromophores with different absorption and emission spectra can be easily detected in the excitation and emission spectra. The emission spectra in **Figure 13c−f** obtained on varying λ_{exc} from 440 up to 480 nm, show clearly two bands, the first-one centered at 652 nm and the second at about 675 nm. On increasing λ_{exc}, starting from 440 nm, the emission band at 652 nm gradually emerges until, with $\lambda_{exc} = 460$ nm, its intensity becomes equal to the 675 nm band. At higher λ_{exc}, the 652 nm band decreases until it becomes a small contribution, for $\lambda_{exc} = 480$ nm, **Figure 13c**. The FF excitation spectra of **Figure 13a, b** confirm the presence of at least two chromophores. On the basis of literature data (*3*), we attribute the 652 nm fluorescence band to pheophytin-b and the 675 nm band to pheophytin-a. Their concentration, in olive oils, is up to 3 and 55 ppm, respectively (*3*).

Finally, we note that both fluorescence emission and excitation spectra allow us to evaluate the concentrations of the two pheophytin fluorophores with an analysis of spectra of native oils similar to that described by Galeano Diaz (*3*) for acetone-diluted oils.

Fluorescence of Different Olive Oils. The emission spectra with $\lambda_{exc} = 280$ or 300 nm of O1, O2, and O3, olive oils which differ in origin, cultivar, and age, are compared in **Figures 14** and **15**. The higher content of chlorophyll pigments in O1 is well depicted by the fluorescence band at 675 nm in both figures; with $\lambda_{exc} = 300$ nm, the same oil shows a higher intensity fluorescence also in the antioxidants' spectral zone. In contrast, the fluorescence with $\lambda_{exc} = 280$ nm reveals that the oldest oil, O3, has an higher content of the shorter-wavelength absorbing and emitting fluorophores of the antioxidants' zone. Somehow less remarkable, but still well-defined, differences among the three oils are found also in the spectral zone between antioxidants and pheophytins.

We think that it is possible to use fluorescence spectra to characterize an olive oil of a definite age, geographical origin, and cultivar, an important task pursued with high-field ^{1}H NMR analysis of minor volatile components (*26*) or with HS−SPME/GC-Ms analysis of volatile components (*27*) in extra virgin olive oils. In our case, fluorescent substance content would be used for characterization purposes.

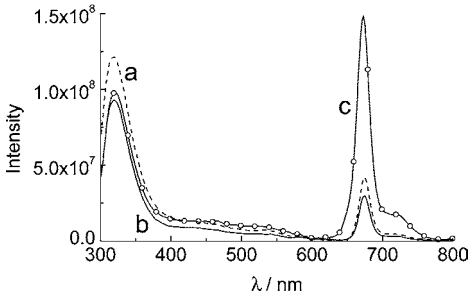

Figure 14. FF Emission spectra of olive oils with $\lambda_{exc} = 280$ nm. (a) oil O3; (b) oil O2; (c) oil O1. The spectra are corrected for second-order effects.

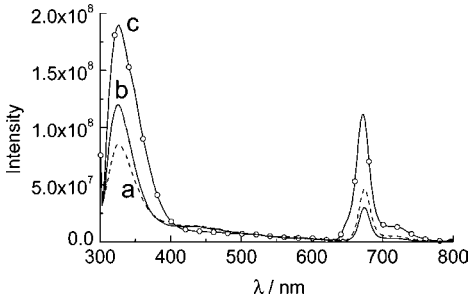

Figure 15. FF Emission spectra of olive oils with $\lambda_{exc} = 300$ nm. (a) oil O3; (b) oil O2; (c) oil O1. The spectra are corrected for second-order effects.

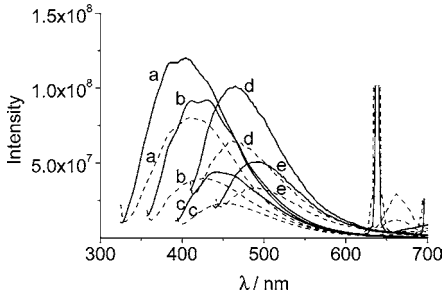

Figure 16. FF Fluorescence emission spectra of peanut oil (solid lines) and a sunflower oil (dashed lines). Spectra were recorded at the excitation wavelengths: (a) $\lambda_{exc} = 320$ nm; (b) $\lambda_{exc} = 350$ nm; (c) $\lambda_{exc} = 385$ nm; (d) $\lambda_{exc} = 400$ nm; (e) $\lambda_{exc} = 430$ nm. Spectral bandwidths were 1.5 and 3 nm for excitation and emission, respectively.

Fluorescence of Edible Seed Oils. It is also interesting to present the FF fluorescence spectra of two common seed oils, namely peanut and sunflower oil (**Figure 16**) and to compare them to the spectra of olive oil. As for olive oils, we took into account only the seed oil FF fluorescence spectra with $\lambda_{exc} = 320$, 350, 385, 400, and 430 nm, corresponding to ABS(λ_{exc}) < 12 AU. These spectra look quite different from the ones of olive oils. In particular, on varying the excitation wavelength in the above spectral region, we observe fluorescence bands with maxima in the range 400−500 nm and from 2- to 6-fold higher intensity than the olive oil bands in the same spectral region (**Figures 5**, **8**). In addition, the bands of olive and seed oils differ clearly in shape. The emission bands of sunflower oil that result are less intense than peanut oil bands at each λ_{exc}, and the ratio of the intensities for the bands of the two seed oils is almost constant at all the λ_{exc}. Interestingly, a weak emission band, centered at about 666 nm in the spectrum of sunflower oil, reveals the presence of fluorescent chlorophyll derivatives. Such a band is undetectable in peanut oil. A more detailed analysis of these spectra, to discover the origin of the

observed bands, however, is beyond the scope of the present work.

ABBREVIATIONS USED

UV−vis, ultraviolet−visible; AU, absorbance unit; RA, right-angle; ABS, absorbance; FF, front-face; RBF, riboflavin; IPA, isopropyl alcohol; DMSO, dimethyl sulfoxide; HCA, hydrocaffeic acid; TFA, trifluoroacetic acid; FAD, flavin adenin dinucleotide.

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