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CORRESPONDENCE/REBUTTAL

Comment on Excitation–Emission Fluorescence Spectroscopy Combined with Three-Way Methods of Analysis as a Complementary Technique for Olive Oil Characterization

Sir: In a recent work (*1a*) the authors presented a rapid method of the authentication of olive oils using three chemiometric analyses of fluorometric data. Twelve of 13 extra virgin olive oils (EVOO) were differentiated from 16 olive (OO), 2 virgin olive (VOO), and 2 olive pomace (OPO) oils. The EVOOs were distinguished from the OOs on the basis of their fluorescence bands in the 400−600 nm range obtained with the right-angle (RA) technique. Such RA emissions consist of only one band in the OOs and of four weak bands in the EVOOs. The main peak at 522 nm in the EVOOs was assumed to be due to vitamin E (*1a*), of which EVOOs are typically much richer than OOs. We claim that such differences in the RA emission, and thus the ability to distinguish the EVOOs and the OOs, are not due to intrinsic fluorescence of the oils but to their light absorption features together with the artifacts associated to the RA technique, namely, *primary and secondary inner filter effects* (*2*). We note that also other recent studies (*1b*−*f*) present RA fluorescence data of oils which are heavily affected by the artifacts mentioned above.

Thus, we purified lutein and pheophytin a through HPLC and verified that they were both *free* of vitamin E and *nonfluorescent* in the 400−600 nm spectral range, and we added them to a sample of a commercial OO. The rationale for this addition is that we know (*3*, *4*) that these substances are the most important pigments in the 400−600 nm range present in EVOOs (**Figure 1A**g) and that they are practically lacking in the OOs (**Figure 1A**a). Upon addition of the pigments, the absorption bands progressively appear (**Figure 1A**a−f) and, simultaneously, the overall intensity of the RA fluorescence reduces (**Figure 1B**a−f) with formation of maxima at 382, 441, 472, 522, and 543 nm, each corresponding exactly to one of the *minima* of absorption of both EVOO and enriched OO. In particular, on increasing the concentration of lutein, practically absent in the starting OO, to the final value of ≈7.8 mg/kg, we note the dramatic reduction of the intensity of the fluorescence, particularly in correspondence to the absorption maxima of lutein. Addition of pheophytin a to ≈11.8 mg/kg results in a further reduction of emission intensity, especially in correspondence to its absorption maximum at 414 nm. A new minimum becomes evident at 538 nm, better defining the 522 nm "vitamin E" band (*1a*). Similar effects are present in the emission curves at all excitation wavelengths in the range of 320−400 nm, resulting in RA excitation−emission matrices similar to those shown in refs *1a*, *1b*, *1d*, and *1e* for EVOOs.

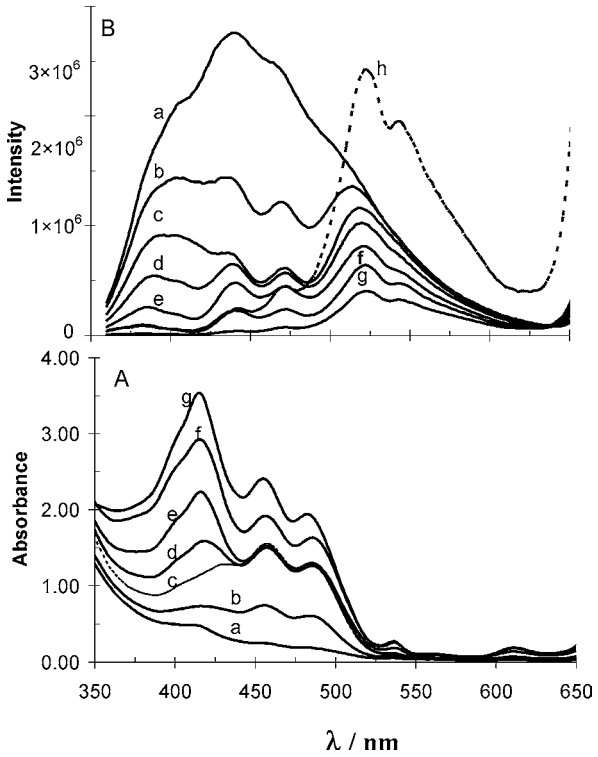

Figure 1. Absorbance (**A**) and RA-fluorescence (**B**) spectra: (a) original OO sample progressively added with lutein and pheophytin a; (b, c) additions of lutein, ca. 2.5 and 3.5 mg/kg, respectively; (d, e) additions of pheophytin a, ca. 3.5 and 4.6 mg/kg, respectively; (f) final addition of lutein, ca. 2 mg/kg, and of pheophytin a, 3.8 mg/kg. Curve h in (**B**) is 6-fold the spectrum of curve g for comparison purposes. Spectra g in both (**A**) and (**B**) are of a typical Italian EVOO (Moraiolo from Carapelli-Florence).

Clearly, the absorbance of an OO could be finely tuned to perfectly emulate the absorption, and *the correspondent RA emission*, of any EVOO by the addition of the mentioned pigments and, also, of other nonfluorescent minor pigments such as chlorophylls, pheophytin b, and β-carotene. In fact, the observed RA fluorescence bands are simply the minor part of the original fluorescence of OO surviving to the self-absorption caused by the added pigments. In EVOOs these bands are *not* due to vitamin E or its oxidation derivatives, in contrast to the suggestion originally proposed by Kiriakidis and Skarkalis (*1c*)

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and adopted by all of the authors of the mentioned studies (*1a*−*f*). Indeed, the emission of vitamin E in olive oils is centered at ≈327 nm (*3*, *5*) and not at 522 nm, whereas the known products of oxidation of α-, *β*-, *γ*-, and *δ*-tocopherol, the α-, *β*-, *γ*-, and *δ*-tocopherolquinones, are nonfluorescent substances (*6*).

The faults in the fluorescence spectra reported in ref *1a* are due to the "right angle" technique, by which the exciting light hits perpendicularly to the sample cell window and fluorescence is collected from another window orthogonal to the first one. Thus, both excitation light and fluorescence beams travel inside the sample. Light absorption of the fluorescence beam *inside* the sample affects the *shape* of the intrinsic fluorescence of EVOOs much more than it does the shape of the OO intrinsic fluorescences, because of their very different absorbance values, ca. 2.5 versus 0.3 on average, respectively (**Figure 1A**a,g). Finally, these effects depend also on the optics of the spectrometer, being maximal for *vertical beam* RA instruments, as the one used here. RA fluorometric techniques applied to oils have already been treated (*3*, *5*) and commented on (*7*). The front-face (FF) technique was shown to be the method providing the most accurate results for the fluorescence of edible oils in nondiluted samples (*3*, *5*). Disappointingly, the FF spectra of EVOOs and OOs seem to be too similar, in the 400−600 nm range, to be used to distinguish these oils.

In conclusion, we have seen that the absorbance (and not the "real fluorescence") of OOs and EVOOs is very different between 400 and 600 nm: we have tested that such absorbance can be easily manipulated, even adding leaves extracts, rich in carotenoids and chlorophylls, to OOs, thus providing OOs that are indistinguishable from EVOOs by fluorometry in the above spectral range. A more reliable distinction between EVOOs and OOs resides in the absorbance at $200 < \lambda < 300$ nm, where the massive presence of conjugated molecules in OOs results in much stronger absorbance values, typically from 12 to 70 au compared to 3−12 au in EVOOs, on passing from 300 to 250 nm. Although it is easy to add material rich in carotenoids and chlorophylls to an OO to increase its absorbance at 400−600 nm, it may be uneconomical to subtract the above conjugated substances from OOs in the attempt to simulate EVOOs through a convenient reduction of the OO absorbance at $\lambda < 300$ nm.

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