3D front-face fluorescence spectroscopy for characterization of olive oil

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Abstract: 3D front-face fluorescence spectroscopy and principal component analysis (PCA) were used to differentiate between Extra Virgin Olive (EVOO) and Olive Oil (OO). The results showed that 3D front-face fluorescence could be an effective tool to characterize EVOO and OO. Fourteen samples of olive oils were acquired directly from producers and from retail markets and their 3D fluorescence spectra were measured and analyzed. The first principal component of the PCA model retained the most important variability as a combination of freshness due to the presence of antioxidant compounds and chlorophyll; and of oxidation stage due to the presence of oxidation products. Olive oil samples presented different spectral patterns providing different scores for EVOO and OO in the PCA model. EVOO samples selected according to the differences in their scores in the PCA were exposed to indirect light. The evolution of their emission spectra was monitored with a right-angle set-up showing differences in concordance with their scores. This technique, the 3D front-face, is useful for characterization of olive oil samples according to their fluorescent compounds. The combination with other techniques (1H HR-NMR, HPLC, NIR spectrometry, etc.) may also be useful in protocols devoted to decision making processes with regard to olive oil quality specifications.

Keywords: fluorescence spectroscopy, olive oil, front-face, oxidation stage

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1 Introduction

Olive oil trade has spread all over the world due to its beneficial effect on human health which is related to the characteristic fatty acid composition, the presence of certain minor components and the antioxidant properties of phenolic compounds (García-González et al., 2008).

In addition, the regulated designations (EEC No 2568/91) extra virgin olive oil (EVOO) and virgin olive oil (VOO) present a high resistance to oxidative deterioration due to the triacylglycerol composition with high content of monoinsaturated fatty acids (oleic acid), and to a group of phenolic antioxidants, mainly polyphenols and tocopherols. However, for virgin olive oil (EVOO, VOO and Lampante) such compounds are drastically reduced during storage and refining process, where new products appear due to oxidation (Velasco and Dobarganes, 2002).

In fact, in many retail markets, olive oils are stored for long periods without any control of the storage conditions, which can induce their oxidation and can possibly cause the development of undesirable flavours (Frankel, 2010).

The oxidation leads to the formation of primary oxidation products such as hydroperoxides that can be decomposed to secondary oxidation products like aldehydes, alcohols and ketones (Velasco and Dobarganes, 2002). These latter compounds are responsible for the characteristic off-flavour of degraded edible oils. Oxidation depends on light exposure, heat, endogenous metals, pigments, phospholipids and antioxidants content (Choe and Min, 2006). Moreover, the fatty acid composition i.e. saturated versus mono- and

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poly- unsaturated fatty acids strongly affects the oxidative process (Poulli et al., 2009).

The guarantee of authentication of EVOO is one of the most challenging issues of the olive oil sector. Among others, high resolution nuclear magnetic resonance ^{1}H HR-NMR (Hernández-Sánchez et al., 2014), high-performance liquid chromatography HPLC, Fourier Transform Infrared FT-IR, visible VIS, near infrared NIR, mid infrared MIR spectroscopy methods have been applied to adulteration detection, geographical origin determination and oxidation status characterization (Frankel, 2010; Aparicio et al., 2013). Currently, there is a need to improve and harmonize the existing normative and regulations concerning olive oil. Several international institutions (e.g. International Olive Council, IOC) are actively involved in the developing of normative for olive oil products, labeling regulations, and rapid, easy and accurate instrumental techniques and analytical methodologies.

Different types of fluorescence spectroscopy methods have been used for analysis of EVOO quality because they are less time-consuming and more cost-efficient compared to other analytical procedures (Karoui and Blecker, 2011).

In addition, fluorescence spectroscopy is a noninvasive, highly selective and sensitive technique. Other outstanding advantages are the absence of solvents and reagents, and the requirement of small amounts of sample. Classic fluorescence spectroscopy, front-face fluorescence spectroscopy, synchronous scanning fluorescence spectroscopy have provided chemists with a sensitive approach to determine the oil quality for soybean, corn, sunflower, and olive oils (Ammari et al., 2012a; Kyriakidis and Skarkalis, 2000; Sikorska et al., 2004; Sikorska et al., 2005; Sikorska et al., 2008). Application of these methods to EVOO has a high potential because most of the interesting components, chlorophyll, antioxidants compounds such as polyphenols and α-tocopherol, and primary and secondary oxidation products are fluorescent molecules.

The front-face approach provided accurate measurements for edible oils in non-diluted samples (Ammari, 2012a; Ammari et al., 2012b, 2012c).

Despite the interpretation of fluorescence spectral data is complex due to the presence of many fluorophores, different multivariate analysis methods (Independent Component Analysis –ICA-, Principal Component Analysis –PCA–, Partial Least Square –PLS– regression, PLS Discriminant Analysis) could successfully facilitate the interpretation and of olive oils (Kassouf et al, 2014; Ammari et al., 2012b, 2012c; Valderrama et al., 2011; Sádecká J., Tóthová J., 2007).

The objective of the present work is to study the characteristic spectral pattern of EVOO and OO on the basis of the differences between signal patterns and intensities arising from different fluorescent compounds. 3D front-face fluorescence spectroscopy was used to identify the spectral regions of interest for EVOO and OO samples. PCA was applied to facilitate the interpretation of the fluorescence data and the characterization of the samples.

Finally, two EVOO samples selected according to the PCA model were exposed to indirect light during five months. Their emission spectra were monitored with a right-angle set-up in order to evaluate the relationship between the PCA model characterization and the further evolution of the spectra.

2 Materials and methods

2.1 Materials

Fourteen samples of olive oils were acquired directly from producers and from retail markets. These samples were grouped according to the indications of producers and of commercial labelling: seven EVOO samples (E1 to E7) and seven OO samples (O1 to O7).

Analytical analyses were carried out at an external laboratory according to European Regulations, EEC No 2568/91 and amendments.

Table 1 summarizes the relevant characteristics of these samples. Note that K_{232} values are not available for samples O1 to O3.

Table 1 Description and analytical measurements of the olive oil samples

Sample	Quality	Variety	Commercial Origin	K_{232}	K_{270}	α-tocopherol, mg / kg	Total Polyphenols, mg /kg
E1	EVOO	Arbequina	Market	2.8	0.12	181.3	122.2
E2	EVOO	Blend: Hojiblanca Arbequina Picual Cornicabra	Market	2.06	0.13	179.1	260
E3	EVOO	Arbequina	Market	1.39	0.14	185.1	255
E4	EVOO	Hojiblanca	Market	2.18	0.13	195.5	175.4
E5	EVOO	Picual	Market	2.04	0.14	159.4	210.3
E6	EVOO	Arbequina	Producers	1.71	0.11	174.8	257.1
E7	EVOO	Picual	Producers	1.73	0.12	209.5	350.7
O1	Non EVOO	Blend	Market	Non available	0.41	242.3	53.7
O2	Non EVOO	Blend	Market	Non available	0.28	215	145.9
O3	Non EVOO	Blend	Market	Non available	0.34	207.2	65.5
O4	Non EVOO	Blend	Market	1.30	0.41	151.7	138
O5	Non EVOO	Blend	Market	2.01	0.48	32.5	66
O6	Non EVOO	Blend	Market	1.21	0.50	202.1	79
O7	Non EVOO	Blend	Market	2.38	0.41	141.4	142

The two groups of oils, EVOO and OO, accomplished the limits for K_{232} and K_{270} according to EEC No 2568/91 ($K_{232} \leq 2.5$ and $K_{270} \leq 0.22$ for EVOO; $K_{270} \leq 0.9$ for non-EVOO). However, the K_{232} value of E1 sample is higher than the limit; note that this sample has been obtained from a retail market, where it could have undergone uncontrolled oxidation.

2.2 3D front-face fluorescence spectroscopy and chemometric analysis

3D front-face fluorescence spectra were measured directly on the oil samples without prior preparation using a spectrofluorometer (LS45, Perkin-Elmer) equipped with a xenon lamp source, excitation and emission monochromators and a front-face sample-cell holder.

Measurements were carried out using quartz cuvettes (10 mm × 10 mm × 45 mm). The excitation wavelengths ranged from 230 to 646 nm (step 4 nm) and emission wavelengths ranged from 250 to 698.5 nm (step 0.5 nm). Excitation and emission monochromator slit widths were set at 10 nm. Emission monochromator scan speed was 800nm·s^{-1}. A photomultiplier of voltage 650 V was used.

The data corresponding to each sample were arranged in a [105 x 898] matrix. All the elementary matrices of the different samples of olive oil were pooled in a [14 x 105 x 898] 3-way cubic array (14 spectra from 14 oil samples, 105 excitation wavelengths and 898 emission wavelengths). Data were then unfolded to give a [14 x 94290] matrix in order to apply the chemometric analysis.

The unfolded 3D spectra of the seven EVOO samples were mean centered (with respect to the mean of these seven spectra) and then submitted to PCA, obtaining the corresponding loadings and scores. The loadings were afterwards refolded for further interpretations.

In addition, the unfolded 3D spectra of the seven OO samples were also mean centered (considering the mean of the EVOO spectra) and then, the loadings previously computed were used to obtain their corresponding scores.

2.3 Right-angle fluorescence spectroscopy

A right-angle set-up prototype devoted to fast inspection was designed and assembled by LPF-TAGRALIA.

The fluorescence spectra were obtained using a photonic multi-channel spectrometer (Hamamatsu, Japan) with detection wavelengths ranging from 196.9 nm to 958.8 nm (step 0.75 nm, 1024 wavelengths). A UV-VIS

light source (L10290, Hamamatsu, Japan) was used with a deuterium lamp, with spectral range from 200 nm to 400 nm. Two optical filters were coupled to the right-angle set-up so as to constrain both the excitation and the emission wavelength ranges. An optical filter limited the incident light to wavelengths lower than 400 nm. The other optical filter limited detected light to wavelengths higher than 400 nm. Measurements were carried out using quartz cuvettes (10 mm × 10 mm × 45 mm).

The two EVOO samples showing the highest and the lowest PCA scores in the front-face model were exposed to indirect light in transparent glass bottles. The evolution of the emission spectra was monthly monitored throughout five months with this set-up.

The emission spectra were normalized by computing the ratio of the signal intensity at each wavelength to the sum of the signal along the whole spectrum.

3 Results and discussion

3.1 3D front-face fluorescence spectra

The 3D fluorescence spectra exhibited several well-defined regions of intense fluorescence. Figure 1 shows the 3D spectra for the 14 samples of olive oils depicted with comparable color scale. The left column corresponds to EVOO group and the right one to OO group. In a first approach, both types of olive oil samples present different 3D fluorescence patterns.

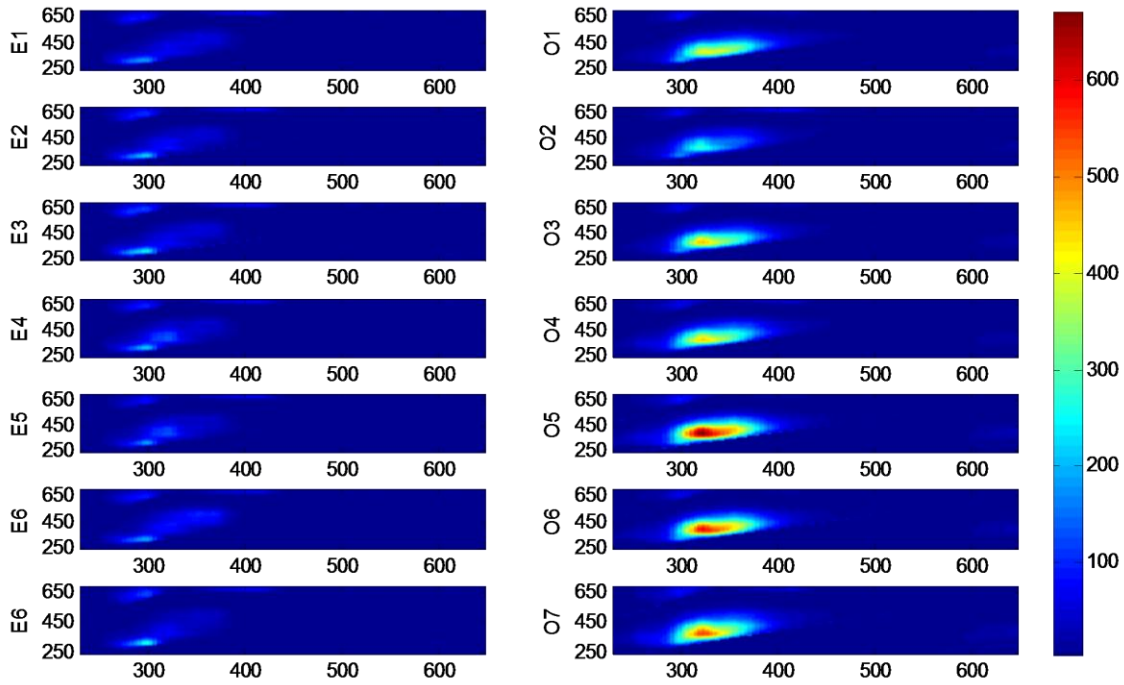
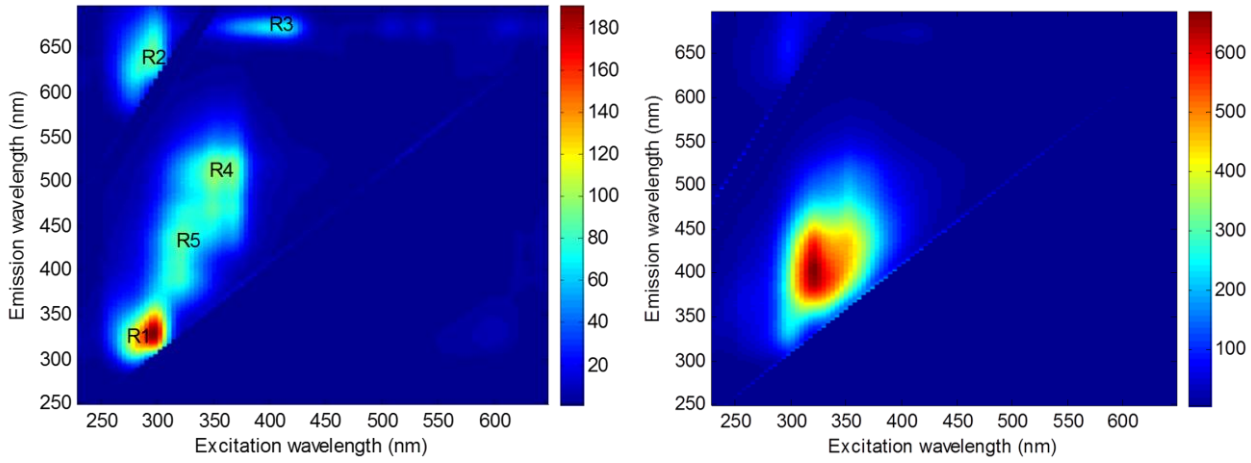


Figure 1 3D front-face fluorescence spectra, left column corresponds to the seven EVOO samples (E1 to E7) and right column to the seven OO (O1 to O7). Y axes: wavelengths of emission from 250 to 698.5 nm. X axes: wavelengths of excitation from 230 to 646 nm.

Two examples corresponding to an EVOO (E6) and to an OO (O5) samples have been considered for the detailed description of the most important regions comprising the characteristic spectral patterns, as illustrated in Figure 2a and 2b respectively.



(a) (b)

Figure 2 (a) Example of a 3D front-face fluorescence spectrum of an EVOO (E6); (b) Example of a 3D front-face fluorescence spectrum of OO (O6). The regions of high intensity emission are labeled from R1 to R5. Note the different color scale.

The spectral emission is the result of different signals emitted by the corresponding different fluorophores present in the sample. Previous researches provide ranges of excitation and emission wavelengths for the main fluorophores of olive oils that are related to freshness and oxidation conditions. Table 2 summarizes these emission and excitation bands as well as the corresponding references.

Table 2 Excitation-emission bands corresponding to the main compounds related to freshness and oxidation conditions in olive oil.

COMPOUNDS	Excitation- emission bands
Polyphenols and tocopherols (Zandomeneghi et al., 2005)	excitation 270-310nm; emission 300- 390 nm
Polyphenols and tocopherols (Ammari, 2012)	excitation 290–315nm; emission 320–360nm
Oxidation products (Ammari, 2012)	excitation 320–420nm; emission 400–500nm
Hydrolysis products (Kyriakidisand Skarkalis, 2000)	excitation 365 nm; emission 445 - 455 nm
Hydroperoxides (diluted sample in propanol) Cheikhousman et al. (2005)	excitation 290 nm emission 450 nm
Oxidation products (Guimet et al., 2004; Kyriakidisand Skarkalis, 2000)	excitation 365 nm; emission 440 and 455 nm emission 410 - 540 nm
Vitamin E (KyriakidisandSkarkalis, 2000)	excitation 365 nm; emission 525 nm
Chlorophylls and pheophytins (Kyriakidisand Skarkalis, 2000, Sikorska et al., 2005)	excitation 365 nm; emission 600 and 750 nm
Chlorophyll (Sikorska et al. , 2008)	excitation 405 nm; emission 681 nm

In the EVOO samples, five regions of interest are revealed. The area of the highest intensity emission signal (region 1, R1) ranges approximately from 300 to 370 nm, corresponding to an excitation band from 270 to 310 nm (Figure 2a). This emission area is ascribed to tocopherols and polyphenols according to literature summarized in Table 2. Second area of emission (region 2, R2) ranges approximately from 615 to 680 nm corresponding to an excitation band from 270 to 310 nm. These excitation and emission bands are not referenced in bibliography as produced by a specific compound of olive oil. However,

it could be attributed to antioxidant compounds according to the loadings in the PCA computation (see below).

The third area of emission (region 3, R3) ranges approximately from 665 to 680 nm corresponding to an excitation band from 380 to 420 nm. This area is related to chlorophylls a and b and pheophytins a and b according to literature (Table 2).

A fourth region of emission (region 4, R4) ranges approximately from 500 to 530 nm for an excitation band from 325 to 375 nm. This emission is attributed to vitamin E in the literature (Table 2). Finally, a weak area of fluorescence (region 5, R5) ranges from 360 to 480 nm corresponding to an excitation band from 318 to 400 nm. This region has been attributed to oxidation products (Table 2). Specifically this spectral emission may be produced by hydroperoxides as indicated by Cheikhousman et al. (2005).

3D spectra of OO samples are characterized by a single and wide high intensity emission region ranging from 360 to 480 nm, for an excitation band from 318 to 400 nm (Figure 2b). This broad region could arise from the partial merging of the fluorescence bands corresponding to primary and secondary oxidation products.

Three rather less intense regions appear in concordance to R1, R2 and R3 described for EVOO samples. Such signals of these regions may arise from the fraction of virgin olive oil (either extra or non-extra) contained in the OO samples, which are blends of refined and virgin olive oils.

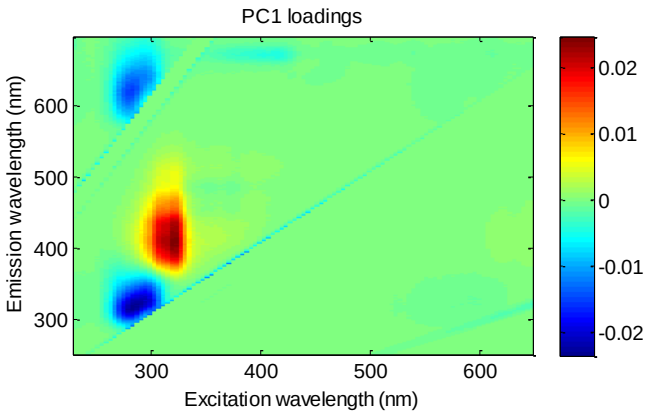
In view of these results, the 3D front-face fluorescence spectroscopy may be useful, by itself or in combination with other techniques (^{1}H HR-NMR, HPLC, NIR spectrometry, etc.) in protocols devoted to decision-making processes with regard to olive oil quality specification and to the detection of fraudulent blends. This technique could be used in the pre-classification of oil samples with significant savings of analytical measurements specified in the regulations.

3.2. Principal component analysis

A principal component analysis was performed in order to provide combined information from the different sources of variability that comprise the 3D spectral pattern of EVOO.

The first principal component, PC1, retains the 49% of the total variance, and it captures the variability as a combination of the presence of antioxidant compounds and oxidation products. As illustrated in Figure 3 (a) the matrix of PC1 loadings shows four main regions.

(a)



(b)

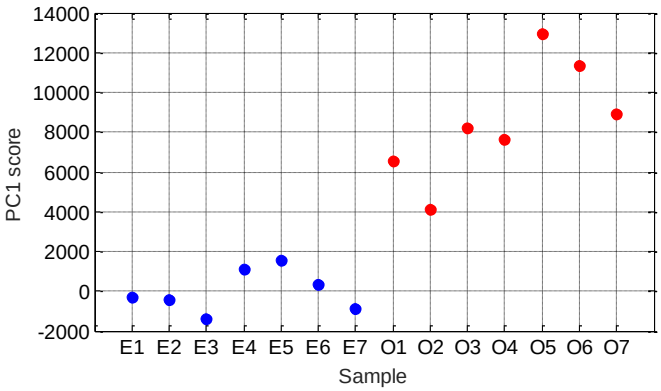


Figure 3 (a) PC1 loadings obtained from PCA on EVOO samples. (b) PC1 scores computed from PC1 loadings.

Three regions, approximately spatially concordant with observed R1, R2 and R3 in 3D fluorescence spectra (Figure 2a), are related to antioxidant compounds, and present negative loading values. The most important of these four regions is the R1, which is related to tocopherols and polyphenols fluorescence. In addition, a

fourth region appears with positive loading values, whose location is concordant with the fluorescence region R5 arising from oxidation products.

The different signs of loadings related to antioxidants (negative) and oxidation products (positive) may enhance the identification of the highest quality EVOO through the computed score. Such EVOO would be characterized by high antioxidant content with beneficial effect on human health and on potential olive oil extended shelf-life.

The PC1 scores are computed for both EVOO and OO groups (Figure 3b). In the case of the EVOO samples (from E1 to E7), the scores present the lowest values derived from their highest fluorescence of antioxidant compounds. The EVOO sample E3 presents the lowest score, whereas the sample E5 shows the highest value (Figure 3b), which reflects the differences in their 3D spectral patterns (Figure 4 a y b).

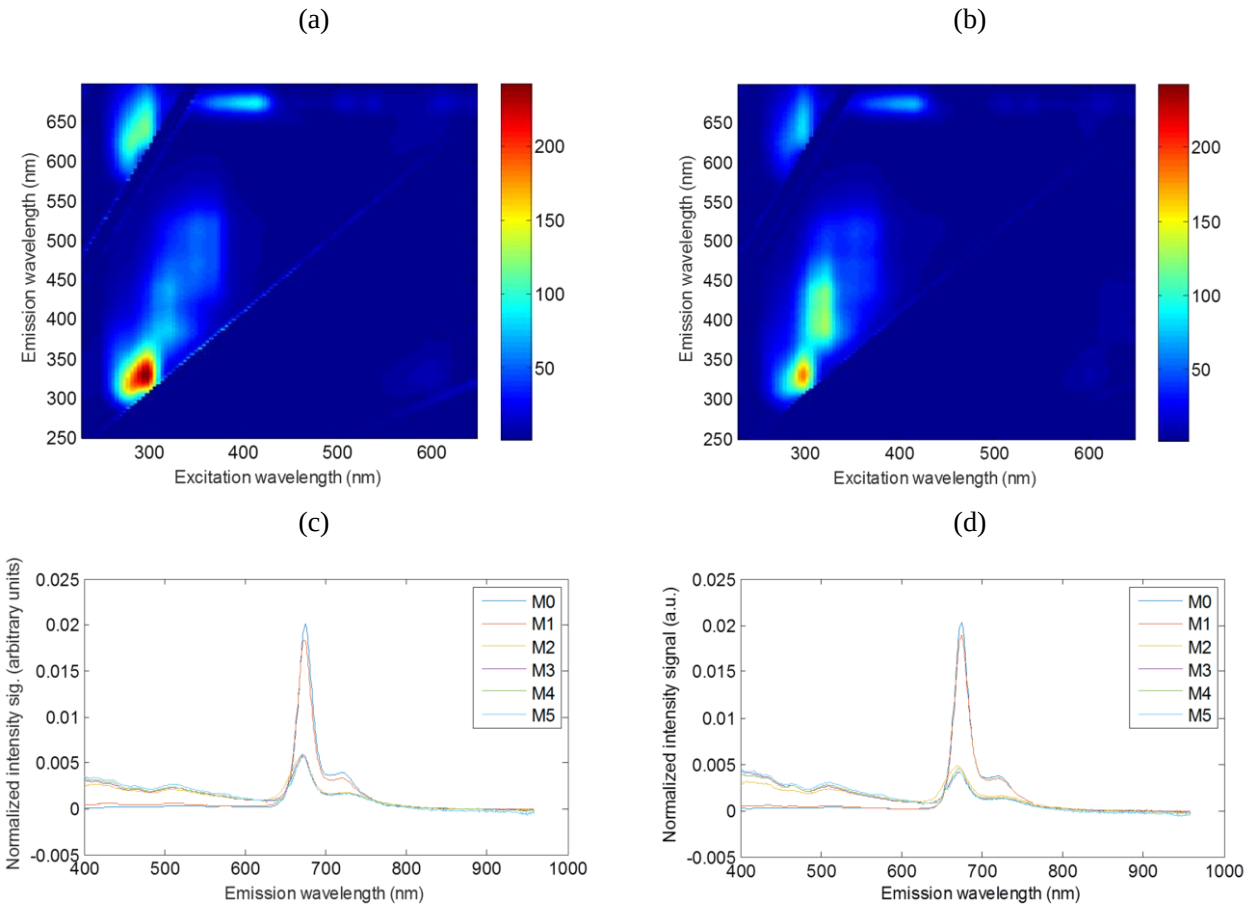


Figure 4 (a) 3D front-face fluorescence spectra of the EVOO with the lowest PC1 score (E3) among EVOO group; (b) 3D front-face fluorescence spectra of the EVOO with the highest PC1 score (E5) among EVOO group; (c) Evolution of the emission spectra along five months (M0 to M5) of the E3 sample (excitation from 200 to 400 nm); (d) Evolution of the emission spectra along five months (M0 to M5) of the E5 sample (excitation from 200 to 400 nm).

The scores of OO group are higher than those of the EVOO group, in agreement with their higher fluorescence of the oxidation compounds (Figure 1). Sample O2 presents a score value close to those of the EVOO group (Figure 3b), whereas O5 shows the farthest score, which also reflects the differences in their spectral patterns (Figure 5).

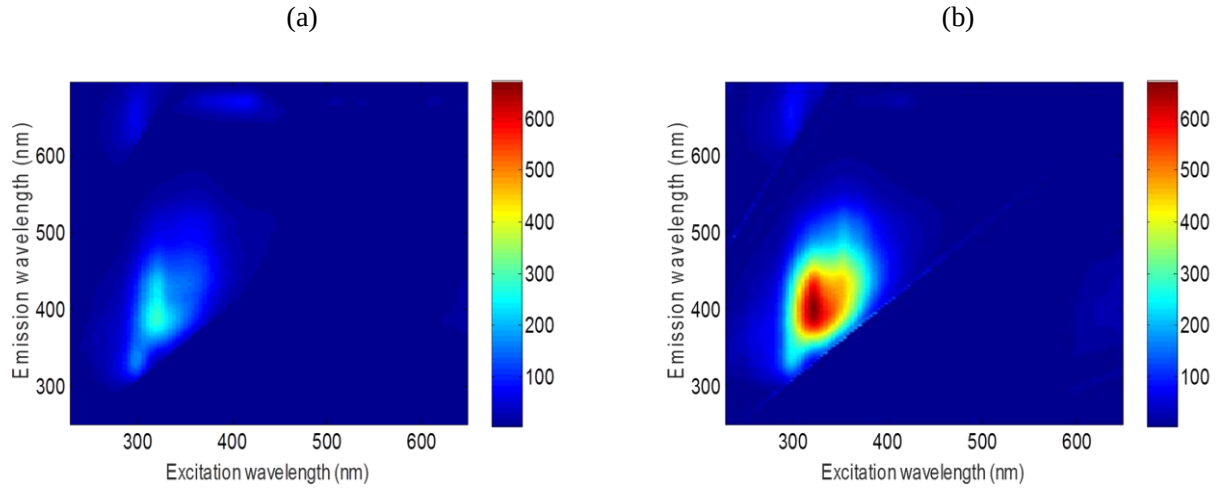


Figure 5 (a) 3D front-face fluorescence spectra of the OO with the lowest PC1 score (O2) among OO group; (b) 3D front-face fluorescence spectra of the OO with the highest PC1 score (O5) among OO group.

For the EVOO group, the value and the narrow dispersion of the scores highlight a predominant content of antioxidant compounds, and a slight rate of degradation. In contrast, the scores within the OO group show a much higher value and variability, reflecting different oxidation status. Such behavior could be expected as OO samples are blends of refined and virgin olive oils (either extra or non-extra). The refined olive oils had undergone a more intense oxidation (Velasco and Dobarganes, 2002).

According to Table 1, the two groups of samples EVOO and OO present differences in their analytical measurements. The observed K_{270} values are lower for EVOO samples (ranging from 0.12 to 0.14) than for OO samples (ranging 0.28 to 0.50), reflecting a higher status of oxidation in the latter group, as expected. In addition polyphenols content is usually higher for EVOO group than for OO group, although some non-EVOO samples present high values. As for $K_{232,}$ the ranges of the values are overlapped. The same fact is observed in α-tocopherol content. However, for α-tocopherol, it could be not necessarily related to the oxidation stage since its addition to non-extra virgin olive is allowed by the European regulation.

These analytical measurements summarized in Table 1 are in agreement with the observed fluorescence features. As illustrated in Figure 3, generally those oils with high polyphenol content present low value of PC1 scores, such as E3, E6 and E7 samples.

Within OO group, oil sample O2 presented the highest polyphenol content, whereas those oils with much lower polyphenol content, presented higher score value, such as O1, O3, O5 and O6 samples. The K_{270} value of O2 sample is the lowest of the OO group (close to the upper limit of the normative, $K_{270} \leq 0.22$) and the total content of polyphenols is the maximum value within its group. In contrast, olive oil O6 appears as the most degraded sample and shows low polyphenol content and high K_{270}value (Table 1).

3.3 Eevolution of EVOO samples

The EVOO samples showing the extreme PC1 scores were exposed to indirect light, and the evolution of the emission spectra was monitored for five months with a right-angle set-up (Figure 4).

This set-up assures the detection of the chlorophyll fluorescence signal, which is a major indicator of the oil evolution under different storage conditions since it acts as antioxidant under dark and as pro-oxidant under light conditions. In Figure 4c and 4d the peak at 670 nm is ascribed to chlorophyll fluorescence. The evolution of the products of oxidation, from both primary and secondary processes, can be monitored as well. In Figure 4c and 4d

the region ranging from 400 to 550 nm is ascribed to such fluorophores.

The evolution of the signal intensity along the time shows a significant decreasing at the chlorophyll region, and a significant increasing at the oxidation products region, when compared to the initial values in the month zero (M0, Figure 4c and 4d). The major changes are detected for sample E5 (Figure 4d), which presented the highest PC1 score derived from a noticeable low fluorescence signal of the antioxidant compounds and high signal for oxidation products in 3D front-face spectra (Figure 4b).

In view of this result, tentatively it could be expected that the 3D front-face spectra are useful as a reference to anticipate the subsequent degradation in EVOO samples.

4 Conclusions

3D front-face fluorescence spectroscopy with excitation wavelengths ranging from 230 to 646 nm and emission wavelengths ranging from 250 to 698.5 nm provides consistent spectral patterns of EVOO and OO samples. According to literature, antioxidant compounds such as polyphenols and tocopherols, chlorophylls and pheophytins and oxidation products give rise to the characteristic regions of interest.

Further characterization of the oil samples can be faced by obtaining models that retain simultaneously the spectral variability of such regions of interest. The behavior captured is a combination of freshness due to the presence of antioxidant compounds and of oxidation conditions due to the presence of oxidation products.

3D front-face fluorescence spectroscopy is a potential screening tool for characterization of EVOO and OO samples. This technique may be complementary and useful in combination with more detailed analytical measurements.

Tentatively it could be expected that the 3D front-face spectra are useful as a reference to anticipate the subsequent degradation in EVOO samples.

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