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Analytical Methods

Looking through the qualities of a fluorimetric assay for the total phenol content estimation in virgin olive oil, olive fruit or leaf polar extract

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ABSTRACT

As an alternate to the Folin-Ciocalteu assay (F-C) we propose a fluorimetric estimation of the total phenol content in virgin olive oil (VOO), olive fruit and leaf polar extracts. Phenol content was determined at excitation/emission wavelengths set at 280/320 nm. Standard operational procedures (slit widths, temperature, pH) and method validation were carried out according to Eurachem guidelines. The qualities of the proposed assay are better than those of the F-C one, as the procedure is more sensitive (LOD and LOQ values 10-fold lower), three times faster, needs no reagents and most importantly, is not destructive for the sample that can be further used in HPLC or other assays. Data for VOO extracts correlated well with the colorimetric ones ($r = 0.69$, $n = 65$). HPLC coupled with diode array and fluorescence detectors supported the above findings. Good correlations were also found between the respective data for olive fruit and leaf extracts ($r = 0.96$, $n = 18$).

1. Introduction

The continuous interest in the phenolic compounds of virgin olive oil (VOO) has also been extended to all products of the olive tree that can be consumed as common foods (table olives, olive paste) or used as sources for them (raw olives, olive leaves). Phenolic compounds identified so far in methanol or aqueous-methanol extracts of all of the above-mentioned olive tree products belong to various categories (simple phenols, secoiridoids, flavonoids, isochromans and lignans) (Boskou, Blekas, & Tsimidou, 2005; Boskou, Tsimidou, & Blekas, 2006; Boskou & Visioli, 2003; Servili et al., 2004). Such extracts have commercial interest in the preparation of infusions and dietary supplements.

In all of these extracts, estimation of the level of "total polar phenols" (TPP) is exclusively carried out using the Folin-Ciocalteu (F-C) assay (Carrasco-Pancorbo et al., 2005; Robards, 2003; Tsimidou, 1999). This assay is accomplished in a rather standard procedure after the extract is obtained from the starting material. The analytical protocols for the colorimetric assay are modifications of the one presented by Gutfinger (1981) for VOO. Despite the wide use of the F-C assay, owed to its simplicity, some inherent drawbacks – i.e. low specificity towards phenolic compounds, use of reagents destructive for the sample – have been repeatedly discussed. Over the years, analysts worked towards reduction of oil sample quantity, replacement of liquid–liquid extraction (LLE) with solid-phase extraction (SPE), or result expression using various standard phenols. Extraction of TPPs from VOO is a straightforward process but the same does not apply to the other olive tree materials. Thus, extraction from olives (raw and processed) is rather complicated and tedious, whereas quantitative recovery of phenols from leaves can be rather time consuming (Antolovich, Prenzler, Robards, & Ryan, 2000; Robards, 2003; Tura & Robards, 2002). Profiling of the bioactive ingredients in the polar extracts is achieved using various separation techniques, RP-HPLC being the method of choice so far. The latter is usually coupled with a diode array detector (Carrasco-Pancorbo et al., 2005; Robards, 2003).

Fluorescence spectroscopy is one of the most promising techniques of increasing importance for complex food analysis (Christensen, Nørgaard, Bro, & Engelsen, 2006). Few are the articles that explore application of fluorimetry to the analysis of vegetable oils and virgin olive oil with or without prior separation of the tested compounds with regards to quality and authenticity issues (Brenes, García, Rios, García, & Garrido, 2002; Cartoni, Coccioli, Jasionowska, & Ramires, 2000; Dupuy et al., 2005; Kyriakidis & Skarkalis, 2000; Nicoletti, 1990; Poulli, Mousdis, & Georgiou, 2005; Sayago, Morales, & Aparicio, 2004; Selvaggini et al., 2006; Sikorska, Górecki, Khmelinskii, Sikorski, & Kozioł, 2005; Zandomeneghi, Carbonaro, & Caffarata, 2005). To our knowledge, no published work has presented a procedure for the estimation of phenol content in the polar fraction using fluorimetry, though, very recently, fluorimetric detectors have been used in conjunction to diode array ones in the RP-HPLC of VOO (Brenes et al., 2002; Cartoni et al.,

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2000; García, Brenes, García, Romero, & Garrido, 2003; Selvaggini et al., 2006) and olive fruit and leaf polar extracts (Ryan, Lawrence, Prenzler, Antolovich, & Robards, 2001; Ryan, Robards, & Lavee, 1999; Ryan et al., 2002).

In search of replacement of the F-C assay by a more convenient procedure suitable for the assessment of TPP content in the polar extract of VOO or in polar extracts of other olive tree products, we propose a fluorimetric approach as most of the phenolics, commonly detected at 280 nm in RP-HPLC separations, also fluoresce. Our assay was developed for the polar extract of VOO, because this is the most well investigated one, and then, was validated according to the Eurachem guidelines (Eurachem Guide, 1998). Applicability of the method was extended to extracts from olive fruits (raw and processed) and leaves. In all of the extracts the TPP content was also determined using the F-C assay. HPLC coupled in series with a diode array and a fluorimetric detector was used to support discussion.

2. Materials and methods

2.1. Samples

The materials used in the study were as follows:

2.1.1. VOO

Two sets of VOO samples were used; the first one (training set) consisted of 40 Greek commercial VOOs collected as has been previously described (Grigoriadou, Androulaki, & Tsimidou, 2005).The second set (test set) consisted of 10 Spanish commercial VOOs, (Dpto. De Tecnología de Alimentos, Facultad de Químicas, Universidad Castilla-La Mancha), 7 Greek VOOs (ELAIS SA, Piraeus, Greece) and 8 Tunisian VOOs obtained using an Abencor system (Laboratoire Caractérisation et Qualité de l'Huile d'Olive, Centre de Biotechnologie de Borj-Cedria, Hammam-Lif, Tunisia). All samples of the test set were from the current crop season. Samples were stored at −20 °C until analysis.

2.1.2. Olives (raw and processed)

Fruits belonging to Chondrolia Chalkidiki and Koroneiki cultivars were collected on three different dates (18/7; 21/8 and 17/10/2006) from an experimental orchard (Chalkidiki, Greece). Sampling was performed between 10 and 12 a.m. and the fruits were picked from branches within arms reach. After sampling fruits were immediately cleaned from dust, crushed with a hammer mill MM (MC2 INGENIERIA Y SISTEMAS, S.L., Sevilla Spain) and subsequently freeze dried. Finally, dried samples were placed under nitrogen in airtight opaque glass jars and were stored at −20 °C until analysis. Processed olives (black, green and Kalamon) in the form of commercial olive paste products were also used for the preparation of the respective extracts.

2.1.3. Olive leaves

Olive leaves were collected from the same orchard and in the same way as previously described. Mature leaves (from the middle of 1-year-old shoots) were collected from the whole perimeter of four trees of cultivars Amfissa, Chondrolia Chalkidiki and Koroneiki on the 22/12/2006. The leaves were immediately cleaned from dust and then freeze dried. Dried samples were placed under nitrogen in airtight opaque glass jars and were stored in a dry dark place till analysis.

2.2. Standards, reagents and solvents

Oleuropein (98% purity) (OL) was purchased from Extrasynthèse (Genay, France); tyrosol (98% purity) (TY) and caffeic acid (98% purity) (CA) were from Sigma–Aldrich (Steinheim, Germany). Folin-Ciocalteu reagent was from Panreac Quimica S.A. (Barcelona-Spain). All other common reagents were of the appropriate purity from various suppliers. HPLC grade methanol (MeOH) and acetonitrile (ACN) were from Merck KgaA (Darmstadt, Germany). Ultrahigh-purity water was produced using a Millipore-Milli-Q system. Solvents used in the extraction of plant material were of analytical grade from various suppliers.

2.3. Fluorimetric estimation of phenolic compounds

The method was developed for the polar extract of VOOs and then was further applied to other plant material.

2.3.1. Extraction of phenolic compounds

(a) Oil (1.000 ± 0.001 g) dissolved in 5 ml *n*-hexane was extracted with 5 ml MeOH/H_2O (60:40, v/v). The mixture was vortexed and centrifuged at 3500 rpm for 10 min. After the removal of the hexane layer, the polar extract was used for further analysis.

(b) Phenol extraction from processed olive paste was according to Amiot, Fleurìet, and Macheìx (1986): 1 g of lyophilized material was homogenized in 15 ml 80% (v/v) ethanol. After agitation at room temperature for 20 min followed by filtering, the residue was treated twice in the same way and the filtrates were combined. Ethanol was evaporated from aqueous alcohol extracts under vacuum (~35 °C). Two successive petroleum ether (2 × 15 ml) extractions allowed removal of pigments and most lipids. After addition of MeOH to the aqueous phase (1:5, v/v), the phenolic compounds were extracted (4 × 20 ml) by ethyl acetate in the presence of ammonium sulphate (20%, w/v) and metaphosphoric acid (2%, w/v). The mixture was then dried over Na_2SO_4 and then the ethyl acetate was evaporated under vacuum (~35°C). The extract was finally made up in 5 ml MeOH prior to further analysis.

(c) A quantity of dry sample (0.5 g of raw fruits and 0.25 g of leaves) was extracted with MeOH (20 and 10 ml, respectively) in an ultrasonic bath at room temperature for 5 min and then the extraction was continued under shaking in the dark at room temperature for 48 h. The methanolic extract was filtered, evaporated to dryness under reduced pressure (~35 °C) and redissolved in MeOH prior to further analysis.

2.3.2. Fluorimetric determination

The polar fraction of VOO was transferred in a 10 ml volumetric flask and the volume was made up to 10 ml with MeOH/H_2O (60:40, v/v) (stock solution, C_0); an aliquot (1.25 ml) from C_0 was diluted in 5 ml of the solvent (C_1) and finally triplicate working solutions (C_2) were prepared using 0.5 ml (or other suitable aliquot) from C_1 in 5 ml volumetric flasks. Similar practice was followed in the preparation of the respective working solutions of olives (raw and processed) and olive leaves. In the case of raw olives and leaves the sample solvent was MeOH. The fluorescence of each C_2 solution was recorded at 280 nm excitation (ex) and 320 nm emission (em) with a fluorimeter RF-1501, (Shimadzu Co., Kyoto, Japan) at a scan speed of 2775 nm min^{-1}. Instrument response time was automatically set. The system was thermostated at 29 ± 1 °C with the aid of an outer water-circulating bath. OL was used as an external standard dissolved in MeOH/H_2O in the case of VOO and processed olive extracts and in MeOH for all the other extracts. Spectra of both samples and standards were corrected for background according to recommendations reported in relevant handbooks (Guilbault, 1973; Hercules, 1966). Corrections were made using the software facilities of the instrument (PC1501 1.2, Shimadzu Scientific Instruments, Inc.).

2.3.3. Method validation

Intra-laboratory method validation was performed by examining parameters such as method linearity, limit of detection (LOD, calculated as $3.3 \times \sigma/S$, where σ is the standard deviation of the y-intercept and S is the slope of the calibration curve), limit of quantification (LOQ, calculated as $10 \times \sigma/S$), intra and inter day precision, recovery and ruggedness. These parameters were checked using two standard solutions (OL, TY), and VOO polar extracts at concentration levels corresponding to the mid of instrument full recording scale, in order errors due to high sample concentration to be eliminated. Tests were done in triplicate and according to Eurachem Guidelines (Eurachem., 1998). Recovery was examined by spiking refined olive oil free of phenolic compounds with (a) a polar extract at different levels of addition (80, 160, 480, 930 of TPP as mg CA/kg refined oil) and (b) 200 mg TY or OL/kg refined oil. The extract had been obtained from VOO (100 g) dissolved in n-hexane (200 ml) using MeOH/H_2O (60:40, v/v; 200 ml) following the procedure presented by Hrncirik and Fritsche (2004) with certain adjustments. The TPP content of the VOO sample used was 220 mg/kg. The spiking procedure was repeated in triplicate for each level of addition and percent recovery was checked colorimetrically and fluorimetrically as previously described.

2.4. HPLC analysis of phenolic compounds

2.4.1. Sample preparation

VOO (2.500 ± 0.001 g) was dissolved in 5 ml of *n*-hexane and 5 ml of MeOH. The mixture was vortexed and then centrifuged at 3500 rpm during 10 min. The polar extract was evaporated to dryness at ~35 °C (Rotavapor, Büchi, Switzerland), redissolved in MeOH/H_2O (60:40, v/v); washed with hexane (3 × 1 ml) to remove completely the oily phase and finally filtered through a 0.45 μm PTFE filter (Waters, Milford, MA, USA) just before HPLC analysis.

2.4.2. Reversed phase HPLC analysis

The HPLC system consisted of a pump, model P4000 (Thermo Separation Products, San Jose, CA, USA), a Midas autosampler (Spark, Emmen,The Netherlands), a UV 6000 LP Diode Array Detector (DAD; Thermo Separation Products) in series with a SSI 502 Fluorescence Detector (FLD; Scientific Systems Inc., State College, PA, USA). The data were processed by the ChromQuest Version 3.0 software (Thermo Separation Products). Phenolic compounds were monitored at 280, 240 and 225 nm by DAD and by fluorescence at 280 nm (ex) and 320 nm (em). Separation was achieved on a Spherisorb ODS-2 column (250 × 4.6 mm i.d., 5 μm) (Alltech, Rigas Labs, Thessaloniki, Greece) using water/acetic acid (95:5, v/v) (solvent A) and ACN (solvent B) as eluents at a flow rate of 1.0 ml/min. The elution protocol was as follows: 0–4 min, 25% B; 4–40 min, 25–50% B; 40–45 min, 50–95% B; 45–50 min, 95% B; 50–60 min, 25% B; 60–70 min, 25% B and the injection volume was 20 μl. Peak identification was based on literature data, relative retention times, spectra matching and standards available.

2.5. Colorimetric assessment of phenolic compounds

Suitable aliquots of the polar extracts were transferred in a 10 ml volumetric flask and, subsequently, water (5 ml) and the Folin-Ciocalteu reagent (0.5 ml) were added. After 3 min, 1 ml of saturated (35%, w/v) sodium carbonate solution was added to the reaction mixture. The solution was diluted with water to 10 ml and after 1 h the absorbance at 725 nm was measured against a blank solution with a spectrophotometer UV-1601 (Shimadzu Co., Kyoto, Japan). CA was used as an external standard ($y = bx + a$, $b = 0.1011 \pm 0.0004$ and $a = 0.0077 \pm 0.0026$; $R^2 = 0.99$). The determination was performed twice for each extract (TPP 147 mg/kg VOO, CV% = 7, $n = 5$). The software package UVPC 3.9 (Shimadzu Scientific Instruments, Inc.) was used for data acquisition and processing.

2.6. Statistical analysis

Statistical comparisons of the mean values for each experiment were performed by one-way analysis of variance (ANOVA), followed by the multiple Duncan test ($p < 0.05$ confidence level) using SPSS 14.0 software (SPSS Inc., Chicago, IL).

3. Results and discussion

3.1. Groundwork

The observation that the most representative compounds of the VOO polar extract (e.g. simple phenols, secoiridoids, lignans) fluoresce, was the initiative to examine whether a fluorimetric estimation of TPP content is feasible. In advance, we had to clarify whether a linear response of fluorescence signal to phenol content could be justified. The phenolic extract, as described in Section 2.3, is a matrix less complex than VOO as it is devoid of other fluorophores (tocopherols, chlorophylls). In such a case, intrinsic fluorescence intensity is expected to be linear with the concentration of the respective fluorophores (Christensen et al., 2006; Zandomeneghi et al., 2005). The choice of the excitation/emission wavelengths was based on experimental trials according to fluorescence theory and practice (Guilbault, 1973; Hercules, 1966) and on the information that the "best compromise for general profiling" of the VOO phenols by HPLC-fluorimetry is that of 280/320 nm (Ryan et al., 1999). Besides, reports on fluorescence characteristics of polyphenols indicate that these compounds absorb around 260–310 nm and emit around 310–370 nm (Zandomeneghi et al., 2005). Therefore, the excitation and emission wavelengths chosen for the aim of our study were the same as those used repeatedly in HPLC-fluorimetry. These wavelength settings were considered as a standard operational procedure throughout the method development part.

The chromatographic analysis for 10 of the VOO samples of the training set and for the majority of the samples of the test set verified the presence of phenolic compounds (Table 1) that can be monitored by both spectrometry (280 nm) and fluorimetry (Fig. 1a and b, respectively). Fluorescent compounds were present in different quantities or even absent in certain extracts as shown in Table 1, where the frequency of appearance of each individual one is given. This finding should be related to all reasons that may affect phenol content and composition of a VOO sample, e.g. freshness, cultivar of olives used, extraction technology, storage time till analysis (Di Giovacchino, Sestili, & Di Vincenzo, 2002) and implies that fluorimetric estimation of TPP content can reveal these variations from sample to sample. On accounts of this finding and hypothesis, we proceeded further in method development and validation.

3.2. Method development

Photochemical decomposition, concentration, temperature, pH, emission/excitation wavelength and solvent are parameters that can affect the fluorimetric response (Guilbault, 1973; Hercules, 1966; Lakowicz, 1999). The polar extract as prepared for the colorimetric estimation of phenol content was also used for the fluorimetric assay after successive dilutions. Thus, in a series of experiments it was found that extraction of the polar fraction using only one (1.0) g of VOO was a sufficient quantity for the preparation of the working solution described in Section 2.3. The results obtained though statistically different were of similar size [147 ± 10 and 176 ± 10 mg CA/kg oil (F-C assay) for the 1.0 and

Table 1
Frequency of appearance of fluorescent phenolic compounds in the VOO extracts analyzed by RP-HPLC

Peak number	Compound[a]	Frequency of appearance of fluorescent phenolic compounds (% of samples analyzed)		
		Training set	Test set	
		10 random samples	7 Greek samples	10 Spanish samples
1	Hydroxytyrosol	100	100	100
2	Tyrosol	100	100	100
3	Unidentified	60	100	100
4	3,4-DHPEA-EDA	30	100	100
5	Unidentified	80	100	40
6	*p*-HPEA-EDA	100	100	100
7_I	Pinoresinol	100	100	100
7_{II}	Acetoxypinoresinol	100	86	20
8	Unidentified	100	100	100
9	3,4-DHPEA-EA	80	86	80
10	*p*-HPEA-EA	40	86	90
11	Unidentified	100	86	20
14	Unidentified	70	86	10
15	Unidentified	70	86	10

[a] Peaks identified as described in Section 2.4.

2.5 g oil, respectively, $n = 5$]. The storability of both stock (C_0) and working solution (C_2) was checked at 4 °C and it was found that C_0 was stable for at least 4 days after preparation but the working solution should preferably be used within an hour after preparation (data not shown).

In order to select the standard operational parameters (SOP) of the analytical procedure, slit widths, cell temperature, solvent and pH effects were studied. The effect of these parameters was checked in triplicate for two standards (OL, TY) and VOO extracts at concentration levels within the mid of instrument full recording scale.

The slit width is the most important parameter in determining the resolution of a fluorimeter. Although there is no linear relationship between band pass and slit width, better resolution is obtained by decreasing the latter. Indeed, between the two slit widths available, namely 10 and 20 nm, after trial and error assays the lowest ones were found appropriate for both excitation and emission. The repeatability of the measurements was satisfactory (CV% = 1–5, $n = 3$).

Fluorescence measurements are also temperature dependent. In many samples, temperature rise of 1 °C, as indicated by the manufacturers, leads to a loss of 1–2% in fluorescence intensity, while for some other biochemical samples the respective change can be much higher (~10%). Therefore, a constant temperature cell holder seems to be a necessary accessory of the instrumentation. The temperature effect was tested at 27, 28, 29, 30, 31 °C and the data indicated the importance of standardization of this parameter (Table 2). The operational temperature chosen was 29 ± 1 °C, a condition that can be maintained in the laboratory regardless the season.

The nature of solvent is a critical operational parameter as fluorescence strongly depends on interactions of the fluorophore with its surrounding molecules. No generic model can be used for prediction of enhancement or quenching of fluorescence signal in a certain environment. Hydrogen bonding, acid-base chemistry and charge transfer interactions are the cause of the above effects. For a certain molecule, effects in solvent mixtures are less studied than those in pure solvents (Guilbault 1973; Hercules 1966; Lakowicz, 1999). In the case of a number of fluorescent compounds present simultaneously in a natural extract, e.g. VOO polar extract, interactions are expected to be more complex and difficult to interpret. Thus, taken for granted that the composition of solvent mixture for the VOO extract is MeOH/H_2O (60:40, v/v), we focused on the effect of the pH of the solvent used. In the case of analysis of VOO phenolic compounds by HPLC, pH control is critical for the chromatography, regardless the detection system. Addition of minute amounts of acids (acetic, formic, phosphoric) to the mobile phase helps non-dissociation of phenolic compounds, improving, thus, peak asymmetry by reduction of peak tailing. Calculation of TPP content by HPLC coupled with a diode array or fluorescence detection system is, thus, accomplished in acidic environment. On the other hand, colorimetric determination of total phenols using the Folin-Ciocalteu reagent takes place in high alkali environment whereas calculation of the bitter index based on absorbance at 225 nm of the VOO polar extract is estimated without any pH control (Gutiérrez Rosales, Perdiguero, Gutiérrez, & Olías, 1992). In view of the importance of pH control in fluorimetry a series of measurements for the two standard solutions and the VOO extract at selected pH values were carried out. The data shown in Table 2 revealed the expected complexity and difficulty in optimization of this parameter. As findings indicate OL fluorescence depended on pH in a way different to that observed for TY. The lowest value for the former was recorded in alkaline environment whereas acidic conditions seemed to affect TY fluorescence severely. At pH 6.5 both standards and VOO extract exhibited higher fluorescence, which remained almost unchanged when distilled water replaced the phosphate buffer. Our decision, not to adjust the pH value of the aqueous-methanol extract of VOO (~6.5) was considered as an acceptable compromise in an effort to develop a simple method for estimation of total polar phenols obtained by this solvent mixture.

For the rest of the analyses the SOPs were set as follows: slit widths, 10 nm; temperature, 29 ± 1 °C; sample solvent, MeOH/H_2O, 60:40 (v/v).

3.3. Method validation

Linearity between the concentration and fluorescence intensity under the described SOP conditions was determined by analyzing eight concentration levels of OL and TY in triplicate. The respective regression equations were: $y = a + bx$ ($R^2 = 0.99$), $b = 234.5 \pm 1$, $a = 50.4 \pm 3$ and $y = a + bx$ ($R^2 = 0.99$), $b = 417.6 \pm 4$, $a = 8.7 \pm 4$. The LOD and LOQ values for OL and TY were found to be similar (0.04 and 0.11 μmol/L, respectively). LOD and LOQ values were 10-fold lower than those recorded in the course of the present study for the F-C assay (data expressed in mol CA/L). The repeatability of measurement calculated for one single working solution C_2 of OL, TY and a VOO extract was found satisfactory (CV% = 0.6, 0.2, 3.9, $n = 5$, respectively). Repeatability of C_2 solution preparation was satisfactory, too (CV% = 4.9, 4.2 and 4.3, $n = 5$, respectively). Extraction repeatability was examined for 16 extracts of the same VOO sample (CV% = 9.4, $n = 48$). Then, for four consecutive working days between day repeatability of extraction was checked (each day three freshly prepared extracts of a VOO, CV% = 13, $n = 36$). Extraction repetabilities were found satisfactory, verifying the negligible effect of scattered light due to the potential presence of suspended particles (Zandomeneghi et al., 2005).

Recovery studies of VOO phenolics are rather limited even for the F-C assay. Hrncirik and Fritsche (2004) found that LLE is less selective than SPE systems towards individual phenols. The reported recovery for total phenol content estimated by HPLC at 280 nm was ~85% (2.5 g oil, 425 and 850 μg VOO phenolics/g oil) which was improved only when a second extraction step was introduced (up to 92–94%). In our work the recovery was first checked colorimetrically and was found to range between 62% and 125% for the four levels of addition used (80, 160, 480 and 930 mg VOO phenolics/kg oil). Using fluorimetry, recovery was found to be 144 ± 7%, 134 ± 6%, 124 ± 6% and 112 ± 3% (external standard OL) or 112 ± 6%, 118 ± 5%, 110 ± 5% and 109 ± 4% (external standard TY). Recovery of OL (200 mg/kg level of addition)

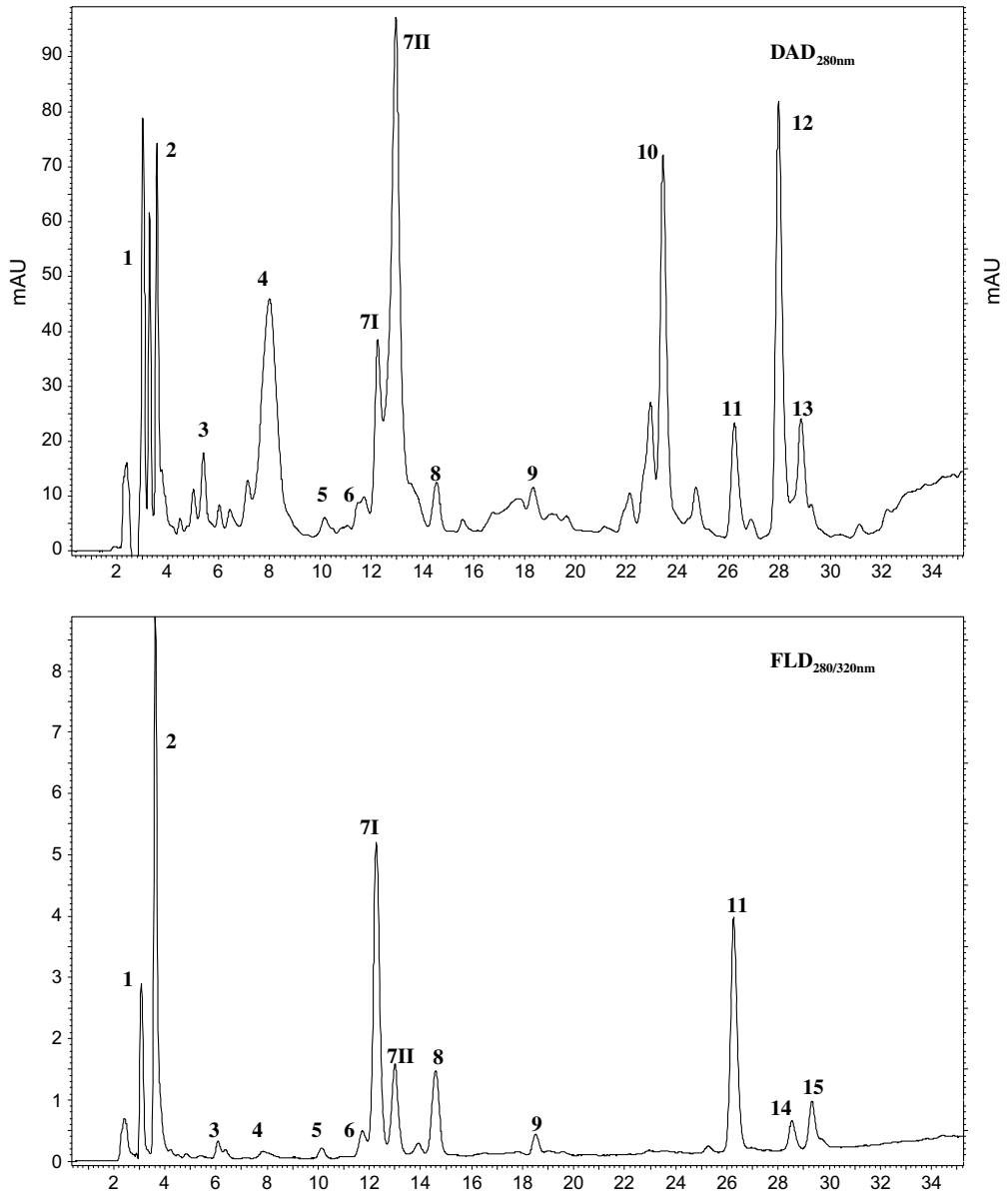

Fig. 1. HPLC/DAD chromatogram (280 nm) and HPLC/FLD chromatogram (280 nm excitation/320 nm emission) of a representative VOO extract, peak numbers correspond to compounds given in Table 1.

Table 2
Effect of temperature and pH on fluorescence intensity measurements[a] for OL and TY standard solutions and a VOO extract

Temperature (°C)	OL	TY	VOO
27	487 ± 38[b]	344 ± 45[a]	264 ± 12[a]
28	434 ± 22[a]	363 ± 31[a]	304 ± 13[b]
29	431 ± 3[a]	307 ± 20[a]	294 ± 4[b]
30	426 ± 7[a]	297 ± 36[a]	312 ± 15[b]
31	447 ± 8[a]	321 ± 29[a]	337 ± 18[c]
Solvent (pH value)[b]	OL	TY	VOO
MeOH/H_2O (2.0)	255 ± 13[b]	46 ± 10[a]	172 ± 27[a]
MeOH/H_2O (2.5)	305 ± 8[c]	246 ± 16[b]	209 ± 4[b]
MeOH/H_2O (11.0)	43 ± 2[a]	237 ± 20[b]	280 ± 16[c]
MeOH/phosphate buffer (6.5)	470 ± 10[e]	390 ± 31[d]	564 ± 19[d]
MeOH/H_2O (~6.5)	431 ± 3[d]	378 ± 12[d]	552 ± 12[d]

[a] Mean values ± standard deviation ($n = 3$); values in each row bearing the same superscripts are not significantly different from one another ($p < 0.05$).

[b] The pH of the solvent (MeO/H_2O; 60:40, v/v) was adjusted to values 2.0 and 2.5, with the addition of the appropriate amount of acetic acid; to value 11.0 with a saturated solution (35%) of sodium carbonate.

was satisfactory using the fluorimetric assay (97 ± 7%, $n = 9$) though colorimetric estimation resulted in rather low value (41 ± 2%, $n = 6$). Similar studies for TY showed comparative% recoveries (~80 ± 7%) using both assays. Due to lack of relevant literature data it was not possible to commend further on the accuracy of the method. Nevertheless, both assays seem suitable for quantitative estimation of total phenol content.

In order to estimate small changes in the experimental conditions that might occur in day to day application of the method the ruggedness of the assay was checked in-house for the two aforementioned standards and a representative VOO extract. Ruggedness was examined against small, deliberate variations of some critical parameters such as excitation wavelength, temperature, and analyst. Taking into account that the accuracy of the instrument was ±5 nm, measurements were recorded at 270, 275, 280, 285 and 290 nm (ex). Data indicated (Table 3) that variations of λ excitation even within the standard deviation range may affect the final result (>than 15% of the estimated level). Small but deliberate variations of the standard operational temperature

Table 3
Ruggedness of fluorescence intensity measurements[a] of an OL and TY standard solution and a VOO extract

λ excitation (nm)	OL	TY	VOO
Effect of excitation wavelength			
270	329 ± 6[b]	365 ± 26[c]	440 ± 35[b]
275	380 ± 4[c]	402 ± 7[d]	496 ± 44[c]
280	431 ± 3[d]	366 ± 18[c]	552 ± 12[d]
285	335 ± 11[b]	244 ± 1[b]	397 ± 26[b]
290	233 ± 3[a]	125 ± 10[a]	254 ± 5[a]

[a] Mean value ± standard deviation ($n = 3$); values in each row bearing the same superscripts are not significantly different from one another ($p < 0.05$).

(29 ± 1 °C) indicated that variations around the error range of the thermostated bath (±0.5 °C) were not significantly different (Table 2).

The effect of analyst on the performance of the method was checked for a VOO extract. The method was rugged towards this non-procedure related factor, as the respective intensity measurements (743 ± 9, 721 ± 13, 744 ± 12, $n = 3$) conducted by three analysts on the same sample did not differ significantly ($p < 0.05$).

3.4. Expression of results

In quantification studies, all of the fluorimetric measurements for VOO extracts were expressed as OL equivalents using a calibration curve within the range 0.04–3.70 μmol/L or 20–2000 μg/L with the exception of studies on TY in which the calibration curve was constructed with that standard (0.04–2.17 μmol/L or 5–300 μg/L). The respective equations of TY and OL calibration curves used are those reported in Section 3.3. TY and OL were chosen as external standards as they are commercially available and can be considered as typical representatives of simple and complex olive phenols, respectively.

3.5. Application to VOOs

The wide application of F-C assay for more than 20 years in olive oil analysis pointed it out as equivalent to a "reference method" that can be recommended as a useful industrial tool for routine VOO control (Blekas, Psomiadou, Tsimidou, & Boskou, 2002). This view is also reinforced by the fact that good correlations have been also reported between colorimetric and HPLC (280 nm) data (Hrncirik & Fritsche, 2004). On the other hand, HPLC, although sensitive and specific, is an expensive, sophisticated and time consuming method that can hardly be applied for real time routine analysis. One of the main advantages of our assay is the further simplification and acceleration of the determination of TPP content. Indeed, the time needed to accomplish analysis of 10 samples via fluorimetry is less than an hour. This analysis time is the one third of that required for the F-C assay. Additionally, the fluorimetric approach is more sensitive in comparison to the latter, as indicated by the respective LOD and LOQ values, whereas is also simple, inexpensive, needs no reagents and, most importantly, is not destructive for the sample that can be further used in HPLC or other assays. Since in the case of VOO extracts there are no interferences from other groups of compounds (Blekas et al., 2002), the measured values via fluorimetry can be used as a reliable estimate of the overall TPP content. Indeed, the fluorimetric data for a number of VOO samples (training set) correlated in a satisfactory way with the colorimetric ones when a linear model was used ($r = 0.70$, $p < 0.05$, $n = 40$). Inclusion of data for more samples of different origin, cultivar, ripening index or extraction technology (test set), did not change this trend ($r = 0.69$, $p < 0.05$, $n = 65$). The fact that the correlation, although promising, was not improved by the increase of data can be partially attributed to the different principle of each method. Moreover, taking into account that fluorescence signal depends on quantum efficiency f, and molar absorptivity ε of a compound, except for concentration, the estimation of TPP content cannot be expected to correlate too strongly with the results of the colorimetric assay. To highlight this point, the TPP content for a selected number of oil samples was determined by HPLC-fluorimetry. The data were compared to those by either F-C or the proposed assay. The TPP content, estimated by addition of fluorescent peak areas, as mg OL/kg oil, was found to correlate better with fluorimetric data estimated without prior separation of individual phenols ($r = 0.86$, $p < 0.05$, $n = 17$) than with colorimetric ones ($r = 0.41$, $p < 0.05$, $n = 17$). Samples that presented higher peaks in fluorescence chromatograms had also higher total phenol content as determined by the new method even when the TPP content estimated colorimetrically was the same.

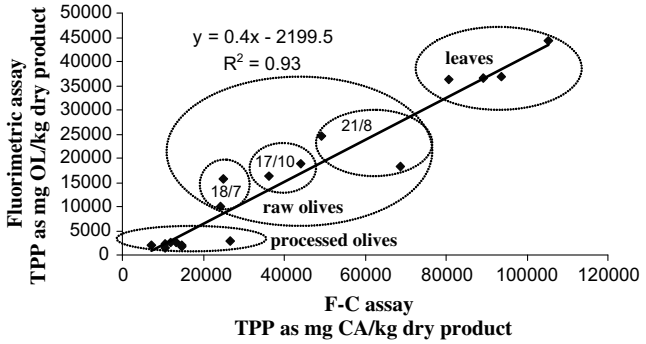

Fig. 2. Correlation between the phenolic content of olive fruit and leaf extracts estimated by the proposed and the F-C assay.

3.6. Further application to olive fruit and leaf extracts

Both olive fruit and leaf extracts contain fluorescent phenolics similar and/or different to those found in VOO, as indicated in relevant studies (Ryan et al., 2002). The TPP content of such extracts is also estimated by the F-C assay, so that it was interesting to investigate whether our method could be also applied to such extracts. Thus, a number of polar extracts ($n = 18$) prepared from olives (raw and processed) and leaves as already mentioned in Section 2.3 was analyzed by fluorimetry and the F-C assay. Data acquired correlated in a satisfactory way ($r = 0.96$, $p < 0.05$, $n = 18$) with the colorimetric assay (Fig. 2) showing the potential of the proposed procedure to other plant extracts.

4. Conclusion

The fluorimetric assay for the estimation of the TPP content of VOO seems promising in both analytical terms and practice as non-destructive for the extract. It is faster than the established F-C assay and applicable to both olive fruit and leaf polar extracts.

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