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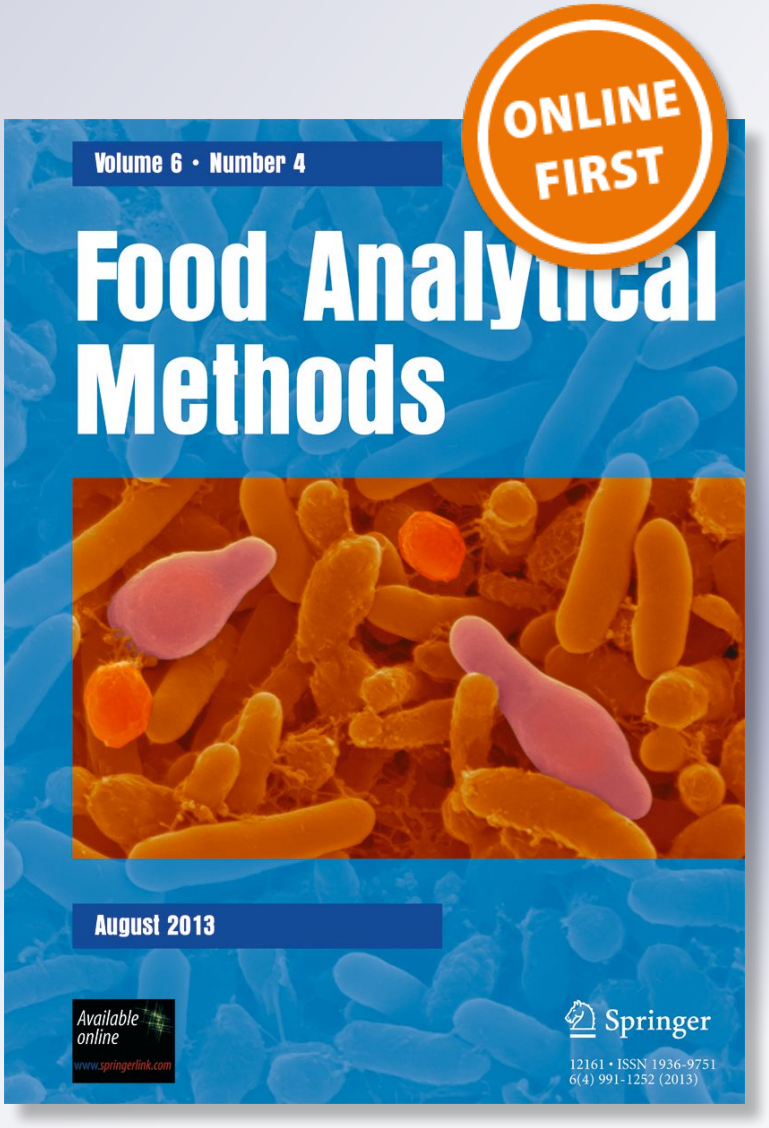

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Micro-scaled Quantitative Method to Analyze Olive Oil Polyphenols

Giovanni Caprioli[1] · Maria Chiara Boarelli[2] · Massimo Ricciutelli[3] · Gianni Sagratini[1] · Dennis Fiorini[2]

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Abstract

This study aims to improve an analytical method to quantify phenolic substances in olive oil. In order to minimize time required and quantity of solvents, sample extract preparation performed for a previously developed high-performance liquid chromatography–diode array detector to quantify olive oil polyphenols has been ten times downscaled and then validated. The new method performs the extraction of phenolic substances from 0.5 g of oil and allows to quantify the phenolic acids vanillic acid, *p*-coumaric acid, and ferulic acid; the phenolic alcohols tyrosol and hydroxytyrosol; secoiridoid derivatives; the lignans pinoresinol and acetoxypinoresinol; and the flavonoids luteolin and apigenin. Recoveries obtained were 66–89% for phenolic alcohols, 64–90% for phenolic acids, 93–96% for oleuropein (used as a reference for secoiridoid derivatives), 71–95% for flavonoids, and 97–100% for lignans. The total quantity of organic solvents used in the sample preparation is decreased from 30 to 3 mL with an important abatement of waste, costs, and working time requested.

Keywords Olive oil polyphenols · Quantitative determination · HPLC-DAD · Secoiridoid derivatives · Green method

Introduction

Olive oil phenolic substances have been recognized to have a key role in determining oil quality. Furthermore, the European Food Safety Authority (EFSA) has allowed the acknowledgement of a health claim on olive oil polyphenols. Thus, there is a great interest, both of the producer, of the consumer, and in organisms demanded to the official analytical control, in knowing the content of polyphenols found in an olive oil, and especially in the phenolic substances allowing the acknowledgement of the health claim, that are "hydroxytyrosol and its derivatives, e.g., oleuropein complex and tyrosol" (EU 2012). There are several methods available in literature to perform this analysis (e.g., Carrasco-Pancorbo et al. 2005; Franco et al. 2014; Capriotti et al. 2014; Flores et al. 2012; Suárez et al. 2008; Caporaso et al. 2015; Bayram et al. 2012; Bakhouche et al. 2013; Bendini et al. 2007) starting from which we developed a new method allowing the analysis of these substances, and other minor phenolic substances, like phenolic acids, flavonoids, and lignans, obtaining some improvements mainly in terms of chromatographic separation (Ricciutelli et al. 2017), making use of a reverse-phase analytical column never used before in this application, the Synergi Polar (4 μm, 80 Å, 250 × 4.6 mm). The application of this method to process a high number of samples led us to the idea of downscaling the quantities of sample and solvents used, in order to have an abatement first of all in terms of time requested to prepare the sample (the evaporation of the low volatile extraction solvents, water and methanol, is in fact a limiting factor), but also an improvement in terms of environmental impact, with reduced quantity of organic solvents used and reduced waste and overall analytical costs. Thus, the aims of the present study were (i) to downscale the previously developed method, (ii) to validate the new method, and (iii) to apply the new method to extra virgin olive oils (EVOOs) and olive oils (OOs).

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Materials and Methods

Reagents and Standards

The analytical standards of hydroxytyrosol (CAS number 10597-60-1), tyrosol (CAS number 501-94-0), vanillic acid

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(CAS number 121-34-6), oleuropein (CAS number 32619-42-4), luteolin (CAS number 491-70-3), and apigenin (CAS number 520-36-5) were purchased from Extrasynthese (Genay, France). *p*-Coumaric acid (CAS number 501-98-4), ferulic acid (CAS number 537-98-4), and pinoresinol (CAS number 487-36-5) were purchased from Sigma-Aldrich (Milano, Italy). Syringic acid was purchased by Fluka (CAS number 530-57-4) and used as internal standard (IS). Standard stock solutions of each compound were prepared by dissolving 10 mg of pure analytical standard in 10 mL of MeOH. Standard working solutions, at various concentrations, were prepared when needed by appropriate methanol dilution of stock solution aliquots.

HPLC-grade methanol, hexane, and isopropanol were purchased from Sigma-Aldrich (Milano, Italy). Water (resistivity above 18 MΩ cm) was obtained from a Milli-Q SP Reagent Water System (Millipore, Bedford, MA, USA). All the solvents and solutions were filtered through a 0.45-μm PTFE filter from Supelco (Bellefonte, PA, USA) before use.

Sample Collection

Ten EVOOs and ten OOs were purchased from a supermarket and stored at room temperature away from light until the analysis.

Sample Extraction

An amount of 0.5 g of oil was weighed in a 2-mL vial with Teflon screw cap, the sample was dissolved in 0.5 mL of hexane, and a fixed aliquot of IS (i.e., 20 μL of syringic acid methanolic solution at a concentration of 100 mg L^{-1}) was added. The solution is extracted with 4 × 0.5 mL of methanol: water (60:40, *v*/*v*) by means of a vortex device. After each extraction, samples were centrifuged (5 min, 5000 rpm) and the hydro-alcoholic solutions were collected in a 4-mL screw cap vial. Hexane (1 mL) was added to the final hydro-alcoholic solution (in order to remove traces of acylglycerols left), vortexed and centrifuged for 5 min at 5000 rpm. The hexane phase is removed and the hydro-alcoholic solution was transferred into a 10-mL round bottom neck flask and evaporated to dryness by means of a rotary evaporator under reduced pressure. The dried extract was reconstituted with 0.25 mL of HPLC-grade methanol, filtered through a 0.45-μm PTFE filter, and collected into a 2-mL high recovery vial before HPLC-DAD-ESI/MS analysis.

HPLC-DAD-MS Analysis

Chromatographic analyses were performed by means of a high-performance liquid chromatograph coupled with a diode–array detector (DAD) and a mass spectrometer detector (ion trap) equipped with an electrospray ionization (ESI) source (Agilent 1100, Santa Clara, CA, USA), in the same conditions reported by Ricciutelli et al. (2017), using a chromatographic column a Synergi Polar (250 × 4.6 mm, 4 μm). The mobile phase was water with 0.1% formic acid (A) and methanol/*i*-PrOH 90:10 *v*/*v* with 0.1% formic acid (B) working in gradient mode at a flow rate of 1 mL min^{-1}. The solvent composition varied as follows: 0 min, 30% B; 0–40 min, 60% B; 40–50 min 95% B; then the column was reconditioned. The column temperature was set at 35 °C and the injection volume was 10 μl. HPLC-DAD analysis, that was used for the quantification, was performed monitoring different wavelengths: 260 nm for vanillic acid, 280 nm for tyrosol, hydroxytyrosol, secoiridoids derivatives (namely the dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol or to tyrosol, an isomer of the oleuropein aglycon, and ligstroside aglycon), pinoresinol, acetoxypinoresinol, and syringic acid; 310 nm for *p*-coumaric acid, 325 nm for ferulic acid, 338 nm for apigenin, and 350 nm for luteolin. Quantification was performed as reported in the above mentioned study with the only exception of secoiridoid derivatives which were quantified using tyrosol calibration curve that resulted to provide very similar results as compared to the previously reported approach. In the present study, the mass spectrometer was used only for confirmation of the identity of the analytes and to identify the most abundant secoiridoid derivatives mentioned above.

Method Validation

The method was validated by determining linearity, repeatability, reproducibility, recovery at two fortification levels, limits of detection (LOD), and limits of quantification (LOQ). The main validation parameters obtained are reported in Table 1. For the assessment of the validation parameters related to the quantification of secoiridoid derivatives, oleuropein was used due the lack of their commercial availability and since it has a structure similar to secoiridoid derivatives found in higher amount in EVOO (especially those deriving from its hydrolysis).

Calibration curves of the analyzed compounds were constructed injecting 10 μl of standard solutions at seven different concentrations (according to each analyte) in HPLC-DAD. Linearity was assessed for each species in the concentration range found in the samples and was very good, with a correlation coefficient within the range 0.9988–0.9999.

Repeatability and reproducibility were evaluated by analyzing both the extract from an EVOO sample and a standard mixture of polyphenols, three times within a day (repeatability) and for 3 days (reproducibility) and measured in terms of relative standard deviation percentage (RSD %).

The recoveries were determined by spiking the olive samples with a standard mixture of polyphenols at two different levels, i.e., spike 1: 0.04 mg kg^{-1} (vanillic acid, *p*-coumaric

Table 1 Method validation parameters

Standard	Linearity	R^2	LOD[a]	LOQ[b]	Repeatability		Reproducibility		Recovery[c]%	
					(sample)	(standard)	(sample)	(standard)	(RSD %, $n = 3$)	
	(mg L^{-1})[d]		(mg kg^{-1})		RSD %[e] ($n = 3$)				Spike 1	Spike 2
Hydroxytyrosol	0.06–100	0.9996	0.007	0.025	2.3	2.2	4.3	2.5	66 (1.9)	76 (3.0)
Tyrosol	0.06–100	0.9996	0.009	0.028	2	1.9	0.7	2	78 (1.6)	89 (4.1)
Vanillic acid	0.12–10	0.9988	0.017	0.056	2.7	4.2	2.8	4	64 (5.7)	86 (3.4)
p-Coumaric acid	0.02–10	0.9988	0.002	0.008	3.2	2.5	3.6	2.8	82 (4.6)	90 (3.3)
Ferulic acid	0.07–10	0.9996	0.01	0.033	0.3	1.8	5.5	2	64 (4.3)	70 (2.9)
Oleuropein	0.7–1000	0.9993	0.1	0.35	/ [f]	2.2	/ [f]	2.5	93 (5.8)	96 (1.2)
Pinoresinol	0.2–100	0.9996	0.03	0.1	2.5	0.9	4.6	1.3	97 (2.3)	100 (3.0)
Luteolin	0.16–100	0.9996	0.023	0.077	2.1	5.3	2.4	4.6	71 (8.3)	85 (3.5)
Apigenin	0.09–100	0.9999	0.013	0.044	3.6	1.4	2.2	1.7	87 (6.1)	95 (4.0)

[a] *LOD* limit of detection (expressed as mg of analyte in kg of oil), [b] *LOQ* limit of quantification (expressed as mg of analyte in kg of oil), [c] determined by analyzing an olive oil sample spiked at two levels of concentrations; spike 1: 0.04 mg kg^{-1} (vanillic acid, *p*-coumaric acid, ferulic acid), 10 mg kg^{-1} (hydroxytyrosol, tyrosol, luteolin, pinoresinol, apigenin), and 100 mg kg^{-1} (oleuropein); spike 2: 2 mg kg^{-1} (vanillic acid, *p*-coumaric acid, ferulic acid), 20 mg kg^{-1} (hydroxytyrosol, tyrosol, luteolin, pinoresinol, apigenin), and 200 mg kg^{-1} (oleuropein); [d] mg of standard analyte in L of solution; [e] RSD: relative standard deviation. [f] Oleuropein was not detected in the analyzed samples

acid, ferulic acid), 10 mg kg^{-1} (hydroxytyrosol, tyrosol, luteolin, pinoresinol, apigenin), and 100 mg kg^{-1} (oleuropein) and spike 2: 2 mg kg^{-1} (vanillic acid, *p*-coumaric acid, ferulic acid), 20 mg kg^{-1} (hydroxytyrosol, tyrosol, luteolin, pinoresinol, apigenin), and 200 mg kg^{-1} (oleuropein).

The repeatability of the method was also evaluated on the replicated analyses performed with the spiked samples used to assess recovery. The limits of detection and the limits of quantification of the different analytes were estimated on the basis of 3:1 and 10:1 signal to noise ratios.

Results and Discussion

The developed method applies to 0.5 g of EVOO that is dissolved in 0.5 mL of hexane and added with internal standard solution and then extracted four times, each with 0.5 mL of a solvent mixture methanol/water 6/4. The final volume of hydro-alcoholic extract is washed with 1 mL of hexane to remove triacylglycerols traces and finally dried and dissolved in 250 μl of methanol. As compared to the method we previously developed (Ricciutelli et al. 2017), there is 10-fold decrease of the solvents and sample needed, leading to a great reduction of the time requested for sample preparation, that is largely due to the long time needed to evaporate the relatively low volatile solvent extraction mixture used (6/4 methanol/water). Furthermore, in comparison to other methods reported in literature, where purification by solid phase extraction (SPE) (e.g., Franco et al. 2014; Capriotti et al. 2014) or acid/alkaline hydrolysis (Carrasco-Pancorbo et al. 2005) is exploited, that is usually time-consuming steps, a further reduction of time is provided. Additionally, the method gives a substantial improvement in terms of environmental impact, with reduced quantity of organic solvents used and reduced waste. The common amount of oil used is higher with respect to our method (i.e., 0.5 g), being in the range 1–10 g (Caporaso et al. 2015; Pirisi et al. 2000; De La Torre-Carbot et al. 2005; Selvaggini et al. 2006) as well as the corresponding solvent volume used for dissolving the oil, usually in the range 6–10 mL or more (Franco et al. 2014; Caporaso et al. 2015; Pirisi et al. 2000; De La Torre-Carbot et al. 2005; Selvaggini et al. 2006; Pirisi et al. 1997).

The amount of extraction solvent used in the present work (2 mL as total amount) is lower with respect to the solvent volumes used in other methods, which often are in the range 10–25 mL (Caporaso et al. 2015; Montedoro et al. 1992) or even more (about 40 mL) if considering the total solvent amount used for the SPE process (Franco et al. 2014; De La Torre-Carbot et al. 2005).

Thus, the developed method aimed to extract and quantify polar-phenolic substances in olive oils with an important abatement of waste, costs, and working time requested for sample preparation.

In Fig. 1, a chromatogram obtained from the HPLC-DAD analysis of a standard mixture is reported and in Fig. 2, typical chromatograms obtained from the analysis of EVOO and OO extracts are reported. The most abundant secoiridoid derivatives found in EVOO (the dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol or to tyrosol, an isomer of the oleuropein aglycon, and ligstroside aglycon) were quantified by using tyrosol as external standard as also done in other studies (Caporaso et al. 2015; IOC 2009)

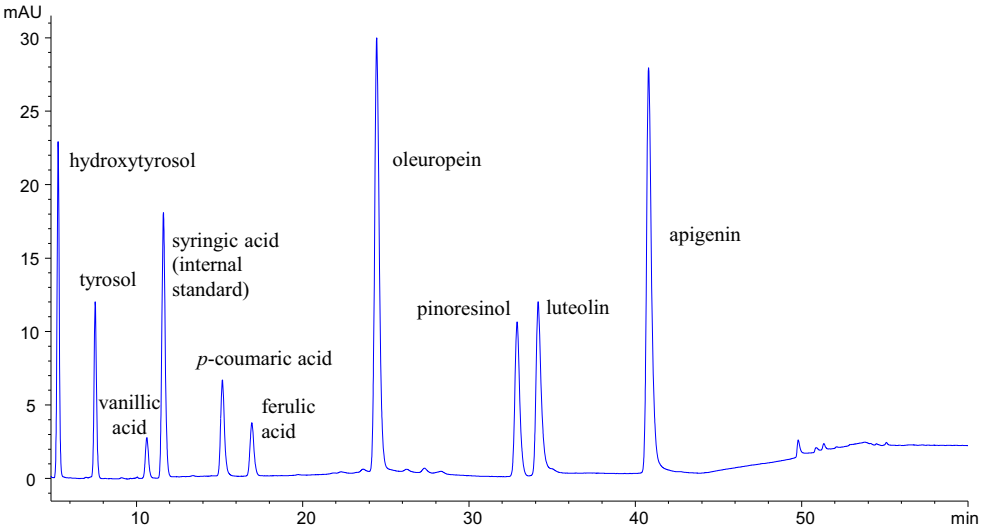

Fig. 1 HPLC-DAD chromatogram ($\lambda = 280$ nm) of a standard mixture composed by hydroxytyrosol, tyrosol, vanillic acid, syringic acid (internal standard), *p*-coumaric acid, ferulic acid, oleuropein, pinoresinol, luteolin, and apigenin, obtained by applying the optimized downscaled method

and based on our previous results (Ricciutelli et al. 2017). Due to the lack of the commercial availability of the specific secoiridoid derivatives found in higher amount in the oil, the use of standards different from them was made in many studies (e.g., Franco et al. 2014; Caporaso et al. 2015; De La Torre-Carbot et al. 2005; Alessandri et al. 2014), unless the isolation of the above mentioned secoiridoid species is performed (Suárez et al. 2008; Selvaggini et al. 2006). The other analytes (phenolic alcohols, phenolic acids, lignans, and flavonoids) were quantified in the present study at their specific wavelengths by using their specific calibration curves. The micro-scaled method was validated by determining LOD, LOQ, recovery, linearity, repeatability, and reproducibility (Table 1). Linearity was very similar to the reference method and sensitivity was comparable or, in several cases, improved, as compared to the reference method. In fact, LOQ range for phenolic alcohols is 0.025–0.028 mg kg^{-1} while it was 0.17–0.32 mg kg^{-1} in the reference method; for phenolic acids, it is 0.008–0.056 mg kg^{-1} and it was 0.033–0.061 mg kg^{-1} in the reference method; for pinoresinol, it is 0.1 mg kg^{-1} and it was 0.39 mg kg^{-1}; for flavonoids, it is 0.044–0.077 mg kg^{-1} and it was 0.096–0.109 mg kg^{-1}; for oleuropein, it is 0.35 mg kg^{-1} and it was 1.15 mg kg^{-1} in the reference method. For most of compounds, LODs and LOQs obtained are similar or lower than values reported in recent literature by using HPLC-DAD (e.g., Franco et al. 2014; De La Torre-Carbot et al. 2005; Selvaggini et al. 2006) or HPLC with fluorimetric detection (HPLC-FLD) (Selvaggini et al. 2006). For example, with respect to Selvaggini et al. (2006), who reported a very convenient method in terms of sample preparation, our method presents lower LOQs for hydroxytyrosol (14-fold), tyrosol (11-fold), and pinoresinol (10-fold) as compared to the HPLC-DAD method, and 3.4, 3.6, and 35-fold respectively, as compared to the HPLC-FLD method. For oleuropein, we obtained a LOQ of 350 μg kg^{-1} (or 700 μg L^{-1} if expressed in the analyzed solution), thus similar to the value obtained in the

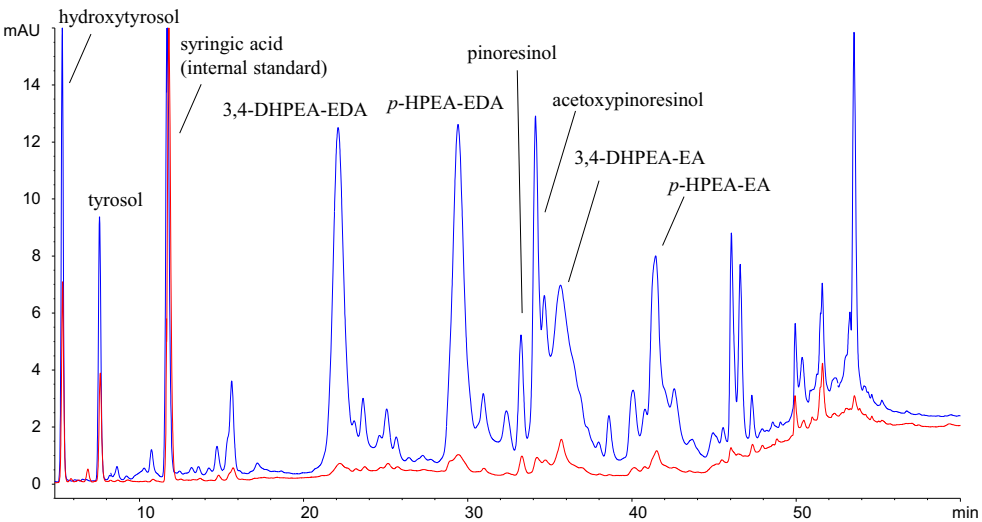

Fig. 2 Overlaid HPLC-DAD chromatograms ($\lambda = 280$ nm) obtained from an extra virgin olive oil hydro-alcoholic extract (blue) and from an olive oil hydro-alcoholic extract (red) by applying the optimized method. The phenolic substances and the main secoiridoid quantified at 280 nm are indicated (*3,4-DHPEA-EDA* dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol, *p-HPEA-EDA* dialdehydic form of decarboxymethylelenolic acid linked to tyrosol, *3,4-DHPEA-EA* isomer of oleuropein aglycone, *p-HPEA-EA* ligstroside aglycon)

Table 2 Application of the proposed micro-scaled HPLC-DAD method to extra virgin olive oils (EVOOs) and olive oils (OOs). Average concentrations of the phenolic substances investigated in the oils are reported

	Hydroxytyrosol	Tyrosol	Vanillic acid	*p*-Coumaric acid	Ferulic acid	Luteolin	Apigenin	Secoiridoid derivatives[a]	Pinoresinol	Acetoxypinoresinol[b]	Total polyphenols
	(mg kg^{-1}) [c]										
EVOO-1	19.93	17.85	0.71	0.133	0.058	2.78	1.15	252.91	5.65	18.45	319.63
EVOO-2	8.59	7.82	0.35	0.146	nq	1.78	0.73	275.23	5.78	15.51	315.97
EVOO-3	12.82	13.53	0.55	0.215	0.036	3.08	1.32	316.72	6.94	20.95	376.16
EVOO-4	34.95	28.73	0.26	0.200	0.078	1.59	0.71	128.78	11.30	16.59	223.22
EVOO-5	3.40	5.20	0.30	0.069	0.034	1.56	1.15	360.21	5.11	26.64	403.68
EVOO-6	18.43	17.55	0.26	0.142	0.035	2.10	1.02	238.06	6.03	11.49	295.13
EVOO-7	12.88	13.35	0.41	0.163	0.045	2.39	1.17	231.81	6.14	15.96	284.31
EVOO-8	13.81	14.57	0.27	0.185	0.045	1.99	1.10	206.16	6.26	12.56	256.95
EVOO-9	25.40	34.94	0.40	0.151	0.047	3.06	1.62	144.38	7.96	15.83	233.79
EVOO-10	18.48	13.92	0.38	0.173	0.050	3.24	1.48	104.63	7.35	20.66	170.37
OO-1	1.58	1.76	nq	0.014	nd	nq	nq	15.62	0.47	0.66	20.20
OO-2	1.44	1.83	nq	0.015	nd	0.08	0.09	20.12	0.97	0.93	25.50
OO-3	3.52	3.51	nq	0.034	nd	0.21	0.11	25.38	0.83	1.02	34.63
OO-4	1.35	1.34	nd	0.008	nd	nq	nq	17.05	0.33	0.44	20.60
OO-5	2.85	2.18	nq	0.018	nd	0.10	0.07	17.21	0.60	0.84	23.88

[a] Sum of the dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol and to tyrosol, the isomer of the oleuropein aglycon, and ligstroside aglycon, quantified using tyrosol as external standard. [b] Quantified using pinoresinol as external standard. [c] Average concentration ($n = 3$; % relative standard deviations range is 0.3–3.6% in EVOOs and 1.8–15.3% in OOs). nd: below limit of detection, nq: above limit of detection and below limit of quantification (cf. Table 1)

study of De La Torre-Carbot et al. (2005) (600 μg L^{-1}). LOQ values found for tyrosol, *p*-coumaric acid, and for the flavonoids luteolin and apigenin are also slightly lower (7-, 2-, 2-, and 4-fold respectively) in comparison to those reported by De La Torre-Carbot et al. (2005). On the other side, LOQ found for vanillic acid in our study is 3-fold higher with respect to that reported by De La Torre-Carbot et al. (2005). Recovery, a parameter which could result more affected by the downscaled procedure due to both the lowered amounts of sample and solvents, as well as to the different glassware used, actually resulted to be only slightly lower as compared to the reference method (Ricciutelli et al. 2017) in which it ranged from 73 to 102%; it remained in fact within values higher than 64%, with a range of 64–97% for the first fortification level and of 70–100% for the second fortification level, similarly to what obtained by Capriotti et al. (2014), in which it ranged from 75 to 105%.

In the recovery experiments, the reproducibility of the method, in terms of % RSD, was in the range 1.6–8.3% for the first fortification level and 1.2–4.1% for the second fortification level. Repeatability, in terms of RSD %, was within the range 0.9–5.3% in the analysis of the analytical standard and 0.3–3.6% in the analysis of the sample; reproducibility gave comparable results to repeatability: 1.3–4.6% for the standard and 0.7–5.6% for the sample. Regarding sensitivity, it has been shown that it was widely sufficient to quantify all the analytes considered in all of the ten commercial EVOO samples investigated to which the method was applied. Instead, the quantification of the analytes was not always allowed in OOs due to the very low concentration of phenolic substances in this type of sample, where the refining process, in which the most part of the oil composing an OO is undergone, leads to a great reduction of the phenolic content.

The results obtained from the application of the method to 10 EVOOs and 5 OOs are reported in Table 2. In the EVOOs investigated, the total polyphenol content ranged from 170.37 to 403.68 mg kg^{-1}; secoiridoids were found in the range 104.63–360.21 mg kg^{-1}, free hydroxytyrosol and tyrosol at concentrations of 3.40–34.95 mg kg^{-1} and 5.20–34.94 mg kg^{-1} respectively, phenolic acids at 0.41–0.90 mg kg^{-1}, lignans at 17.52–31.76 mg kg^{-1}, and flavonoids at 2.31–4.72 mg kg^{-1}. The results are in agreement with several other studies where the phenolic substances content in EVOOs is reported (e.g. Caporaso et al. 2015; Selvaggini et al. 2006; Alessandri et al. 2014; Servili et al. 2014). In the case of OOs, where the content of the hydrophilic phenolic compounds is much lower due to the refining process to which most part of OO is undergone, the proposed method does not allow the quantification of vanillic and ferulic acids, and sometimes of the flavonoids, while it is still adequate to quantify *p*-coumaric acid, free hydroxytyrosol and tyrosol (that are found in similar amount as compared to EVOOs), secoiridoid derivatives, and lignans, found in all the investigated samples in concentrations above LOQ values. In conclusion, the proposed micro-scaled method, while allowing the shortening of the working time and reducing solvent quantities, is still adequate for the quantification of phenolic substances in EVOO.

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Compliance with Ethical Standards

Conflict of Interest Giovanni Caprioli declares that he has no conflict of interest. Maria Chiara Boarelli declares that she has no conflict of interest. Massimo Ricciutelli declares that he has no conflict of interest. Gianni Sagratini declares that he has no conflict of interest. Dennis Fiorini declares that she has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent Not applicable.

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