Research Article

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An effective HPLC-based approach for the evaluation of the content of total phenolic compounds transferred from olives to virgin olive oil during the olive milling process

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ABSTRACT

BACKGROUND: Several studies demonstrate a strong interest in learning more about phenolic transfer during oil extraction, with the main goal of increasing the phenolic concentration in olive oils. We aimed to propose and apply a new methodological approach for evaluating phenolic transfer from olives into oil during milling, based on the quantification of phenolic content in whole lyophilized fruits and the corresponding oils and considering the oil extraction yields.

RESULTS: We investigated the phenols transferred into the oil during olive milling in continuous extraction systems in Tuscany. In 2012, oils were extracted from cultivar Frantoio by a two-phase extraction system; in 2016, oils were extracted from cultivars Leccio del Corno and Arbequina by a three-phase extraction system. Results highlighted very low percentages of extracted phenols: up to 0.40% by the two-phase system and up to 0.19% by the three-phase system (0.08% for cultivar Arbequina and 0.19% for cultivar Leccio del Corno).

CONCLUSION: The usefulness of a simple and effective methodological approach for evaluating the extracted phenols was highlighted. Values of extracted phenols were up to 25 times lower than previous literature data. The proposed approach is applicable in all types of milling processes.

Keywords: phenols extraction yield; secoiridoid; partition; HPLC–DAD-MS; oleuropein

INTRODUCTION

The interest for olive and olive oil phenols has been boosted in recent years thanks to their numerous health properties, such as anti-inflammatory,[1] antioxidant,[2,3] anticancer,[4] anti-angiogenic,[5] and anti-aterogenic[6] activities, and no adverse effect known to date.[7] The use of olive phenols was even patented for its capability in improving the management of type 2 diabetes.[8] In light of *in vivo* tests on humans,[9,10] the European Food Safety Authority approved an important health claim for virgin olive oils rich in phenolic compounds giving the possibility to insert 'the olive oil polyphenols contribute to the protection of blood lipids from oxidative stress' in the label.[11]

Olive fruits are very rich in phenolic compounds,[12] with the exact composition depending on different variables, the more relevant of which are cultivar, climatic conditions and degree of maturation.[13–16] Nevertheless, it is well known that only a minor part of this phenolic fraction passes in the olive oils during the extraction process, mainly depending on their predominant hydrophilic nature and the enzymatic activities.[17,18] Anyway, some technological conditions seem to be crucial to determine the percentage of phenols passed in the oil or lost in by-products as olive mill wastewater and solid pomace.[7,19,20] Nowadays, oil production can be carried out by both the traditional batch approach or as a two- or three-phase continuous process.[21,22] However, the type of technology seems to influence only the quantitative aspects on the phenolic transfer during oil extraction, while the qualitative changes mainly depend upon the enzymatic activities, especially during the malaxation.[22–25]

Literature data report that only up to 2% of the phenols available in the olive fruits are transferred to the oil due to the greater affinity of phenolic compounds towards the water phase.[7,26,27]

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Table 1. List of all the samples analyzed

Sample analyzed	Crop season	Cultivar	DAFB	Oil extraction system
Olive fruit and virgin olive oil	2012	Frantoio	150th	Two phase
Olive fruit and virgin olive oil	2012	Frantoio	164th	Two phase
Olive fruit and virgin olive oil	2016	Leccio del Corno	190th	Three phase
Olive fruit and virgin olive oil	2016	Arbequina	190th	Three phase

DAFB: day after full blooming.

Klen and Vodopivec[22] evaluated the percentage of phenols transferred from olive to oil obtained both in a traditional press and in two- and three-phase centrifuge systems. It was stated that up to 1.5% of olive phenols was transferred to the oil by the two-phase centrifuge system, up to 1.2% by the traditional press and up to 0.5% by the three-phase centrifuge system. No phenolic compounds content of oil was shown, and the olive fruits phenolic content appeared to be very low with respect to the literature data.[12,28] In addition, the percentage of phenolic compounds was not normalized with respect to the relevant oil yield extraction of the aforementioned process systems; thus, its overestimation presumably occurred.

Goldsmith *et al.*[29] investigated the phenolic compounds transfer during olive oil processing by a traditional press. The total phenolic content (TPC), measured by the Folin–Ciocalteu method, resulted in 250 mg kg^{-1} for olive oil and 18 470 mg kg^{-1} for olives; 1.4% of olive phenolic compounds content was transferred to oil, but also in this case the aforementioned value was not normalized with respect to the relevant oil yield extraction.

Klen *et al.*[20] evaluated that only 0.53% of the phenolic amount of the olive fruit passed into the oil. The phenolic mass balance was evaluated only at a laboratory scale, without evaluations of the oil extraction in a real mill.

In our previous work,[12] the crucial effect of the freeze-drying of whole olives immediately after harvesting was demonstrated to preserve the 'native' phenolic profile of olives. Measurement of phenolic compounds content on crushed or cut olives strongly modified olive phenolic profile due to enzymatic transformations. Therefore, a difference in calculating the yield of phenolic compounds transfer may occur if the 'native' phenolic profiles of olives is lost.

Talhaoui *et al.*[30] studied the transfer of single phenolic compounds from olives to oil by working at laboratory scale for six different cultivars. The total phenol transfer rate varied markedly among cultivars, with values between 0.38% and 1.95%. A very low amount of olive fruits phenolic content, characterized by only traces of oleuropein, appeared in disagreement with the literature data.[12,22]

All these attempts demonstrate a strong interest in learning more about the phenolic transfer during oil extraction, with the main goal of better knowledge of this process to increase the phenolic concentration in the olive oils. The aim of this work was to improve the methodological approach for evaluation of phenolic compounds transferred from olives to oil by (i) a measurement of the native phenolic compounds working on the whole freeze-dried olives, (ii) a determination of the phenolic compounds in virgin olive oils by the official International Olive Council (IOC) method, and (iii) an evaluation of the oil yields after milling. The proposed approach was then applied to two different continuous milling systems, working on two different crop seasons in Tuscany.

EXPERIMENTAL

Chemicals

All chemicals for analysis were of analytical grade. Formic acid and hexane were from Sigma Aldrich (Steinheim, Germany), and phosphoric acid was from Merck (Darmstadt, Germany). Methanol and acetonitrile of HPLC grade were from Baker (Phillipsburg, NJ) and Panreac (Barcelona, Spain) respectively. Deionized water was produced by the Milli-Q-system (Millipore SA, Molsheim, France). Syringic acid and tyrosol from Sigma Aldrich and oleuropein from Extrasynthese (Genay, France) were the standard compounds, stock solutions of which were prepared in hydroalcoholic solution.

Samples

All the samples analyzed are summarized in Table 1.

Olive fruits

During the 2012 crop season, 10 olive plants (*Olea europea* L.) of the cultivar Frantoio were selected from a farm located in Fiesole (Florence, Italy). Regular irrigation of the orchard was applied, and full blooming occurred by 15 June. Olive fly attacks were under 1%. Ripe olive fruits were sampled on the 150th and 164th day after full blooming (DAFB), and the whole fruits were freeze-dried immediately after they arrived in the laboratory.

During the 2016 crop season, 10 olive plants (*Olea europea* L.) for each of the two cultivars, Leccio del Corno and Arbequina, were selected from farms located in the province of Florence (Italy). No irrigation of the orchard was applied, and full blooming occurred by 28 April for the two cultivars. Olive fly attacks were under 1%. Ripe olive fruits were sampled on the 190th DAFB and were freeze-dried immediately after they arrived in the laboratory. Sampling was carried out by picking olives from all the selected plants along all their circumference at a height close to 170 cm.

Freeze-drying of olive samples was carried out as previously described by Cecchi *et al.*[12] Briefly, olives were deep-frozen in liquid nitrogen and then placed into the freeze-dryer at −20 °C under 0.1 atm until they reached a constant weight. Freeze-dried samples were stored at −20 °C until analysis.

Olive oils

During the 2012 crop season, three batches of olives at the 150th DAFB and four at the 164th DAFB were collected; the olive fruits were milled in a two-phase continuous extraction system within 24 h after harvest. Each batch of olives was about 600 kg and was milled according to the scheme in Fig. 1A. Briefly, olives were defoliated and debranched, washed and drained using a vibrating table; then, a hammer mill was used to crush the fruits, and the pastes obtained were malaxed in two vertical tank kneaders equipped with a heating jacket, each of capacity of

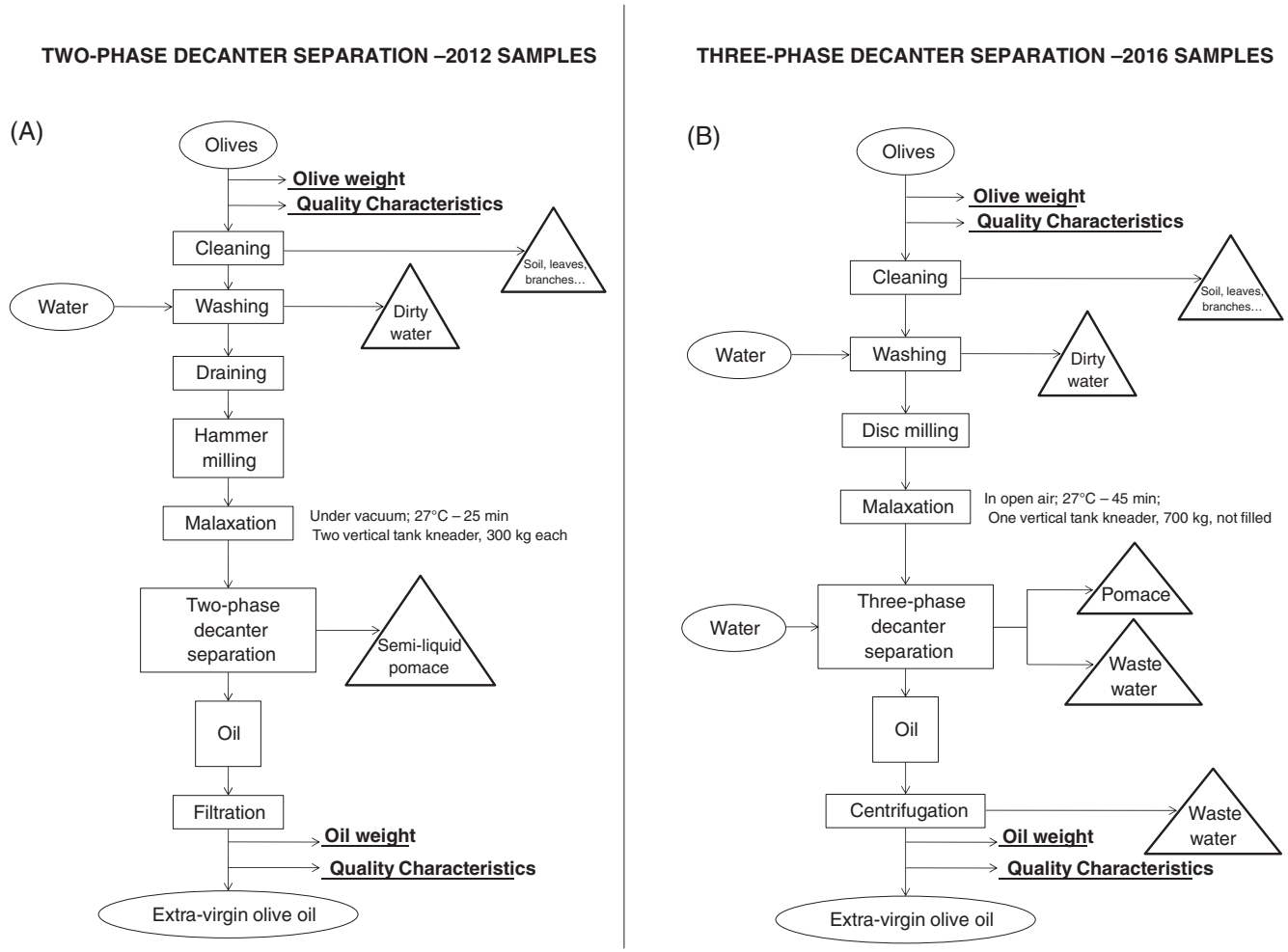

Figure 1. Comparison between the two-- and three-phase continuous extraction system used for the virgin olive oil extraction.

300 kg; malaxation was carried out for 25 min at 27 °C under vacuum. After malaxation, the virgin olive oil was extracted by a two-phase decanter (i.e., no water was added) and filtered by a filter press.

During the 2016 crop season, three batches of olives of each cultivar (Leccio del Corno and Arbequina), harvested on the 190th DAFB, were milled in a three-phase continuous extraction system within 24 h after harvest. Each batch of olives was about 600 kg and was milled according to Fig. 1B. The olives were washed, and then a disc crusher was used to crush the olives; the pastes obtained were malaxed in a horizontal tank kneader equipped with a heating jacket; malaxation was carried out in open air for 45 min at 27 °C, with the kneader not completely filled. After malaxation, the virgin olive oil was extracted by a three-phase decanter (~30 kg of water was added for 100 kg of olives) and were centrifuged at 7000 rpm in a vertical centrifuge; no filtration was applied.

Measurements and determinations

Oil extraction yield

Oil extraction yield (OY) was determined during the olive milling processes by measurement of olive and oil weights, as shown in Fig. 1. The olives were weighed before washing, while the oils were weighed after filtration for the 2012 samples (Fig. 1A) and after centrifugation for the 2016 samples (Fig. 1B).

The yields were calculated both as actual yield (OY) obtained in the process and as extractability index (EI) or olive mill efficiency[31] as follows:

$$OY = \frac{OE_x}{OI_m} \times 100 \quad (1)$$

$$EI = \frac{OE_x}{OC_{om}} \times 100 \quad (2)$$

where OE_x (kg) was the extracted olive oil, OI_m (kg) was the milled olive fruits and OC_{om} (kg) was the oil content of milled olive fruits.

Yield of phenolic compounds transfer from olive to oil

The yields of phenolic compounds transfer were determined during the olive milling processes by measurement of olive and oil phenolic compounds content as shown in Fig. 1. Phenolic yield (PY) was calculated in percent as a normalized value with respect to the aforementioned actual OY as follows:

$$PY = \frac{PO_{Ex} \times OY}{POI_m} \quad (3)$$

where PO_{Ex} (mg kg^{-1}) was the TPC of the extracted olive oil and POI_m (mg kg^{-1}) was the TPC of milled olive fruits.

Water, oil and sugar content of olive fruits

Moisture content (grams per kilogram) was measured by gravimetry between the fresh and freeze-dried samples. The oil content (grams per kilogram) was measured on freeze-dried olives by extraction with hexane in an automatic extractor (Randall mod.148, VELP Scientifica, Milan, Italy), following the method of Cherubini *et al.*[32] Sugar content (grams per kilogram) was measured enzymatically, and expressed as sum of glucose and fructose, as previously reported by Trapani *et al.*[15]

European legal quality characteristics of virgin olive oil

Acidity (percentage oleic acid), peroxide value (milliequivalents of O_2 per kilogram) and spectroscopic indices were measured according to the EU official method.[33] Sensory evaluation of olive oil was performed by a panel test according to the EU official method.[34]

Phenolic compounds content

Olive fruits. Freeze-dried olives were crushed in a small laboratory crusher (Zeutec, Germany), so obtaining an olive cake as homogeneous as possible, from which phenolic compounds were extracted as previously described.[28] Briefly, 4 g of the olive cake was cold extracted twice with 30 mL of EtOH: H_2O 80: 20 solution added with 0.5 mL of the internal standard (syringic acid 1.5 mg mL^{-1}). The solution obtained was concentrated, washed twice with hexane, centrifuged at 7000 rpm and 10 °C and filtered in a 10 mL flask by a cellulose acetate membrane of 0.45 μm; the final volume of 10 mL was reached adding MeOH:H_2O 50:50. The solution obtained was immediately used for the chromatographic analysis, which were carried out using an HP1100 liquid chromatograph equipped with diode array detector (DAD) and mass spectrometry detector with HP1100 MSD API-electrospray interface (all by Agilent Technologies, California, USA). For the 2012 samples a Hypersil Gold QRP-18 (250 mm × 4.6 mm internal diameter (id), 3 μm particle size; Thermo Electron Corp., Austin, TX) column with a pre-column of the same phase was used. The oven temperature was 30 °C. Elution was performed using H_2O (pH 3.2 by formic acid), acetonitrile and methanol at the condition previously described,[12] and the chromatograms were acquired at the following wavelengths: 240, 280, and 330 nm. For the 2016 samples a new-generation Poroshell 120, EC-C18 (150 mm × 3.0 mm id, 2.7 μm particle size; Agilent, USA) column with a pre-column of the same phase was used. The oven temperature was 26 °C. Elution was performed using H_2O (pH 3.2 by formic acid) and acetonitrile with a flow rate of 0.4 mL min^{-1} with the following multistep linear gradient: the organic solvent, acetonitrile (A), changed from 5% at 0.1 min to 40% at 40 min, then remained at 40% until 45 min and changed to 100% at 50 min; after remaining at 100% until 53 min it returned to 5% at 55 min. The chromatograms were acquired at the following wavelengths: 240, 280, and 330 nm. Before using the new-generation column for the 2016 samples, the two columns (Hypersil Gold QRP-18 and Poroshell 120, EC-C18) were preliminarily compared and the results were the same (data not shown).

Quantification of phenolic compounds was carried out by the internal standard method, according to our previous work.[12] Briefly, syringic acid was the internal standard, and the relative response factor (RRF) were evaluated with the following standards: oleuropein, verbascoside, tyrosol and luteolin-7-*O*-glucoside. Consequently, single phenolic compounds were expressed as follows: hydroxytyrosol, hydroxytyrosol glucoside and tyrosol glucoside as milligrams of tyrosol per kilogram (mg_{tyr} kg^{-1}); chlorogenic acid, caffeic acid, verbascoside and verbascoside isomers as milligrams of verbascoside per kilogram (mg_{verba} kg^{-1}); demethyloleuropein, nuzhenide, caffeoyl-6′-secologanoside, oleuropein aglycones, oleuropein, comselogoside and ligstroside as milligrams of oleuropein per kilogram ($mg_{oleurop}$ kg^{-1}); rutin and luteolin-7-*O*-glucoside as milligrams of luteolin per kilogram (mg_{lut} kg^{-1}). TPC was calculated by the integration of all the peaks present in the chromatogram at 280 nm and was expressed as milligrams of oleuropein per kilogram, taking into account that the main peaks in the phenolic profiles are oleuropein and similar secoiridoids.

Virgin olive oil. Phenolic compounds from olive oils were extracted and analyzed according to the IOC official method.[35] Briefly, phenolic compounds were extracted by an MeOH:H_2O 80:20 solution and immediately analyzed. Analyses were performed by an HP1200 liquid chromatograph, equipped with an HP 1200 auto-sampler and HP1200 DADs (all by Agilent Technologies, California, USA). A LiChrospher 100 endcapped RP-18, 5 μm, 250 mm × 4.6 mm id column was used; elution was performed by using the acid H_2O (0.2% H_3PO_4)/acetonitrile/methanol gradient reported in the official method and by an injection volume of 20 μL; identification was carried out at 280 nm. Quantification was carried out by the internal standard method, for which syringic acid was used as internal standard and tyrosol as reference compound. As a consequence, TPC and the content of single secoiridoids, lignans, flavonoids and phenolic alcohols and acid were expressed as milligrams of tyrosol per kilogram of oil (mg_{tyr} $kg_{oil}{}^{-1}$).

Data processing. The precision of the procedure for the quantitation of phenolic compounds of the olive fruits was previously reported by Cecchi *et al.*[12] Regarding the olive oil samples, the standard deviation of TPC was determined according to the official method.[35] To evaluate the precision of extraction and quantitation of each phenolic compound, one oil was selected and, starting from different aliquots of it, quantitation of phenols was repeated eight times and the results obtained were used to calculate the variation coefficient (CV%).

RESULTS AND DISCUSSION

Table 2 shows the quality characteristics of olives used for the oil extractions; cultivars Arbequina and Leccio del Corno had a significant higher moisture content (~60%) than cultivar Frantoio in 2012; this moisture value could cause 'difficult' olive pastes for oil extraction.[36] Phenolic content varied from 24 000 $mg_{oleurop}$ kg^{-1} for cultivar Frantoio at the 164 DAFB to 31 000 $mg_{oleurop}$ kg^{-1} for cultivar Arbequina.

Quality characteristics of cultivar Frantoio olives were congruent with the ripening degree expressed by the DAFB values: higher DAFB values reflected higher oil content values and lower TPC values.[15]

Figure 2 shows two examples of chromatographic profiles at 280 nm of 2016 olive samples. All the main peaks were well resolved and they corresponded to the typical glycosylated phenols of the 'native' fruits (e.g., oleuropein, demethyloleuropein, ligstroside, nuzhenide, verbascoside, rutin, luteolin-7-*O*-glucoside).[12,37,38] Minor or undetectable amounts of the degradation products (e.g., oleuropein aglycones, tyrosol, hydroxytyrosol, caffeic acid) were measured, confirming that our method preserved the olive 'native' phenolic profile.

Table 2. Olive quality characteristics

Cultivar	Year	DAFB	Sugar (g kg^{-1})	Oil (g kg^{-1})	Moisture (g kg^{-1})	TPC (mg$_{oleurop}$ kg^{-1})
Frantoio	2012	150th	24.9^{a} ± 1.7	175^{b} ± 5	530^{b} ± 20	28 643^{a} ± 980
Frantoio	2012	164th	25.6^{a} ± 1.8	193^{a} ± 6	500^{b} ± 20	23 693^{c} ± 810
Arbequina	2016	190th	15.3^{b} ± 1.1	123^{d} ± 4	600^{a} ± 20	30 633^{a} ± 1084
Leccio del Corno	2016	190th	17.6^{b} ± 1.2	133^{c} ± 4	590^{a} ± 20	26 302^{b} ± 900

Different lower case letters in the same column indicate significant differences ($p = 0.01$) for the different samples.

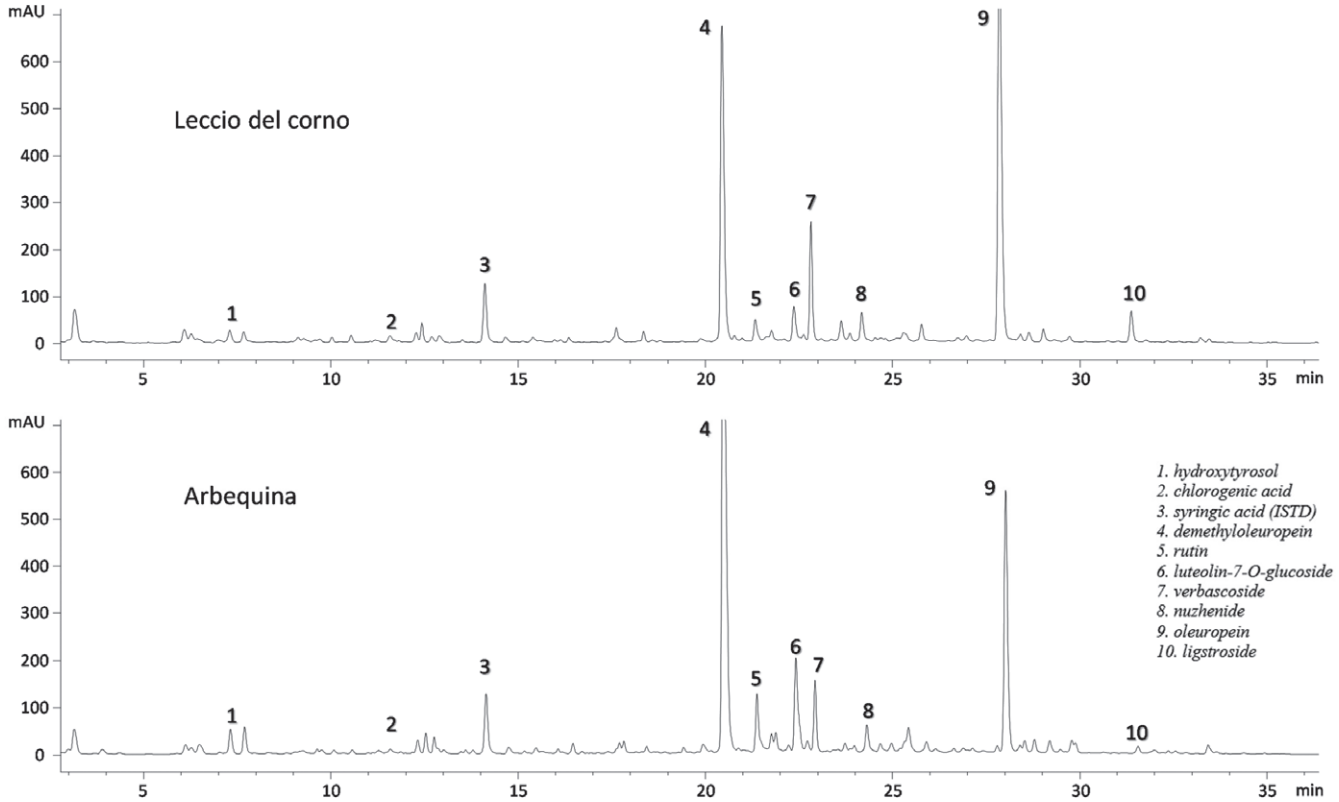

Figure 2. Examples of chromatographic profiles at 280 nm of 2016 olive samples with the main peaks identified.

Table 3 shows the amounts of all the phenolic compounds that were identified in the olive samples. Olive samples had higher values of TPC than have been reported in literature.[20,22,29,30] The glycosylated secoiridoids were the predominant phenolic compounds; they represented ~75% of the identified phenols and they were even 90.6% in cultivar Leccio del Corno olive sample in 2016. The high content of demethyloleuropein seemed to confirm that this phenolic compound was formed from endogenous esterase activity on oleuropein during olive ripening.[12]

Cultivar Arbequina olive sample in 2016 had a particular phenolic profile, which was characterized by a higher percentage of demethyloleuropein content (~33%) and a lower percentage of oleuropein content (~13%) than the other olive samples tested, which were characterized by ~17% of demethyloleuropein content and by ~33% of oleuropein content.

All virgin olive oil samples extracted from olives were classified as extra virgin, and their yields data and oil TPCs are shown in Table 4. The TPC was significantly higher in oil samples extracted from cultivar Frantoio olives in 2012 than in oil samples extracted from cultivar Arbequina and cultivar Leccio del Corno olives in 2016, although a similar difference did not occurred in their olive TPC (Table 2). The application of a three-phase oil extraction system may explain this difference, related to the added water. The dilution of the aqueous phase of olive paste changed the partition equilibrium of phenolic compounds and most of phenolic compounds flushed away with the produced wastewater according to previous studies.[22,39]

Oil samples from cultivar Arbequina olives in 2016 also had the lowest TPC (238 mg$_{tyr}$ kg^{-1}). The particular phenolic profile of cultivar Arbequina olives, previously described in the text, could explain this behavior, since the phenolic compounds with high hydrophilic nature were predominant.[27]

The yields of phenolic compounds transfer (PY) from olive fruits to olive oils was calculated from data in Tables 2 and 4. In 2012 from 1 kg of cultivar Frantoio olives at the 150th DAFB, a mean of 0.158 kg of olive oil was extracted with a total phenolic concentration of 687 mg kg.$^{-1}$ This means that only ~109 mg of the potential 28 643 g of TPC in the fruit were transferred and, then PY was 0.38% (see also Eqn (3)). Following the same approach, PY at the 164th DAFB was 0.40%. These data were one order of magnitude lower than the 2% value reported in the literature,[7,26] and they were also lower than the 0.53% value determined at the laboratory scale.[20]

Table 3. Amount of the phenolic compounds identified in the olive samples[a]

Phenolic compound	Frantoio cv. 2012 DAFB 150	Frantoio cv. 2012 DAFB 164	Arbequina cv. 2016 DAFB 190	Leccio del Corno cv. 2016 DAFB 190
Hydroxytyrosol	159.2	176.2	138.5	86.7
Hydroxytyrosol glucoside	n.d.	n.d.	147.9	66.6
tyrosol glucoside	n.d.	n.d.	22.3	29.7
Chlorogenic acid	62.5	38.5	19.2	31.5
Caffeic acid	n.d.	n.d.	14.2	n.d.
Demethyloleuropein	2 939.2	4 367.6	9 992.5	5 635.6
Rutin	115.4	74.3	160.6	67.4
Luteolin-7-*O*-glucoside	130.2	104.3	296.1	114.2
Verbascoside	630.2	637.6	211.4	389.9
Nuzhenide	577.0	458.8	383.8	438.6
Sum of isoverbascoside isomers	92.3	117.9	5.9	5.4
Caffeoyl-6′-secologanoside	274.3	299.3	64.0	9.8
Sum of oleuropein aglycone isomers	n.d.	692.4	83.0	96.3
Oleuropein	12 286.5	6 522.3	4 115.6	7 339.6
Comselogoside	324.6	391.4	175.9	n.d.
Ligstroside	626.1	321.4	110.8	509.9
Total phenolic compounds	28 643.2	23 693.0	30 633.1	26 302.4

n.d.: not determined.
[a] Data expressed as mg kg^{-1} on fresh fruit basis as explained in the 'Materials and methods' section.

Table 4. Yields data and oil TPCs of extracted extra-virgin olive oils

Cultivar	Year	Oil extraction system	OY (%)	EI (%)	TPC (mg_{tyr} kg^{-1})	PY (%)
Frantoio	2012	Two-phase	15.8	90.3	$687^{a} \pm 78$	0.38
Frantoio	2012	Two-phase	16.1	83.4	$593^{a} \pm 64$	0.40
Arbequina	2016	Three-phase	9.8	79.7	$238^{c} \pm 32$	0.08
Leccio del Corno	2016	Three-phase	11.0	82.7	$445^{b} \pm 48$	0.19

Different lower case letters indicate significant differences ($p = 0.01$) for the different samples.

Furthermore, it should be taken into account that the oils from the 2012 crop season had a high phenolic content (i.e., 593 mg kg^{-1} and 687 mg kg^{-1}), higher even than the mean phenolic concentration of high-quality extra-virgin olive oil which usually did not exceed 350 mg $kg.^{-1}$ [40] These data suggested that for common extra-virgin olive oils extracted by a two-phase system the yields of phenolic compounds transfer could be lower than 0.4%.

In 2016, from 1 kg of cultivar Leccio del Corno olives, a mean of 0.110 kg of olive oil was extracted with a TPC of 445 mg $kg,^{-1}$ indicating that only ~49 mg of the potential 26 302 mg in olives was transferred to the oil. Then, a 0.19% PY was determined (see also Eqn (3)). Following the same approach, the PY of cultivar Arbequina was lower and only of 0.08%. The yields were up to 25 times lower than the 2% value reported in the literature and they were up to five times lower than the PY obtained during oil extraction from cultivar Frantoio olives in 2012 by a two-phase system, confirming the effect of a three-phase extraction system on phenolic compounds content as previously reported in the text.

The aforementioned PY values were related to the oil actual yields (OY), which are also dependent on both the olive quality characteristics and the efficiency of processing steps before the oil extraction, such as olive milling and olive paste malaxation. The highest OY values, and consequently the PY values, were for cultivar Frantoio in 2012 (Table 4). In our trials, the effect of the olive quality characteristics on OY values (i.e., the greater the oil content and the less the water content, the greater the oil yield value) seemed to prevail on the effect of processing steps.[22] Indeed, OY differences in Table 4 (about 15.9% for two-phase extraction and 10.4% for three-phase extraction) were much wider than the EI differences.

Comparing the phenolic profiles of the extracted oils (Table 5), one of the main differences was the oleuropein content, which was higher in the oils from the two-phase system, also in terms of percentage on the TPC. On the other hand, the percentage of oleuropein derivatives was higher into the oils from the three-phase system. This behavior could be explained by both the higher amount of water in the olive fruits of 2016 and the added water in the three-phase system: each of these factors could promote a faster hydrolytic and enzymatic degradation of the secoiridoids.

Regarding lignans (i.e., pinoresinol and acetoxypinoresinol), which has attracted much interest in recent years,[4,41] our data showed the higher percentage in the oils from Arbequina (15.3%) and then the other oils (Frantoio 2012: 150th DAFB, 9.2%; Frantoio 2012: 164th DAFB, 10.2%; Leccio del Corno 2016: 12.2%). These data are in agreement with previous results,[42] according to which the lignans content in olive oils mainly depends on the cultivar.

Table 5. Phenolic contents of extracted extra-virgin olive oils

Phenolic compound	Phenolic content (mg_{tyr} kg^{-1})			
	Frantoio 2012 - DAFB 150	Frantoio 2012 - DAFB 164	Arbequina 2016	Leccio del Corno 2016
Hydroxytyrosol	3.2 ± 0.2	1.4 ± 0.1	1.8 ± 0.1	3.3 ± 0.2
tyrosol	2.3 ± 0.1	2.3 ± 0.1	1.3 ± 0.0	1.5 ± 0.1
Caffeic + vanillic acid	1.3 ± 0.0	0.4 ± 0.0	1.0 ± 0.0	0.7 ± 0.0
Vanillin	2.4 ± 0.2	1.9 ± 0.1	0.8 ± 0.1	0.9 ± 0.1
p-Coumaric acid	0.9 ± 0.1	0.0 ± 0.0	0.8 ± 0.1	0.6 ± 0.1
Hydroxytyrosyl acetate	0.7 ± 0.1	2.4 ± 0.2	0.5 ± 0.0	0.4 ± 0.0
Ferulic acid	0.2 ± 0.0	0.7 ± 0.1	1.5 ± 0.3	0.6 ± 0.1
o-Coumaric acid	1.3 ± 0.2	1.3 ± 0.2	1.0 ± 0.2	0.8 ± 0.1
Dyaldehydic form of decarboxymethyloleuropein aglycon oxidized	45.4 ± 3.8	34.8 ± 2.9	16.0 ± 1.3	34.5 ± 2.9
Dyaldehydic form of decarboxymethyloleuropein aglycon	116.8 ± 1.3	101.2 ± 1.1	56.4 ± 0.6	95.7 ± 1.0
Oleuropein	100.4 ± 3.6	95.3 ± 3.4	15.9 ± 0.6	18.7 ± 0.7
Dyaldehydic form of oleuropein aglycon	43.2 ± 2.7	34.3 ± 2.2	22.2 ± 1.4	49.4 ± 3.1
Dyaldehydic form of decarboxymethylligstroside aglycon oxidized	22.1 ± 1.1	19.8 ± 1.0	3.6 ± 0.2	11.4 ± 0.6
Dyaldehydic form of decarboxymethylligstroside aglycon	61.7 ± 1.3	55.6 ± 1.2	10.2 ± 0.2	33.6 ± 0.7
Pinoresinol +1-acetoxypinoresinol	63.3 ± 1.6	60.2 ± 1.6	36.3 ± 0.9	54.3 ± 1.4
Cinnamic acid	14.5 ± 2.2	11.4 ± 1.7	3.6 ± 0.5	9.3 ± 1.4
Dyaldehydic form of ligstroside aglycon	5.2 ± 0.6	7.4 ± 0.8	7.8 ± 0.9	11.1 ± 1.2
Aldehydic and hydroxylic form of oleuropein aglycon oxidized	33.9 ± 1.5	26.3 ± 1.2	13.3 ± 0.6	26.5 ± 1.2
Luteolin	24.4 ± 3.5	19.3 ± 2.8	7.4 ± 1.1	10.2 ± 1.5
Aldehydic and hydroxylic form of oleuropein aglycon	60.5 ± 0.9	57.6 ± 0.8	14.1 ± 0.2	38.2 ± 0.5
Aldehydic and hydroxylic form of ligstroside aglycon oxidized	32.6 ± 3.2	29.6 ± 2.9	8.6 ± 0.9	13.9 ± 1.4
Apigenin	17.5 ± 1.7	6.1 ± 0.6	6.6 ± 0.6	11.0 ± 1.1
Methyl luteolin	17.8 ± 1.4	6.9 ± 0.6	5.6 ± 0.4	11.0 ± 0.9
Aldehydic and hydroxylic form of ligstroside aglycon	15.8 ± 0.6	16.9 ± 0.6	1.6 ± 0.1	7.9 ± 0.3
Total phenolic compounds	**687 ± 78**	**593 ± 64**	**238 ± 32**	**445 ± 48**
Lignans (%)	9.2	10.2	15.3	12.2
Oleuropein (%)	14.6	16.1	6.7	4.2
Oleuropein derivatives (%)	43.6	42.8	51.2	54.9

The last three lines show the percentages of total lignans, oleuropein and oleuropein derivatives of the TPC.

CONCLUSIONS

Health claims related to phenolic compounds in olive oil are permitted in the European Union. They guarantee that a cause and effect relationship has been established between the consumption of olive oil phenolic compounds and protection of low-density lipoprotein particles from oxidative damage. Therefore, both increase and control of yield of phenolic compounds transfer from olive to oil may be aims to an improved extra-virgin olive oil processing.

In this study, a methodological approach for an improved measurement of PY was set up. This approach is independent from the complexity of transformation and transfer phenomena of phenolic compounds during oil extraction.

The measurement of phenolic compounds content on whole lyophilized olive fruits allowed the avoidance of enzymatic transformations of these molecules, and the 'native' phenolic profiles of olives were preserved. In this profile, secoiridoids, and in particular oleuropein, are by far the most abundant compounds; therefore, we proposed to express the TPC as milligrams of oleuropein per kilogram of fresh olives.

Regarding PY, more realistic values were determined by the proposed approach, which allows the expression of PY as percentage normalized values with respect to the OY. By this approach, PY was approximately one order of magnitude lower than previous data in the literature.

In agreement with previous literature, the three-phase extraction system appears less efficient than a two-phase continuous extraction system to allow high phenolic recovery. At the same time, the ripening degree of olive fruits confirmed to have a significant effect on OY and indirectly on PY values, and particularly a lower water content was associated with higher oil yield. The proposed approach is applicable in all types of milling processes.

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REFERENCES

1 Beauchamp GK, Keast RSJ, Morel D, Lin J, Pika J, Han Q *et al.*, Phytochemistry: ibuprofen-like activity in extra-virgin olive oil. *Nature* **437**:45–46 (2005).
2 Achat S, Rakotomanomana N, Madani K and Dangles O, Antioxidant activity of olive phenols and other dietary phenols in model gastric conditions: scavenging of the radical DPPH and inhibition of the haem-induced peroxidation of linoleic acid. *Food Chem* **213**:135–142 (2016).
3 Franco MN, Galeano-Diaz T, Lopez O, Fernandez-Bolanos JG, Sanchez J, De Miguel C *et al.*, Phenolic compounds and antioxidant capacity of virgin olive oil. *Food Chem* **163**:289–298 (2014).

4 López-Biedma A, Sánchez-Quesada C, Delgado-Rodiguez M and Gaforio JJ, The biological activities of natural lignans from olives and virgin olive oil: a review. *J Funct Food* **26**:36–47 (2016).
5 Garcia-Vilas JA, Quesada AR and Medina MA, Hydroxytyrosol targets extracellular matrix remodeling by endothelial cells and inhibits both *ex vivo* and *in vivo* angiogenesis. *Food Chem* **221**:1741–1746 (2017).
6 Covas MI, Olive oil and the cardiovascular system. *Pharmacol Res* **55**(3): 175–186 (2007).
7 Ciriminna R, Meneguzzo F, Fidalgo A, Ilharco LM and Pagliaro M, Extraction, benefits and valorization of olive polyphenols. *Eur J Lipid Sci Technol* **118**:503–511 (2016).
8 De Bock M, Hodgkinson S, Curtfield W and Schlothauer RC, Methods and uses of an extract from olive leaf in management of type 2 diabetes. Patent WO/2014/038962 (2014).
9 Covas MI, Nyyssonen K, Poulsen HE, Kaikkonen J, Zunft HJ, Kiesewetter H *et al.*, The effect of polyphenols in olive oil on heart disease risk factor: a randomized trial. *Ann Intern Med* **145**:333–341 (2006).
10 De la Torre-Carbot K, Chavez-Servin JL, Jauregui O, Castellote AI, Lamuela-Raventos RM, Nurmi T *et al.*, Elevated circulating LDL phenol levels in men who consumed virgin rather than refined olive oil are associated with less oxidation of plasma LDL. *J Nutr* **140**:501–508 (2010).
11 EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), Scientific opinion on the substantiation of health claims related to polyphenols in olive oil and protection of LDL particles from oxidative damage. *EFSA J* **9**:2033 (2011). [Online]. Available: http://www.efsa.europa.eu/en/efsajournal/pub/2044.htm [08 April 2011].
12 Cecchi L, Migliorini M, Cherubini C, Innocenti M and Mulinacci N, Whole lyophilized olives as sources of unexpectedly high amounts of secoiridoids: the case of three Tuscan cultivars. *J Agric Food Chem* **63**:1175–1185 (2015).
13 Koseoglu O, Sevim D and Kadiroglu P, Quality characteristic and antioxidant properties of Turkish monovarietali olive oils regarding stages of olive ripening. *Food Chem* **212**:628–634 (2016).
14 Peres F, Martins LL, Mourato M, Vitorino C, Antunes P and Ferreira-Dias S, Phenolic compounds of 'Galega vulgar' and 'Cobrancosa' olive oils along early ripening stages. *Food Chem* **211**:51–58 (2016).
15 Trapani S, Migliorini M, Cherubini C, Cecchi C, Canuti V, Fia G *et al.*, Direct quantitative indices for ripening of olive oil fruits to predict harvest time. *Eur J Lipid Sci Technol* **118**:1202–1212 (2016).
16 Merchak N, El Bacha E, Bou Khouzam R, Rizk T, Akoka S and Bejjani J, Geoclimatic, morphological and temporal effects on Lebanese olive oils composition and classification: a ^{1}H-NMR study. *Food Chem* **217**:379–388 (2017).
17 Hbaieb RH, Kotti F, Cortes-Francisco N, Caixach J, Gargouri M and Vichi S, Ripening and storage conditions of Chetoui and Arbequina olives: part II. Effect on olive endogenous enzymes and virgin olive oil secoiridoid profile determined by high resolution mass spectrometry. *Food Chem* **210**:631–639 (2016).
18 Delgado-Povedano MDM, Priego-Capote F and Luque de Castro MD, Selective ultrasound-enhanced enzymatic hydrolysis of oleuropein to its aglycon in olive (*Olea europaea* L.) leaf extracts. *Food Chem* **220**:282–288 (2017).
19 Jolayemi OS, Tokatli F and Ozen B, Effects of malaxation temperature and harvest time on the chemical characteristic of olive oils. *Food Chem* **211**:776–783 (2016).
20 Klen TJ, Wondra AG, Vrhovsek U, Sivilotti P and Vodopivec BM, Olive fruit phenol transfer, transformation and partition trail during laboratory-scale olive oil processing. *J Agric Food Chem* **63**:4570–4579 (2015).
21 Artajo LS, Romero MP, Suarez M and Motilva MJ, Partition of phenolic compounds during the virgin olive oil industrial extraction process. *Eur Food Res Technol* **225**:617–625 (2007).
22 Klen TJ and Vodopivec BM, The fate of olive fruit phenols during commercial olive oil processing: traditional press *versus* continuous two- and three-phase centrifuge. *Food Sci Technol* **49**:267–274 (2012).
23 Migliorini M, Mugelli M, Cherubini C, Viti P and Zanoni B, Influence of O_2 on the quality of virgin olive oil during malaxation. *J Sci Food Agric* **86**:2140–2146 (2006).
24 Migliorini M, Cecchi L, Cherubini C, Trapani S, Cini E and Zanoni B, Understanding degradation of phenolic compounds during olive oil processing by inhibitor addition. *Eur J Lipid Sci Technol* **114**:942–950 (2012).
25 Trapani S, Breschi C, Cecchi L, Guerrini L, Mulinacci N, Parenti A *et al.*, Indirect indices of oxidative damage to phenolic compounds for the implementation of olive paste malaxation optimization charts. *J Food Eng* **207**:24–34 (2017).
26 Cardoso MS, Falcao SI, Peres AM and Domingues MRM, Oleuropein/ligstroside isomers and their derivatives in Portuguese olive mill wastewater. *Food Chem* **129**:291–296 (2011).
27 Rodis PS, Karathanos VT and Mantzavinou A, Partitioning of olive oil antioxidants between oil and water phases. *J Agric Food Chem* **50**:596–601 (2002).
28 Cecchi L, Migliorini M, Cherubini C, Giusti M, Zanoni B, Innocenti M *et al.*, Phenolic profiles, oil amount and sugar content during olive ripening of three typical Tuscan cultivar to detect the best harvesting time for oil production. *Food Res Int* **54**:1876–1884 (2013).
29 Goldsmith CD, Stathopoulos CE, Golding JB and Roach PD, Fate of the phenolic compounds during olive oil production with the traditional press method. *Int Food Res J* **21**:101–109 (2014).
30 Talhaoui N, Gomez-Caravaca AM, Leon L, De la Rosa R, Fernandez-Gutierrez A and Segura-Cerretero A, From olive fruits to olive oil: phenolic compound transfer in six different olive cultivars grown under the same agronomical conditions. *Int J Mol Sci* **17**:337–350 (2016).
31 Boskou D, Olive oil quality, in *Olive Oil: Chemistry and Technology*, ed. by D. Boskou. AOCS Press, Champaign, IL, pp. 101–120 (1996).
32 Cherubini C, Migliorini M, Mugelli M, Viti P, Berti A, Cini E *et al.*, Towards a technological ripening index for olive oil fruits. *J Sci Food Agric* **89**:671–682 (2009).
33 European Economic Community, Commission Regulation (EEC) No. 2568/91 of 11 July 1991 on the characteristic of olive oil and olive-residue oil and on the relevant methods of analysis. *Off J Eur Communities Legis* **L24**:1–140 (1991).
34 European Economic Community, Commission Regulation (EEC) No. 640/2008 of 4 July 2008 amending Regulation (EEC) No. 2568/91 on the characteristics of olive oil and olive-residue oil and on the relevant methods of analysis. *Off J Eur Communities Legis* **L178**:11–16 (2008).
35 IOC/T.20/Doc No. 29, Official method of analysis. Determination of biophenols in olive oil by HPLC. International Olive Council, Madrid (2009).
36 Aguilera MP, Beltran G, Sanchez-Villasclaras S, Uceda M and Jimenez A, Kneading olive paste from unripe 'Picual' fruits: I. Effect on oil process yield. *J Food Eng* **97**:533–538 (2010).
37 Morello JR, Vuorela S, Romero MP, Motilva MJ and Heinonen M, Antioxidant activity of olive pulp and olive oil phenolic compound of the Arbequina cultivar. *J Agric Food Chem* **53**:2002–2008 (2005).
38 Servili M, Baldioli M, Selvaggini R, Macchioni A and Montedoro GF, Phenolic compounds of olive fruit: one and two-dimensional nuclear magnetic resonance characterization of nuzhenide and its distribution in the constitutive parts of fruit. *J Agric Food Chem* **47**:12–18 (1999).
39 Servili M, Selvaggini R, Esposto S, Taticchi A, Montedoro GF and Morozzi G, Health and sensory properties of virgin olive oil hydrophilic phenols: agronomic and technological aspects of production that affect their occurrence in the oil. *J. Chromatogr A* **1054**:113–127 (2004).
40 Cecchi L, Migliorini M, Cherubini C, Trapani S and Zanoni B, The case of the 2014 crop season in Tuscany: a survey of the effect of the olive fruit fly attack. *Ital J Food Sci* **28**:352–361 (2016).
41 Cecchi L, Innocenti M, Melani F, Migliorini M, Conte L and Mulinacci N, New isobaric lignans from refined olive oils as quality markers for virgin olive oils. *Food Chem* **219**:148–157 (2017).
42 Oliveras-Lopéz MJ, Innocenti M, Giaccherini C, Ieri F, Romani A and Mulinacci N, Study of the phenolic composition of Spanish and Italian monocultivar extra virgin olive oils: distribution of lignans, secoiridoidic, simple phenols and flavonoids. *Talanta* **73**:726–732 (2007).