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Article Sub-Title		
Article CopyRight	Springer-Verlag Berlin Heidelberg (This will be the copyright line in the final PDF)	
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Schedule		
	Received	29 February 2016
	Revised	24 May 2016
	Accepted	16 July 2016

Abstract	
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Keywords (separated by '-')	Food supplement - Oleuropein - HPLC separation - HPLC/DAD analysis - Tuscany and Apulia olive leaves
Footnote Information	

Eur Food Res Technol
DOI 10.1007/s00217-016-2756-3

ORIGINAL PAPER

Polyphenols and secoiridoids in raw material (*Olea europaea* L. leaves) and commercial food supplements

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Received: 29 February 2016 / Revised: 24 May 2016 / Accepted: 16 July 2016

Abstract Twenty-five compounds, among which flavonoids and secoiridoids, were separated and quantified after extraction from *Olea europaea* leaves. Differences were found in total polyphenols content and in oleuropein depending on cultivar, production area, sampling time (pruning or harvest time), and state of leaves (fresh, refrigerated, dried, frozen, or lyophilized). Polyphenols content in fresh leaves ranged from 34.21 to 7.87 mg/g, while oleuropein content changes from 21.03 to 2.79 mg/g in fresh leaves of different cultivars and decreases after the drying process. The differences are discussed in order to exploit these by-products for food supplements. In addition, five commercial food supplements from olive leaves were analyzed, and their total polyphenol, secoiridoids, and flavonoid contents were detected by HPLC/DAD analysis. In order to provide stable contents of bioactive molecules, all the above-mentioned variabilities should be taken into account.

Keywords Food supplement · Oleuropein · HPLC separation · HPLC/DAD analysis · Tuscany and Apulia olive leaves

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Introduction

Olea europaea L. leaves, a typical herbal drug of the Mediterranean region, have been widely used like traditional remedy as extract, infusion, herbal tea, and powder in countries such as Greece, Spain, Italy, France, Turkey, Israel, Morocco, Albania, and Tunisia. Olive leaves are the source of many bioactive compounds, the main of which is oleuropein, a secoiridoid, which can constitute up to 6–9 % of leaf dry matter. Oleuropein and its derivatives exhibit specific biological activities as antioxidant, antihypertensive, antiatherogenic, anti-inflammatory, hypoglycemic, hypocholesterolemic, antiproliferative, and antifungal [1–10]. The composition of leaves extract has been studied, and active compounds were identified such as secoiridoids, flavonoids, and triterpenes [7, 11–13]. Olive leaves may be regarded as a by-product in the cultivation of olives both for olive oil and table olives during pruning operations and/or during olive harvest; leaves extract is used to prepare commercial affordable dietary supplements [14]. Extraction process in order to obtain commercial supplements needs quite constant starting material while it has been pointed out that leaf polyphenols content depends on cultivar [7], geographic production zone, and time of olive leaf harvesting [15].

From the quantitative determination of flavonoids and secoiridoid derivatives of leaves, subjected to different treatments, the final product, i.e., dietary supplements and/or dry leaves, or extracts used for pharmaceutical purposes, can be achieved with a quite constant content of bioactive compounds. We set up a method, which was tested to characterize and quantify secondary metabolites (oleuropein and its derivatives, flavonoids, hydroxycinnamic acids, hydroxytyrosol, and elenolic acid derivatives) in *Olea europaea* leaves extracts. The aim of this study is the characterization of fresh, refrigerated, frozen, dried, and lyophilized

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Table 1 Elution method

Time (min)	H_2O/HCOOH (%)	CH_3CN (%)	Flow (mL/min)
0.1	100	0	0.8
23	89	11	0.8
33	89	11	0.8
41	87	13	0.8
45	87	13	0.8
55	80	20	0.8
68	80	20	0.8
74	0	100	0.8
82	0	100	0.8

Olea leaves of different cultivars under various extraction conditions. The identification of the best operating conditions, which may help in obtaining a high and almost constant bioactive products yield when *Olea* leaves are used in the achievement of commercial food supplements, is the further goal of the study.

Materials and methods

Plant material

Olive leaves were collected in Tuscany (Siena district), Latium (Rieti district), and Apulia (Foggia district) during the year 2014 and were immediately processed.

Extraction

Fresh **cut** leaves were extracted with water at 70 °C for 30 and/or 60 min. The same conditions were applied to leaves stored in refrigerator (4 °C) and in freezer (−18 °C). Fresh leaves were extracted overnight with ethanol/water (30:70) under stirring. Fresh leaves were dried at room temperature for 15 days, or in ventilated stove at 40 °C for 3 days or lyophilized. Extracts were obtained at different of *Olea* leaves percentages (g leaves/100 g solvent). Five liquid commercial Olea leaves food supplements were analyzed after 1:3 water dilution.

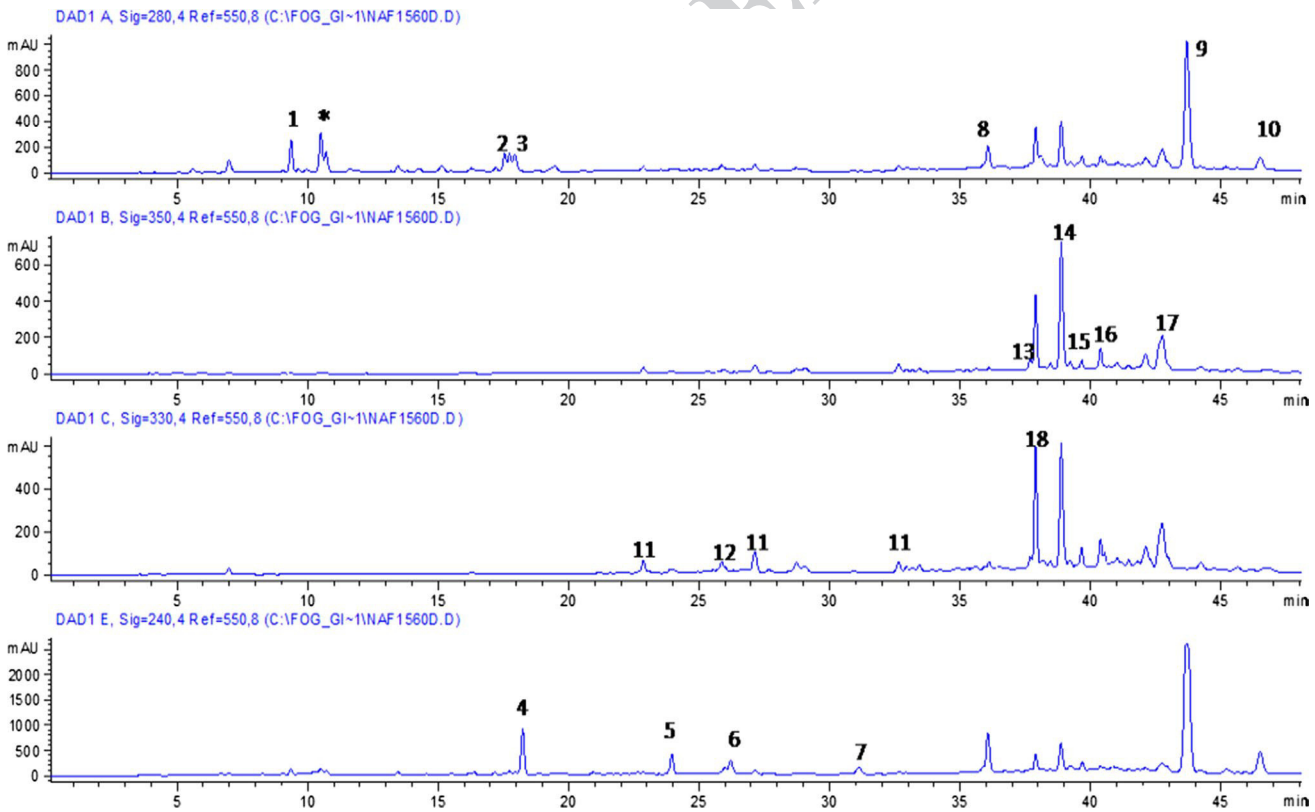

Fig. 1 Chromatograms of the aqueous extract of Frantoio leaves recorded at 240, 280, 330, and 350 nm. *1*. Hydroxytyrosol glicol; *2*. hydroxytyrosol; *3*. hydroxytyrosol glucoside; *4*. oleoside; *5*. elenolic acid diglucoside; *6*. elenolic acid glucoside; *7*. elenolic acid glucoside derivative; *8*. dimethyl oleuropein; *9*. oleuropein *10*. ligustaloside B.; *11*. caffeic acid derivatives; *12*. p-coumaric acid derivatives; *13*. rutin; *14*. luteolin-7-O-glucoside; *15*. quercetin-3-O-glucoside; *16*. apigenin-7-O-glucoside; *17*. luteolin-4′-O-glucoside + Chrysoeriol; *18*. verbascoside; *Asterisk* cinnamic acid derivative

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HPLC/DAD analyses

Analyses of polyphenols were carried out using a HP 1200 liquid chromatograph equipped with a DAD detector and managed by an Agilent HPLC Chemstation (Agilent Technologies, Palo Alto, CA, USA). Compounds were separated using a 250 × 4.6 mm i.d, 5-μm Lichrosorb RP18 column. UV/Vis spectra were recorded in the 190- to 600-nm range and the chromatograms acquired at 250, 280, 330, and 350 nm. The samples were analyzed by gradient elution at a flow rate of 0.8 mL/min. The mobile phase is a multi-steps linear solvent gradient system, starting from 100 % H_2O (adjusted to pH 3.2 by HCOOH) up to 100 % acetonitrile in 82 min. The elution method is reported in Table 1.

Identification and quantification of individual compounds

The identity of polyphenols was ascertained using data from HPLC/DAD analyses, by comparison with bibliographic data [16] and combination of retention times and UV/Vis spectra with those of authentic standards. Hydroxytyrosol, verbascoside, vitexin diglucoside, rutin, luteolin-7-*O*-glucoside, quercetin-3-*O*-glucoside, apigenin-7-*O*-glucoside, apigenin-7-*O*-rutinoside, luteolin-4′-*O*-glucoside, luteolin, **chrysoeriol**-7-*O*-glucoside, and oleuropein were purchased from Extrasynthese (Lyon, France). The following compounds were isolated by preparative HPLC: hydroxytyrosol glycol, hydroxytyrosol glucoside, elenolic acid glucoside, dimethyl oleuropein, 10-hydroxy-oleuropein glucoside, and ligustaloside B. Quantification of individual polyphenolic compounds was performed by HPLC/DAD using a five-point regression curve ($r^2 = 0.998$) in the range of 0–30 □g on the basis of authentic standards. In all cases, concentrations of the derivatives were calculated after applying corrections for differences in molecular weight. Each sample was analyzed in triplicate, to express the analytical results as an average with its standard deviation.

Table 2 Quantitative data of the aqueous extract of four olive cultivars

Compound	Frantoio	Leccino	Moraiolo	Carboncella
Hydroxytyrosol glycol	0.57 (0.11)	0.21 (0.04)	0.22 (0.03)	Traces
Hydroxytyrosol glucoside	1.36 (0.12)	0.60 (0.06)	0.68 (0.07)	1.95 (0.23)
Hydroxytyrosol	0.12 (0.02)	0.06 (0.01)	0.10 (0.02)	0.49 (0.07)
Cinnamic acid derivative	Traces	Traces	Traces	Traces
Oleoside dimethyl glucoside	1.36 (0.24)	0.47 (0.04)	0.83 (0.11)	1.05 (0.15)
Oleoside derivative dimethyl glucoside	1.81 (0.22)	0.86 (0.12)	1.09 (0.09)	1.15 (0.19)
Elenolic acid glucoside	1.55 (0.19)	0.67 (0.1)	0.95 (0.08)	0.18 (0.02)
Elenolic acid glucoside derivative	1.01 (0.18)	0.38 (0.04)	0.53 (0.09)	0.59 (0.09)
Caffeic acid derivatives	0.28 (0.05)	0.12 (0.02)	0.13 (0.02)	0.11 (0.01)
p-coumaric acid derivatives	0.03 (0.006)	Traces	0.01 (0.002)	0.01 (0.002)
Verbascoside	0.73 (0.08)	0.16 (0.03)	0.18 (0.04)	0.30 (0.04)
Vitexin diglucoside	Traces	Traces	Traces	Traces
Luteolin diglucoside	0.07 (0.01)	0.02 (0.003)	0.02 (0.004)	0.04 (0.007)
Rutin	0.51 (0.06)	0.10 (0.02)	0.14 (0.02)	0.09 (0.01)
Luteolin-7-O-glucoside	1.04 (0.21)	0.28 (0.04)	0.33 (0.04)	0.38 (0.03)
Quercetin-3-O-glucoside	0.34 (0.05)	0.03 (0.006)	0.06 (0.005)	0.04 (0.005)
Apigenin-7-O-glucoside	0.32 (0.05)	0.04 (0.003)	0.06 (0.004)	0.04 (0.004)
Apigenin-7-O-rutinoside	Traces	Traces	Traces	Traces
Luteolin-4′-O-glucoside	0.32 (0.05)	0.12 (0.02)	0.17 (0.01)	0.31 (0.04)
Luteolin	Traces	Traces	Traces	Traces
Chrysoeriol-7-O-glucoside	0.12 (0.01)	Traces	Traces	Traces
Dimethyl oleuropein	1.06 (0.08)	0.45 (0.04)	0.65 (0.03)	Traces
10-hydroxy-oleuropein glucoside	0.77 (0.08)	0.23 (0.04)	0.38 (0.04)	Traces
Oleuropein	13.64 (0.71)	2.79 (0.11)	3.83 (0.14)	11.63 (0.59)
Ligustaloside B	1.16 (0.12)	0.28 (0.02)	0.38 (0.04)	1.26 (0.07)
Total polyphenols	28.17	7.87	10.74	19.62

Data are mg/g fresh weight. Standard deviation within brackets

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Results and discussion

In Fig. 1, the chromatograms of the aqueous extract of Frantoio leaves are reported at four different wavelengths. A number marks all the identified compounds. Secoiridoid derivatives are the most abundant compounds in the extract. In Table 2, the quantitative data of Frantoio leaves are compared to those of Leccino, Moraiolo, and Carboncella. These four Italian cultivars are much widely used for olive oil production: Leccino and Frantoio are peculiar Tuscany cultivars, Moraiolo is typical of central Italy regions, and Carboncella is a Latium cultivar from the Sabina area. Frantoio is by far the richest matrix in oleuropein and in flavonoids with regard to Leccino and Moraiolo, while Carboncella exhibited the highest amount of hydroxytyrosol and hydroxytyrosol derivatives and comparable amount of oleuropein. The contents of biofunctional compounds are higher than those reported for Tunisian cultivars [7, 10], while lower than those relative to unknown provenance olive leaves [11]. Oleuropein content is lower than that extracted with methanol/water mixture from Tunisian Chemlali leaves, and hydroxytyrosol content was higher than that reported for the same leaves [12]. With the ethanol/water extraction method, polyphenols amount was much lower in the case of Frantoio and Carboncella (22 and 27 %, respectively) and lower in the case of Moraiolo (52 %) and Leccino (70 %). Other than cultivar, even extraction solvent conditions affect the profile of the starting material so as the production area. In Table 3, biomolecules content of Ogliarola cultivar leaves is reported; for four out of five provenances, oleuropein and polyphenols contents are very close each other; only in the case of Gargano, a lesser amount was found. Leaves from Bicchieri are the richest in hydroxytyrosol and hydroxytyrosol derivatives, while for flavons and hydroxyl-cinnamic acids no important variation was pointed out. Sampling time, on the contrary, has a much larger importance on secondary metabolites content. For Carboncella cultivar, the content changes from 33.9 mg/g fresh

Table 3 Polyphenols content of Ogliarola leaves sampled in different Apulia zones

Compound	Ogliarola Cerignola	Ogliarola Bicchieri	Ogliarola Mattinata	Ogliarola Gargano	Ogliarola standard
Hydroxytyrosol glycol	0.39 (0.02)	0.39 (0.02)	0.25 (0.01)	0.24 (0.01)	0.26 (0.01)
Hydroxytyrosol glucoside	3.20 (0.12)	6.99 (0.07)	5.85 (0.07)	4.93 (0.11)	5.08 (0.12)
Hydroxytyrosol	0.37 (0.02)	0.80 (0.01)	0.24 (0.02)	0.24 (0.01)	0.23 (0.02)
Cinnamic acid derivative	Trace	Trace	Trace	Trace	Trace
Oleoside dimethyl glucoside	1.27 (0.07)	1.60 (0.06)	1.61 (0.07)	1.12 (0.06)	1.82 (0.05)
Oleoside dimethyl glucoside derivative	2.69 (0.10)	2.54 (0.11)	0.73 (0.06)	1.30 (0.08)	0.84 (0.04)
Elenolic acid glucoside	0.28 (0.01)	0.27 (0.02)	0.33 (0.01)	0.19 (0.02)	0.25 (0.01)
Elenolic acid glucoside derivative	0.55 (0.03)	0.67 (0.02)	0.36 (0.04)	0.52 (0.04)	0.45 (0.03)
Caffeic acid derivatives	0.06 (0.002)	0.07 (0.003)	0.10 (0.001)	0.05 (0.002)	0.08 (0.002)
p-coumaric acid derivatives	0.03 (0.001)	0.03 (0.001)	0.03 (0.001)	0.02 (0.001)	0.04 (0.001)
Verbascoside	0.55 (0.02)	0.55 (0.02)	0.25 (0.03)	0.15 (0.01)	0.22 (0.01)
Vitexin diglucoside	Trace	Trace	Trace	Trace	Trace
Luteolin diglucoside	Trace	0.24 (0.01)	Trace	Trace	Trace
Rutin	0.21 (0.01)	0.82 (0.02)	0.19 (0.01)	0.14 (0.009)	0.24 (0.008)
Luteolin-7-O-glucoside	0.60 (0.02)	Trace	0.58 (0.02)	0.30 (0.03)	0.70 (0.03)
Quercetin-3-O-glucoside	Trace	Trace	Trace	Trace	Trace
Apigenin-7-O-glucoside	Trace	Trace	Trace	Trace	Trace
Apigenin-7-O-rutinoside	Trace	Trace	Trace	Trace	Trace
Luteolin-4′-O-glucoside	Trace	Trace	Trace	Trace	Trace
Luteolin	Trace	Trace	Trace	Trace	Trace
Chrysoeriol-7-O-glucoside	Trace	0.24 (0.01)	Trace	Trace	Trace
Dimethyl oleuropein	Trace	Trace	Trace	Trace	Trace
10-hydroxy-oleuropein glucoside	Trace	Trace	Trace	Trace	Trace
Oleuropein	21.03 (1.05)	16.84 (0.88)	17.45 (0.91)	12.77 (0.76)	20.32 (1.01)
Oleuropein derivatives	2.15 (0.08)	2.16 (0.09)	3.32 (0.07)	2.00 (0.09)	3.41 (0.08)
Ligustaloside B	Trace	Trace	Trace	Trace	Trace
Total polyphenols	33.38	34.21	31.29	23.97	33.94

Data are mg/g, fresh weight. Standard deviation within brackets

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Table 4 Total polyphenol content of leaves under different extraction conditions

State of starting material	Extracted leaves (%)	Extraction time	Frantoio	Carboncella
Fresh	15	60′	17.8 (43.9 %)	19.8 (58.7 %)
Fresh	15	30′	13.6 (38.5 %)	
Fresh	10	60′	18.7 (42.4 %)	
Fresh	10	30′	13.3 (27.6 %)	
Freezer—18 °C, 40 days	15	60′	16.7 (47.3 %)	4.2 (17.5 %)
Freezer—18 °C, 40 days	15	30′	11.3 (47.0 %)	
Refrigerator 4 °C, 40 days	15	60′	13.1 (39.8 %)	
Refrigerator 4 °C, 40 days	15	30′	12.3 (42.6 %)	
Ambient temperature, 18–20 °C, 40 days	15	60′	16.3 (46.8 %)	
Ambient temperature, 18–20 °C 40 days	15	30′	11.3 (32.6 %)	
Dry, room temperature, 15 days	15	60′	16.1 (22 %)	
Dry, room temperature, 15 days	15	30′	12.3 (19.5 %)	7.1 (8.3 %)
Ventilated stove 40 °C, 3 days	15	60′	16.8 (41 %)	
Ventilated stove 40 °C, 3 days	15	30′	11.4 (26 %)	

Data of fresh leaves are mg/g, fresh weight; data of dried leaves are mg/g dry weight. Oleuropein percentage within brackets. The percentage of extracted leaves is relative to the weight of fresh or dry leaves expressed as g/100 g solvent

Table 5 Total polyphenol content (mg/g) of lyophilized material from fresh and dried leaves under different extraction conditions

Cultivar	Fresh, 15 %, 60′	Fresh, 15 % 30′	Fresh, 10 % 60′	Fresh, 10 % 30′	Dry, 10 %, 60′	Dry, 10 % 30′
Frantoio	130.5 (47.4 %)	122.6 (43.9 %)	128.7 (45.8 %)	99.0 (30.0 %)	87.6 (25.2 %)	85.9 (23.3 %)
Carboncella	292.0 (58.8 %)	278.2 (51.6 %)			62.1 (2.1 %)	64.2 (1.5 %)

Oleuropein percentage within brackets. The percentage of extracted leaves is relative to the weight of fresh or dry leaves expressed as g/100 g solvent

weight at pruning time to 19.8 mg/g fresh weight at olive harvest time, with oleuropein content changing from 51 to 59 %. This occurrence has already been pointed out [15] when leaves are used for the extraction of biocomponents. It has already been demonstrated that thawing of frozen leaves involves a loss in oleuropein content, while drying at room temperature preserves oleuropein [17]. Our data partly confirm these findings. In the case of Frantoio (see Table 4), there are minor differences depending on the starting material status, while in the case of Carboncella the best results were achieved when fresh leaves are extracted and even the drying process causes a loss in oleuropein content. Along with the increase in extraction time, an increase in extracted biomolecules is generally observed (from 23 to 32 %); this increase, however, involving a longer extraction period, may not justify the production of high extraction volumes in the light of the raw material low cost. When lyophilized material is used, as reported in Table 5, minor differences owing to the extraction time were found. For Carboncella, the polyphenols content decrease, with dry lyophilized leaves respect to fresh ones, is about 80 %, while in the case of Frantoio under the same conditions the decrease is about 33 %. These differences may be ascribed to the different dying condition of the two cultivars (see experimental section). Also dried leaves in many cases are commercialized for industrial production of phytotherapic compounds. We deemed it interesting, therefore, to analyze commercial dried leaves from three different provenances, Morocco, Albania, and Italy. Table 6 lists oleuropein and secoiridoids derivatives contents: Moroccan leaves are the richest in polyphenols. We may assume that the differences are bound not only to raw materials characteristics but also to the different drying conditions, which affect the final product (see Table 3) and to the period in which the leaves were

Table 6 Oleuropein and secoiridoid derivatives content (mg/g) of commercial dried leaves (3 % humidity)

Provenance	Oleuropein	Secoiridoid derivatives	Total
Morocco	15.84 (0.63)	1.94 (0.09)	17.78
Albania	9.35 (0.41)	0.81 (0.04)	10.17
Italy	1.6 (0.05)	0.98 (0.04)	2.58

Standard deviation within brackets.*xx*

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Table 7 Total polyphenol, secoiridoids, and flavonoid contents (mg/L) of commercial food supplements obtained from *Olea* leaves extract

Compounds	Olife lot 3113 expiry date 06/15	Olife lot 4148 expiry date 06/16	Verdepuro expiry date 05/2017	Verdepuro expiry date 05/2018	Farmaderbe expiry date 12/2015
Tyrosol derivatives	198.70 (9.42)	191.50 (6.70)	252.50 (5.35)	318.00 (12.72)	95.50 (8.78)
Elenolic Acid glucoside derivatives	116.14 (4.64)	48.38 (1.98)	182.25 (4.01)	119.93 (4.92)	31.95 (2.78)
Oleuropein	1061.85 (31.82)	682.15 (12.96)	1289.05 (46.44)	1282.40 (54.6)	Traces
Flavonoids	72.00 (5.04)	80.10 (6.44)	101.12 (9.01)	122.69 (11.34)	12.64 (0.63)
Total polyphenols	1448.69	1002.12	1824.92	1843.01	140.09

Standard deviation within brackets

harvested. From oleuropein content, we may assume that Moroccan and Albanian leaves were harvested at the pruning time different from Italian leaves, which were collected at olive technological harvest time. Table 7 lists the quantitative data of commercial food supplements from olive leaves (almost 90 % of the commercial product). Different contents were pointed out; in one case, however, the two lots exhibited comparable values, showing that commercial products with a standardized composition can be achieved.

Conclusions

The commercial products analyzed are used as antioxidants and/or as arterial blood pressure modulators. Oleuropein content and stability has been demonstrated as related to both the drying process and the extraction temperature; this occurrence has never been pointed out before. The bioactive compounds content variability, which was *demonstrated*, does not allow a proven efficacy and biological efficiency. However, from the knowledge of raw material composition, harvest time, drying conditions and extraction procedures, commercial products with a constant and standardized content of active ingredients could be obtained.

Acknowledgments Part of the work presented was funded by the Regione Toscana with the Tuscany Projects—NATURBEN (PRAF 2012–2015) and VOLATOSCA.

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Journal : Large 217 | Dispatch : 23-7-2016 | Pages : 6
Article No : 2756 | LE | TYPESET
MS Code : EFRT-16-0307 | CP | DISK