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Bioaccessibility and Antioxidant Activity Stability of Phenolic Compounds from Extra-Virgin Olive Oils during *in Vitro* Digestion

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The impact of an *in vitro* procedure that mimics the physiochemical changes occurring in gastric and small intestinal digestion on the bioaccessibility and antioxidant activity of phenols from 10 extra-virgin olive oil samples was assessed. Extra-virgin olive oil phenols were totally extracted in the aqueous phase, which reproduces gastric fluids during the digestion procedure. A linear bioaccessibility model, based on tyrosol behavior in model oil samples, was used to estimate the bioaccessibility index (BI%) of extra-virgin olive oil phenols. The BI% varied amongst samples from a maximum of 90% to a minimum of 37%, thus indicating that only a fraction of phenols can be considered bioaccessible. The specific antioxidant activity of olive oil phenols proved to be negatively affected by the digestion procedure. By computing a principal component analysis, it was possible to show that differences in the potential bioactive effect of extra-virgin olive oil samples were related to different phenolic profiles.

KEYWORDS: Health properties; phenolic profile; gastrointestinal digestion; bioaccessibility model

INTRODUCTION

Phenolic compounds in plant-derived foods and beverages have been shown to have important physiological properties and may be responsible for both detrimental and beneficial effects on human health (*1, 2*). Peroxidative chain reactions have been positively linked to the pathogenesis of coronary heart diseases and various forms of cancer (*3*). The strong radical scavenging activity of phenolic compounds probably accounts for their role in preventing diseases related to oxidative stress.

It is well known that the beneficial effects of a Mediterranean diet on human health are mainly attributable to the presence of antioxidant-rich foods. Virgin olive oil is a source of at least 30 phenolic compounds, and it represents the principal fat component of the Mediterranean diet. Oleuropein–aglycone and ligstroside–aglycone are the esters of elenolic acid with 3,4′-dihydroxyphenylethanol (hydroxytyrosol) and 4-hydroxyphenylethanol (tyrosol), respectively. These aglycones and their derivatives, such as hydroxytyrosol and tyrosol (end products of oleuropein– and ligstroside–aglycones hydrolysis), are the most abundant phenols in olive oils (*4, 5*).

The antioxidant activity of olive oil phenols is related to their chemical structure (*6*). Oleuropein-aglycone derivatives with an *ortho*-diphenolic structure are considered the main source of the overall antioxidant activity of extra-virgin olive oils (EVOOs). On the other hand, little or no antioxidant activity has been found for mono-phenols such as ligstroside–aglycone and tyrosol.

The importance of the intake of phenolic compounds through the consumption of olive oil and the possible role of phenols in human health have been extensively investigated and critically reviewed (*1, 4, 5, 7*). A number of studies have shown that these phenols are inhibitors of low-density lipoprotein (LDL) oxidation *in vitro*; thus, their role in *in vivo* protection from the formation of atherosclerotic plaques has been postulated (*8*). On the other hand, it has been suggested that the amount of olive oil phenols in the diet is too low to protect *in vivo* LDLs against oxidative modification to any important extent (*7*). The *in vivo* adsorption of tyrosol and hydroxytyrosol from realistic doses of virgin olive oil has been demonstrated, and it has been suggested that they could exert beneficial effects on human health (*9*). Investigations on intestinal adsorption in perfused rats indicated that oleuropein is capable of permeating the intestine even if the amount that reaches the systemic circulation unchanged is quite small (*10*). Positive health benefits have been postulated due to oleuropein metabolites. Furthermore, oleuropein may act locally to protect other dietary antioxidants from degradation at the intestinal level and thus increase the total antioxidant status of the body.

It is generally accepted that there are two major requirements of a dietary compound for it to be a potential *in vivo* antioxidant. The first requirement is to be bioaccessible, which refers to the compound's tendency to be extracted from the food matrix and then absorbed by intestinal cells. The second requirement is the

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Table 1. EVOO Samples: Main Characteristics of the Olive Oil Fruits and of the Relevant Oil Samples[a]

	olive fruit			oil sample			
sample	area	harvesting time	variety	processing	acidity (oleic acid %)	peroxide value (meq O_2/kg)	K_{232}
s1	A1	H1: Nov 7	OB	W	0.275	7.24	1.62
s2	A1	H2: Nov 20	OB	W	0.205	6.20	1.52
s3	A1	H1: Nov 8	OB	DS	0.305	5.37	1.70
s4	A1	H2: Nov 22	OB	DS	0.285	5.78	1.46
s5	A2		MA	W	0.300	7.20	1.60
s6	A3		MA	W	0.291	6.90	1.70
s7	A4	H1: Nov 21	CO	W	0.325	2.62	1.40
s8	A4	H2: Dec 4	CO	W	0.255	1.61	1.26
s9	A4	H1: Nov 21	CO	DS	0.305	2.75	1.58
s10	A4	H2: Dec 4	CO	DS	0.265	1.75	1.30

[a] Area: A1, Montescaglioso; A2, Corleto; A3, Missanello; A4, Lavello. Varieties: OB, Oliarola del Bradano; MA, Maiatica; CO, Coratina.

persistence of its antioxidant activity in plasma, meaning stability during the digestion process and metabolic pathways to produce derivatives which are still bioactive. Experimental results from *in vitro* cellular systems have shown that olive oil phenols are quantitatively transported into the small intestine by passive diffusion (*11*). Data from *in vivo* experiments on animals and humans confirmed that olive oil phenols are well absorbed at the intestinal level (*12*). However, data regarding the metabolism of olive oil phenols in the human body are very limited, and contrasting results have been obtained regarding the amounts and forms in which they are present in plasma and excreted in urine (*7*). Evidence from *in vitro* experiments indicates that resistance toward the oxidizing conditions of biological fluids as well as interactions with protein and other macromolecular components found in food products and the digestive tract are strongly affected by the phenols' chemical structure (*13*, *14*).

The phenolic profile of extra-virgin olive oil depends on various factors such as olive cultivar, agronomic and pedoclimatic conditions, and the production process technology. From the literature, it is possible to underline the following:

Extra-virgin olive oils with different phenolic compositions would show a different capacity to act as a potential antioxidant after ingestion.

A direct relationship between the biological activity of extra-virgin olive oils and both the quantification of individual phenolic compounds and their metabolic derivatives has not been demonstrated yet.

This work deals with the comparison of the potential biological activity of different extra-virgin olive oils on the basis of both the compositional profile and the behavior after digestion of their phenolic compounds. With this aim, an *in vitro* digestion that had already been used successfully to assess the digestive stability and bioaccessibility of phenolic compounds from different food products was applied (*15*–*19*).

The experimental plan consisted of three stages: (1) the building up of a bioaccessibility model which simulates the modality of *in vivo* tyrosol adsorption, (2) the defining of an index for describing the *in vitro* bioaccessibility of phenols from extra-virgin olive oils, and (3) estimating the effect of the *in vitro* digestion procedure on the antioxidant activity of phenols from olive oil samples.

MATERIALS AND METHODS

1. Chemicals. Tyrosol, porcine pepsin, porcine pancreatin, porcine bile extract, 2,2′azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Reagents used for high-performance liquid chromatography (HPLC) analysis were HPLC-grade and purchased from Carlo Erba (Milan, Italy).

2. Samples. *2.1. Model Oil Samples.* Model olive oil samples (MOs) were prepared by adding tyrosol (Sigma, St. Louis, MO) to commercial peanut (*Arachys hypogea*) seed oil in which phenols were absent. For this purpose, a tyrosol solution in methanol was prepared (6 mg/mL). Aliquots of tyrosol solution were added to seed oil, and four samples with increasing tyrosol concentrations were obtained: MO_1 (250 μg/g), MO_2 (300 μg/g), MO_3 (400 μg/g), and MO_4 (550 μg/g). MO samples were stirred for 18 h under a nitrogen blanket and then used immediately.

2.2. Extra-Virgin Olive Oil Samples. In order to obtain EVOO samples differing in both their total phenolic content and their phenolic profile, three different varieties of olive fruits (Oliarola del Bradano, OB; Maiatica, MA; and Coratina, CO) grown in four different areas (Montescaglioso, A1; Corleto, A2; Missanello, A3; and Lavello, A4) of the Basilicata region (southern Italy) were collected at two different harvesting times (beginning of harvest, H1, and two weeks later, H2). For each area, harvest, H1, dates were determined by technicians of the cooperating producer company. EVOO samples were produced in a UNI EN ISO 9001 certified industrial plant. Olive pastes were obtained from crushing and malaxation of the whole (W) or destoned olive fruits (DS). A continuous centrifugation system was applied for oil extraction. A total of 1000 kg of olive fruits was processed each time. A total of 10 oils was produced. Acidity, peroxide value, and spectroscopic indices K_{232} in the UV region were determined according to the EU official method. All samples conformed to the legal limits for extra-virgin olive oils. The main characteristics of the EVOO samples are summarized in **Table 1**. EVOO samples were stored at 10 °C in amber bottles under a nitrogen blanket and analyzed within a period of 4 months.

3. *In Vitro* Digestion. The *in vitro* digestion method used was a slightly modified version of previously described methods (*20*, *21*).

The oil sample (10 g) was diluted with distilled water (1:8, p/v) and acidified to pH 2.0 using 6N HCl under vigorous stirring. The acidified oil sample was stirred for 15 min; then, the pH value was checked and eventually corrected if necessary with 6N HCl. The oil sample was then mixed with 3 mL of a solution (160 mg/mL) of pepsin from pig gastric mucosa (Sigma Chem.Co., 3.8 units/mg protein) in 0.1N HCl; distilled water was added to reach a final volume of 100 mL. The mixture was stirred for 2 h at 37 °C.

After gastric digestion, the pH of the digesta was increased to pH 5 with 0.9 M $NaHCO_3$ and 24.5 mL of pancreatin–bile solution (pancreatin 4 mg/mL, bile 25 mg/mL in 0.1 M $NaHCO_3$) were added. Then, the pH was increased to 7.0 with 0.1 M $NaHCO_3$, and the mixture was stirred at 37 °C for 2 h. The obtained emulsion was divided into two aliquots.

One aliquot (40 g) was dialyzed using a cellulose dialysis tube (cutoff 12 kDa) containing 25 mL of a NaCl solution (9 mg/mL). The solution was dialyzed under stirring for 2 h at 37 °C. The dialyzed solution phase contained in the tube (D_{IN}) was then removed and stored at 4 °C in amber bottles under a nitrogen blanket.

The other aliquot (25 g) was centrifuged at 5790*g* for 90 min at 20 °C and the solution separated into three layers: the pellet, which was discarded; an aqueous suspension (AS); and an oil phase (O_{SN}) at the top of the centrifuge tube. The AS was removed and filtered through

a 0.45 μm cellulose acetate membrane, covered with a nitrogen blanket, and stored at 4 °C until it was analyzed. The O_{SN} was removed and stored at 4 °C in amber bottles under a nitrogen blanket.

All fractions were analyzed within 24 h. The *in vitro* digestion steps were carried out in amber bottles, under a nitrogen blanket with constant and uniform stirring.

4. Phenols Extraction and Determination. Phenolic compounds were extracted from oil and O_{SN} samples using C18 cartridges (Bond Elut C18, Varian, endcapped sorbent, 1 g sorbent mass, 3 mL). C18 cartridges were activated with methanol (10 mL) and hexane (10 mL). A 1 mL sample, diluted in hexane 1:10 (v/v), was loaded onto the column. Hexane (15 mL) was used to eliminate interference from hydrophobic substances, while phenols were eluted using methanol (10 mL).

Phenolic compounds from AS and D_{IN} samples were extracted in methanol using the C18 extraction optimized for phenolic compound recovery from the aqueous phase. C18 cartridges were activated with 2 mL of methanol and 5 mL of water; then, 1 mL of the sample was loaded. Water (5 mL) was used to wash out interfering substances, and phenols were eluted with methanol (2 mL).

The quantity of phenolic compounds in methanol extracts was determined according to the Folin-Ciocalteau method (*22*) and expressed as tyrosol equivalent (in micrograms).

The tyrosol equilibrium concentration in the dialyzed phases (TyrEQ) was calculated according to the following formula:

$$\text{TyrEQ} = (\text{TyrAS} \times \text{AS volume})/(\text{AS volume} + \text{D}_{\text{IN}}\ \text{volume})$$

where TyrAS is the tyrosol concentration in the aqueous suspension phase (in micrograms per milliliter), AS volume is the volume of the aqueous suspension phase (40 mL), and D_{IN} volume is the volume of the dialyzed phase (25 mL).

5. HPLC Separation of Phenolic Compounds. Determination of the phenolic profile of EVOO samples was performed using the reversed-phase HPLC procedure described by Sacchi and co-workers (*23*). A Shimadzu (Milan, Italy) liquid chromatograph (model LC-10AD) equipped with a diode array detector (model SPD M10A VP) was used. The chromatographic separation was achieved on a 5 mm ODS-3 Prodigy (250 mm × 4.6 mm i.d.) reversed phase column (Phenomenex, Macclesfield, U.K.). Binary gradient elution was employed. Solvent A was water/trifluoroacetic acid (97:3), and Solvent B was acetonitrile/methanol (80:20). Peak quantification was carried out at 279 nm. Elenolic acid and its esters were monitored at 239 nm. Quantification of phenolic compounds was achieved using tyrosol as an external standard.

6. Antioxidant Activity Determination. The assay is based on the ability of antioxidant molecules to decolorize the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS*) (*24*). A stable stock solution of ABTS* was prepared by reacting 5 mL of an aqueous solution of ABTS (7 mM) with 0.088 mL of $K_2S_2O_8$ (148 mM). The mixture was allowed to stand in the dark at room temperature for 16–18 h. An ABTS* working solution was prepared immediately before use by diluting the stock solution in ethanol (~1:88, v/v) to an absorbance value at 734 nm of 0.7 ± 0.02.

Trolox solutions in ethanol (250 to 1500 μM) were used as a reference antioxidant.

Trolox solutions (10 μL), methanol extracts of EVOOs (10 μL), or D_{IN} phases (30 μl) were added to ABTS* working solutions (1 mL) and left to stand for 30 min at 30 °C, and then the absorbance was registered. A test tube containing methanol (10–30 μL) and the ABTS* working solution (1 mL) was used as a reference.

The absorbance inhibition percentage (I%) was calculated according to the following formula:

$$\text{I}\% = (\text{AbsR} - \text{AbsS}/\text{AbsR}) \times 100$$

where AbsR is the absorbance at 734 nm of the reference and AbsS is the absorbance at 734 nm of the sample.

A calibration curve absorbance inhibition percentage versus the Trolox concentration was obtained. The calibration curve was described by the following linear regression: $y = 15.741 \times$ ($r = 0.9963$; $p = 0.000$).

Table 2. *In Vitro* Digestion of MO Samples: Amount of Tyrosol Added to Model Solution (Tyr added), Tyrosol Concentration Determined in Model Oil Samples (TyrMO), and Tyrosol Extracted in the Aqueous Phase (TyrAS) and Permeated in the Dialysis Tube ($TyrD_{IN}$) per Gram of Oil Sample[a]

	Tyr added (μg/g oil)	TyrMO (μg/g oil)	TyrAS (μg/g oil)	$TyrD_{IN}$ (μg/g oil)
MO1	250	242.4 ± 13.2	268.7 ± 16.5	93.6 ± 6.9
MO2	300	306.8 ± 11.1	331.2 ± 25.2	113.1 ± 11.8
MO3	450	420.0 ± 10.7	440.3 ± 28.0	165.7 ± 16.7
MO4	550	530.5 ± 15.5	531.8 ± 22.0	247.0 ± 22.1

[a] Mean values ± SE, $n = 5$.

The antioxidant activity of the samples was expressed in terms of the Trolox equivalent antioxidant capacity (TEAC), defined as the amount of Trolox (in micrograms) necessary to obtain the same antioxidant activity of 1 g of the oil sample ($TEAC/g_{oil}$) or 1 μg of the phenols ($TEAC/\mu g_{phe}$).

RESULTS AND DISCUSSION

1. Bioaccessibility Model of Tyrosol from an Oil Matrix. The bioaccessibility of extra-virgin olive oil phenols was investigated in a model system. MOs were prepared by spiking seed oil with different concentrations of tyrosol which represents one of the extra-virgin olive oil compounds (2.6–27.45 mg/kg) (*4*, *5*, *25*). Moreover, studies on humans after acute olive oil intake have shown that tyrosol is absorbed and partially excreted in urine (*9*, *11*, *26*, *27*). Although tyrosol is not the most abundant phenolic compound in EVOO, it was chosen as a reference olive oil phenolic compound. The main reason for this choice is that, according to previous works (*5*, *14*), tyrosol is characterized by both a lesser antioxidant activity and a lesser tendency to react with other medium macromolecular components compared to those of olive oil secoiridoid derivatives. It is likely that tyrosol remains stable during the digestion process with no dramatic changes in its structure and properties. Thus, it may represent a reference compound for the behavior of other extra-virgin olive oil phenolic compounds. The concentration range of tyrosol in MO samples (250–550 μg/g) was chosen in order to be representative of the whole phenolic content variation range in extra-virgin olive oils used in the present study.

Tyrosol bioaccessibility was estimated by carrying out the *in vitro* digestion process on the MO samples. The obtained data are reported in **Table 2**. Tyrosol concentration values determined in MOs by using the C18 extraction procedure (Tyr MO) resulted in concentration values very close to those expected on the basis of the weighted amount (Tyr added). Mean percentage variation between measured and expected values was lower than 5. Thus, tyrosol was completely eluted by C18 cartridges. The amount of tyrosol extracted from the oil matrix in digestive fluids was determined by measuring the concentration of phenols in AS and in O_{SN} obtained after digesta centrifugation. **Table 2** shows that, from MO samples, tyrosol was totally extracted in the relevant AS fraction. AS fractions contain water-soluble compounds dissolved in the aqueous medium and a micellar suspension formed by oil emulsified by biliar salts (*20*, *21*). Tyrosol detected in the AS phase might in part be dissolved in the aqueous medium and in part be present in the oil core of micelles. On the other hand, no phenols were detected in O_{SN}. Thus, the data obtained indicate that tyrosol was totally extracted from the oil matrix and dissolved in the aqueous medium of AS fractions according to tyrosol solubility, which is greater in the aqueous than in the oil phase (*28*).

A passive diffusion mechanism has been proposed for the intestinal absorption of hydroxytyrosol, which is analogous to

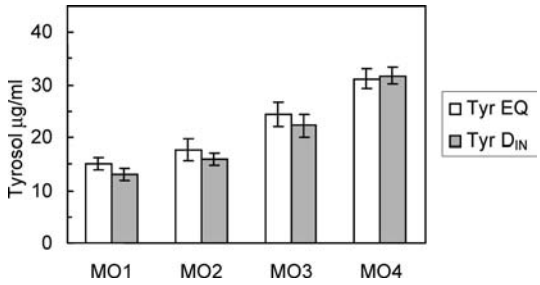

Figure 1. Model oil: tyrosol equilibrium concentration (Tyr EQ) and tyrosol concentration mean values determined in D_{IN} fractions (Tyr D_{IN}). TyrEQ = (TyrAS × AS volume)/(AS volume + D_{IN} volume). Bars represent standard errors, $n = 5$.

tyrosol (*11*). Permeability through a dialysis membrane can be considered as an acceptable simulation of passive intestinal adsorption of water-soluble compounds; the solution outside the dialysis membrane represents material that remains in the gastrointestinal tract, and the solution that enters the dialysis membrane represents the bioaccessible fraction. This method has been used to study the *in vitro* digestion of various phenolic compounds (*15–19*).

According to the literature cited above, the amount of tyrosol available for uptake was estimated on the basis of the amount which permeates from the AS fraction in a dialysis tube (D_{IN} fraction; **Table 2**). The amount of tyrosol in D_{IN} fractions was determined after 2 h. The concentration was not increased by prolonging the dialysis time, thus indicating that the exchange equilibrium between AS and D_{IN} phases was achieved. The tyrosol concentration measured in D_{IN} fractions increased linearly with the tyrosol concentration in the AS phase ($r = 0.9908$; $p = 0.001$), indicating that tyrosol was partitioned depending on the dosage under the *in vitro* conditions used in the present study. A dose-dependent mechanism was also postulated for *in vivo* tyrosol and hydroxytyrosol absorption, given the positive correlation between the intake and excretion of tyrosol and hydroxytyrosol found in humans who consumed oil enriched with these phenols (*12, 26*). On the basis of the AS tyrosol concentration and both AS and D_{IN} fraction volumes used for performing the dialysis, the equilibrium tyrosol concentration (TyrEQ) in the D_{IN} fraction was calculated assuming its free exchange through the membrane. Tyrosol concentration mean values determined in D_{IN} fractions of MO samples do not differ from the relevant TyrEQ values (**Figure 1**), indicating that tyrosol permeates freely through the dialysis membrane. The data obtained therefore indicate that tyrosol from MO samples is totally bioaccessible under the *in vitro* conditions used to simulate the digestion and adsorption of olive oil phenols. A model of tyrosol bioaccessibility was developed by relating its concentration in the MO samples with the tyrosol concentration in the D_{IN} fractions. The proposed *in vitro* model is described by the significant linear regression illustrated in **Figure 2**.

2. Bioaccessibility of Phenols from Extra-Virgin Olive Oil Samples. The *in vitro* digestion procedure was performed on the EVOO samples, and the phenolic content of AS and O_{SN} fractions was measured. The data in **Table 3** show that there were no significant differences between phenolic content mean values of the EVOO samples and those of the relevant AS phases. No phenols were detected in the O_{SN} fractions. The data obtained show that EVOO phenols were completely extracted in the aqueous medium of AS fractions, thus confirming the behavior observed for tyrosol in MO samples. Phenol extraction from EVOOs into the aqueous medium of digesta is in keeping

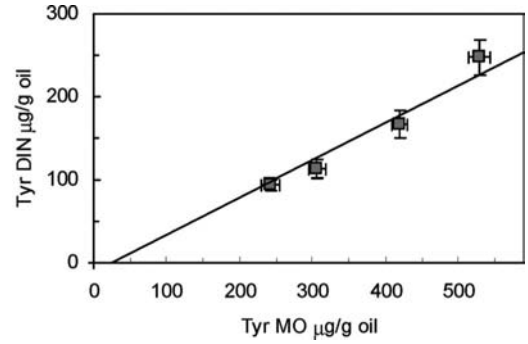

Figure 2. Model oil: tyrosol bioaccessibility model obtained relating tyrosol concentration of model oils (Tyr MO) and tyrosol concentration determined in D_{IN} phase (Tyr D_{IN}). Bars represent standard errors, $n = 5$. Linear equation $y = 0.450x - 10.8$; $r = 0.9868$; $p = 0.0017$.

Table 3. In Vitro Digestion of EVOO Samples: Tyrosol Equivalent Concentration in EVOO Samples (TyrEVOO), Tyrosol Equivalent Extracted in the Aqueous Phase (TyrAS) and Permeated in the Dialysis Tube ($TyrD_{IN}$) per Gram of Oil Sample[a]

EVOO sample	TyrEVOO (µg/g oil)	TyrAS (µg/g oil)	$TyrD_{IN}$ (µg/g oil)	Tyr EXP (µg/ g oil)	BI%
s1	291 ± 8	276 ± 8	46 ± 10	119 ± 3	39
s2	578 ± 9	583 ± 9	165 ± 10	249 ± 4	66
s3	538 ± 8	551 ± 14	113 ± 9	231 ± 3	49
s4	663 ± 9	655 ± 9	231 ± 3	287 ± 4	80
s5	277 ± 6	262 ± 14	46 ± 9	113 ± 2	40
s6	286 ± 11	262 ± 15	47 ± 2	118 ± 5	40
s7	451 ± 11	437 ± 15	72 ± 7	192 ± 5	37
s8	473 ± 15	483 ± 8	122 ± 1	201 ± 6	60
s9	713 ± 13	724 ± 9	208 ± 9	309 ± 5	67
s10	354 ± 15	376 ± 8	134 ± 8	148 ± 6	90

[a] Tyrosol equivalent expected in D_{IN} phase on the basis of bioaccessibility model (Tyr EXP) and bioaccessibility index (BI%). Mean values ± SE, $n = 5$.

Table 4. Mean Antioxidant Activity Values Determined for 1 g of Oil Samples before *in Vitro* Digestion (TEAC/g oil) and after Digestion (TEAC D_{IN}/g oil)[a]

sample	TEAC/g oil	TEAC D_{IN}/g oil
s1	603.39[a]	215.52[a]
s2	829.87[b]	124.04[b]
s3	924.19[bc]	246.86[a]
s4	917.97[bc]	165.32[ab]
s5	594.22[a]	200.39[a]
s6	620.46[ad]	198.21[ac]
s7	768.60[b]	190.81[ac]
s8	971.30[c]	157.35[abc]
s9	1036.00[c]	111.73[bc]
s10	743.18[bd]	170.20[abc]

[a] Mean values followed by different letters are significantly different ($p = 0.000$).

with their low partition oil/water coefficient, which ranges from 10^{-4} to 10^{-1} (*26*). The bioaccessible EVOO phenols were estimated on the basis of the amount detected in the D_{IN} fraction after dialysis (**Table 3**). The dose-dependent bioaccessibility observed for MO was also confirmed for EVOO phenolic compounds. In fact, a significant linear regression was found between the tyrosol equivalent concentration in the AS and D_{IN} phases ($r = 0.82$; $p = 0.003$). The equation describing the tyrosol bioaccessibility model was used to calculate the amount of phenol expected in the D_{IN} phase on the basis of a dose-dependant uptake mechanism in the absence of diffusion limitations (Tyr EXP). The Tyr EXP values were higher than those determined in the relevant DIN phases, except for sample s10 (**Table 3**). The bioaccessibility index of EVOO phenols

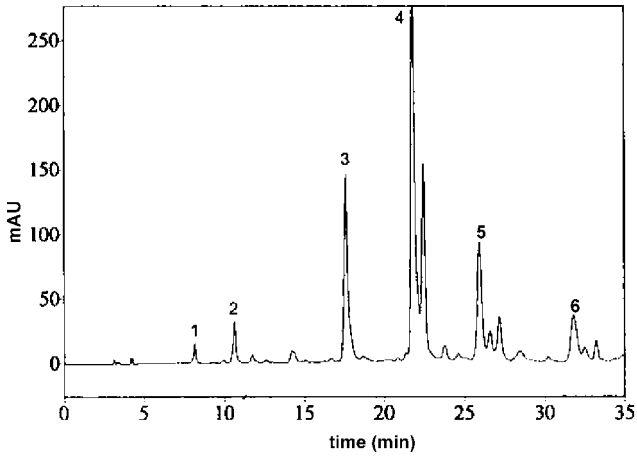

Figure 3. HPLC chromatogram (UV traces at 280 nm) of phenolic compounds extracted from EVOOs (sample 7): peak 1, hydroxytyrosol; peak 2, tyrosol; peak 3, oleuropeine aglycone dialdehydic form; peak 4, ligstroside aglycone dialdehydic form; peak 5, oleuropeine aglycone; peak 6, ligstroside aglycone.

Table 5. Secoiridoids and Phenolic Alcohols Composition of EVVO Samples As Determined by Hplc Analysis[a]

sample	OHTyr	Tyr	OHTyr-EDA	Ty-EDA	OHTyr-EA	Tyr-EA
s1	0.40	6.76	54.25	116.08	40.86	20.69
s2	0.63	21.29	95.06	241.77	89.79	48.48
s3		5.71	123.22	197.55	99.78	47.81
s4		10.05	116.84	273.25	110.78	64.07
s5	0.39	6.45	56.86	121.65	42.82	21.69
s6	0.40	6.86	55.03	117.75	41.44	20.99
s7	1.61	19.55	57.85	172.47	73.92	40.17
s8		7.13	89.19	187.77	89.00	37.93
s9	16.42	45.59	100.48	253.47	139.16	64.39
s10	4.71	20.89	34.40	137.75	57.96	32.04

[a] OHTyr, hydroxytyrosol; Tyr, tyrosol; OHTyr-EDA, oleuropeine aglycone dialdehydic form; Tyr-EDA, ligstroside aglycone dialdehydic form; Tyr-EA, ligstroside aglycone; OHTyr-EA, oleuropeine aglycone. Data expressed as milligrams of tyrosol per kilogram of oil.

(BI%) was estimated from the percentage of phenols determined in the D_{IN} phase ($TyrD_{IN}$) compared with the expected amount (Tyr EXP). The BI% varied among oil samples from a maximum of 90 to a minimum of 37% for s10 and s7, respectively (**Table 3**). These data indicate that, for most of the EVOO samples, only a fraction of the phenols can be considered bioaccessible in the *in vitro* system adopted in the present study. Literature data indicate that phenols can undergo structural modifications during digestion which may reduce their bioaccessible fraction (*18*). Polymeric compounds can be formed as a result of the oxidation of catecolic compounds in slightly alkaline fluid such as the medium utilized to simulate the intestinal juices (*13*, *29*). It has also been hypothesized that the formation of phenol/protein aggregates can explain the lowering of *in vivo* olive oil phenol bioavailability when administered with protein-rich food (*30*). Moreover, experimental evidence indicates that olive oil phenols can bind proteins with different affinities depending on their chemical structure: protein binding capability was not found for tyrosol or hydroxytyrosol, while it was evident in secoiridoid derivatives (*14*).

The comparison of EVOOs obtained from Oliarola del Basento (s1–s4) and Coratina (s7–s10) at two different harvesting times and with two production technologies indicates a positive effect of both a harvesting delay and the destoning process on BI%.

In fact, the comparison of s1 and s3 and of s7 and s9 indicates the positive effect of the destoning technique on both the extra-virgin olive oil phenolic concentration (*5*) and BI%. A delay in the harvesting time (s2 and s4; s8 and s10) reduces the positive effect of destoning on the olive oil phenolic concentration, although its positive effect on BI% is still evident.

3. Effect of *in Vitro* Digestion on the Antioxidant Activity of Phenolic Compounds. Polyphenols are considered to be the main contributors to the antioxidant activity of olive oils (*4*, *5*). Antioxidant activity data from EVOO and D_{IN} samples were independently submitted to a one-way ANOVA model. The sample effect was significant for both series of data ($F_{EVOO9,20} = 11.2$; $p = 0.000$; $F_{DIN9,20} = 5.8$; $p = 0.001$). Results from a least significant difference (LSD) post hoc test show significant differences amongst both EVOO and D_{IN} samples (**Table 4**). It is commonly accepted that differences in the antioxidant activity of phenols are strongly influenced by their chemical structure (*6*, *31*).

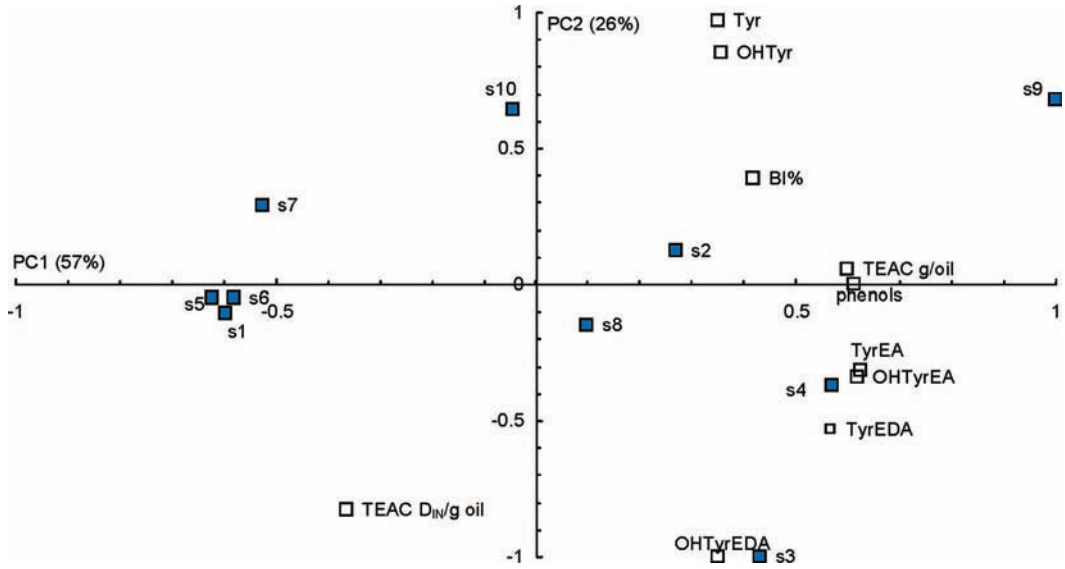

Figure 4. Principal components analysis of EVOO samples' phenolic content (phenols), phenolic profile (Tyr, tyrosol; OHTyr, hydroxytyrosol; Tyr-EA, ligstroside aglycone; Tyr-EDA, ligstroside aglycone dialdehydic form; OHTyr-EA, oleuropeine aglycone; OHTyr-EDA, oleuropeine aglycone dialdehydic form), bioaccessibility index (BI%), antioxidant activity of the oil samples (TEAC g/oil), and antioxidant activity of the solutions that entered the dialysis membrane (TEAC D_{IN}/g oil): scores and loadings.

Compounds with an *o*-diphenolic structure such as hydroxytyrosol and oleuropein aglicone derivatives possess much higher antioxidant activity than tyrosol (*25*). Moreover, synergistic effects among individual phenolic antioxidant molecules have been hypothesized (*32*).

The effect of the digestion process on the specific antioxidant activity of phenols, defined as the amount of Trolox (in micrograms) necessary to obtain the same antioxidant activity of 1 μg of phenols (TEAC/μg_{phe}), was evaluated. A two-way ANOVA model was applied considering the following factors: the sample (10 levels), digestion (two levels, TEAC/μg_{phe} before and after the digestion process), and their interactions. Replications were treated as three observations per cell. As expected, a significant effect on the sample was found ($F_{sample9,36} = 12.8$; $p = 0.000$). The digestion process had a significant negative effect on TEAC/μg_{phe} values ($F_{1,36} = 37.8$; $p = 0.000$). The effect of the digestion procedure on the specific antioxidant activity of phenols seems to be sample-related, as suggested by the significant effect of the interaction sample $\times$ digestion ($F_{9,36} = 7.22$; $p = 0.000$).

4. Relationship between EVOOs Phenolic Profile and *in Vitro* Bioactivity. The prevalent phenols of EVOO are represented by secoiridoids and phenolic alcohols derived from their hydrolytic reactions (*4*, *5*, *25*). HPLC analysis was performed on EVOO samples; peaks relevant to secoiridoids and their hydrolytic derivatives (Tyr and OH-Tyr) were identified (*23*) and the relevant amounts calculated (**Figure 3** and **Table 5**).

Data relevant to the phenolic profile, BI%, and antioxidant activity from EVOO samples were subjected to a principal component analysis (PCA). When the cross validation method was used, two significant components were found (**Figure 4**). Along the first component, which accounts for a 57% of the explained variance, samples positioned on the right side of the biplot are characterized by a higher content of total phenols, oleuropeine, and ligstroside aglycone derivatives and by a higher antioxidant activity. All these variables were found to be correlated with each other. This result was not unexpected since it is very well known that both total phenols and secoiridoid concentrations positively affect olive oil antioxidant activity (*33*). Thus, the first dimension of the PCA model discriminates oils mainly in relation to their antioxidant activity. The second dimension accounts for another 26% of the explained variance. Samples on the upper side of the plot are characterized by a higher content of tyrosol and hydroxytyrosol, a higher phenol bioaccessibility, a lower antioxidant activity of the D_{IN} fraction, and a lower concentration of the dialdehydic form of oleuropeine aglycon.

The performance of the 10 extra-virgin olive oils subjected to the *in vitro* digestion procedure used in the present work was significantly different among the samples, both in terms of phenol bioaccessibility and retention of their antioxidant activity. The phenolic content and the antioxidant activity (TEAC/g oil) of EVOO before digestion did not give significant results correlated to either the phenols' BI% or the antioxidant activity determined in the D_{IN} phase (TEAC D_{IN}/g oil). On the basis of the results obtained, it can be concluded that indices taking into account phenols' behavior during the digestion can be proposed as useful tools to compare the potential biological effect of different EVOOs.

Further studies will be devoted to validating in human studies the findings and implications resulting from the *in vitro* experiments described in the present work.

LEGEND OF USE ABBREVIATIONS

MO, model oil; EVOO, extra-virgin olive oil; AS, aqueous suspension obtained after centrifuging digesta from the *in vitro* intestinal phase; O_{SN}, supernatant oil phase obtained after centrifuging digesta from the *in vitro* intestinal phase; D_{IN}, solution that entered the dialysis membrane after the *in vitro* intestinal phase; TyrEQ, tyrosol equilibrium concentration between the inner and outer phases of the dialysis tube after the *in vitro* adsorption phase; TyrEXP, tyrosol concentration expected in the D_{IN} phase on the basis of the bioaccessibility model equation; BI%, bioaccessibility index; TEAC, Trolox equivalent antioxidant capacity; TEAC/μg_{phe}, phenolic specific antioxidant activity; TEAC/g_{oil}, phenolic antioxidant activity determined per gram of oil sample.

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