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Contribution of the Phenolic Fraction to the Antioxidant Activity and Oxidative Stability of Olive Oil

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Antioxidant activity of the phenolic fraction of extra virgin olive oil (EVOO) was measured by means of a chemical and an electrochemical method. Both methods were tested in predicting the oxidative spoilage and stability to oxidation of 22 EVOO samples and resulted correlated with peroxide values and oxidative stability measured by Rancimat. The main phenolic compounds of EVOOs were detected by HRGC. To study the contribution of single polyphenols (PPs) to antioxidant activity of phenolic fraction and oxidative stability of EVOOs, multivariate statistical analyses were applied on HRGC data. An isomer of oleuropein aglycon was shown to affect significantly antioxidant activity of phenolic fraction but not oil stability to oxidation. No individual compounds was identified as the main cause of the overall antioxidant activity, and the total polyphenol determination by the Folin reagent was better correlated to antioxidant activity and oxidative stability than each tested PP or PPs groups such as *o*-diphenols.

KEYWORDS: Extra virgin olive oil; phenolic compounds; antioxidant activity; electrochemical detection; multivariate analysis

INTRODUCTION

Oxidative stability is a central parameter in the estimation of extra virgin olive oil (EVOO) quality, as it gives a reliable evaluation of the susceptibility to oxidative degeneration, which is the main cause of its adulteration (*1*). The shelf life of olive oil is strictly related to rancidity development, which could depend on the autoxidation of fatty acids (*2*).

This phenomenon takes place in the presence of oxygen, generating some unstable compounds that can modify the sensory and nutritional characteristics of the oil, thus leading to product spoilage. Although unavoidable, the oxidation process can be delayed by endogenous antioxidant that enhance the oxidative stability by preventing the propagation of lipid peroxidation or removing free radicals. Antioxidants are reported as molecules which, when present at low concentrations compared to those of an oxidable substrate, significantly delay or prevent oxidation of that substrate (*3*).

Natural antioxidants exert their antioxidant activity through various mechanisms: preventing first chain initiation by scavenging initiating radicals, metal chelating, decreasing localized oxygen concentration and decomposing peroxides (*4*).

In EVOOs, different classes of compounds having antioxidant activity are present, namely polyphenols (both simple and aglycons) tocopherols, carotenoids, and chlorophylls. The contribution of polyphenols to the virgin olive oil stability and antioxidant activity was estimated to be higher than that of other compounds (*5*−*7*), and various authors have demonstrated a positive linear relationship between oil stability and the total content of polyphenols (*8*, *9*). The antioxidant activity of polyphenols is due to metal chelating properties and radical scavenging activity (*10*−*12*).

An important aspect of the study of antioxidants is the assessment of antioxidant activity. Various methods have been introduced to test antioxidant activity of olive oil; most of them investigate the ability of oil to scavenge a free radical (*7*, *13*−*16*), while only one test is based on the electrochemical properties (*14*).

In the present paper, the polyphenol content, the antioxidant activity, and antioxidant power (AOP), evaluated by a chemical and an electrochemical method, respectively, were measured on 22 samples of EVOOs from different regions of Italy and Croatia. The aim of the work was to test the possibility to use these indexes in the prediction of oil oxidative stability and to asses the effectiveness of the electrochemical method as an alternative method to evaluate the antioxidant power of the phenolic fraction of olive oil. Furthermore, the contribution of single polyphenolic compounds (determined by HRGC) to oxidative stability and antioxidant activity were investigated using multivariate statistical analysis.

MATERIALS AND METHODS

Oil Samples. EVOO samples ($n = 22$) were obtained from fruits of several varieties cultivated in different regions of Italy

Table 1. List of Olive Oil Samples

sample	location	region (state)	variety	ripening stage[a]	extraction technology[b]
S1	Casoli	Abruzzo (I)	Leccino	G	C
S2	Casoli	Abruzzo (I)	Leccino	M	C
S3	Crecchio	Abruzzo (I)	Peranzana	R	C
S4	Loreto	Abruzzo (I)	Dritta	G	C
S5	Loreto	Abruzzo (I)	Dritta	M	C
S6	Loreto	Abruzzo (I)	Dritta	R	C
S7	Rocca San Giovanni	Abruzzo (I)	Gentile	G	C
S8	Rocca San Giovanni	Abruzzo (I)	Leccino	M	C
S9	Rocca San Giovanni	Abruzzo (I)	Gentile	M	C
S10	Rocca San Giovanni	Abruzzo (I)	Leccino	G	C
S11	Rocca San Giovanni	Abruzzo (I)	Gentile	R	C
S12	Rocca San Giovanni	Abruzzo (I)	Leccino	R	C
S13	Monopoli di Sabina	Lazio (I)	Dop sabina	R	T
S14	Scodello	Sicilia (I)	mix: Cerasuola Biancolilla Nocellara	R	T
S15	Firenze	Toscana (I)	mix	R	T
S16	Torremaggiore	Puglia (I)	Peranzana	R	C
S17	Porec	(Croatia)	Picholine	R	C
S18	Porec	(Croatia)	Leccino	R	C
S19	Veglia	(Croatia)	mix: Naska Debella Slatta Rosulia	R	C
S20	Pola	(Croatia)	Biancheria	R	C
S21	Pola	(Croatia)	Buga	R	C
S22	Pola	(Croatia)	Carbonera	R	C

[a] G, green; M, medium; R, ripened. [b] T, traditional; C, continuous.

and Croatia. Olive fruits were harvested in the year 2001 at different ripening stages, and the relative oils were immediately obtained by crushing the olives by continuous (C) or traditional (T) processing techniques (**Table 1**). All the analysis were carried out in the period January − April 2002.

Solid-Phase Extraction of the Phenolic Fraction. Commercially available octadecyl C_{18} cartridges (1 g, 6 mL) (International Sorbent Technology, UK) were used for the extraction of the phenolic fraction according to the following protocol: 1 g of olive oil was dissolved in 10 mL of hexane, and the obtained solution was loaded onto a column previously conditioned with 2 × 10 mL of methanol and 2 × 10 mL of hexane. The column was eluted with 4 × 10 mL of hexane to eliminate all the lipophilic fraction, and the retained polar compounds were recovered by eluting with 4 × 10 mL of methanol. Trapping and release of analytes from C_{18} solid phase was demonstrated to be highly competitive with the liquid/liquid extraction procedure (*17*).

In the extraction procedure for HRGC analysis, a diethyl ether resorcinol solution (100 μL; I. S.) was added to 3 g of sample (0.5 mg/mL), and the mixture was treated as above-described.

Total Polyphenols Determination. The total polyphenol content of the methanol extracts was evaluated colorimetrically using the Folin−Ciocalteau reagent. The method was adapted from Singleton and Rossi (*18*). A diluted extract (0.5 mL of 1:10, v/v) or phenolic standard was mixed with Folin−Ciocalteau reagent (5 mL, 1:10 diluted with Nanopure water) and acqueous Na_2CO_3 (4 mL, 1 M). Solutions were maintained at room temperature for 60 min and the total polyphenol were determined colorimetrically at 725 nm. Gallic acid standard solutions were used to calibrate the method.

Antioxidant Power. The availability of the phenolic extracts to electrochemical oxidation has been reported as a measurement of the "antioxidant power" (AOP) (*19*). The method offers the unique feature to investigate the functional (antioxidant) characteristics of the phenolic compounds without the use of a reactive compound.

The electrochemical behavior of the methanol extract was measured using hydrodynamic voltammetry performed in flow injection analysis (FIA). The apparatus consisted of a Minipuls II peristaltic pump (Gilson, France), a high-pressure injection valve model 7125 (Rheodyne, Rohnert Park, CA), equipped with a 20-μL loop, an electrochemical cell model UniJet (BAS, West Lafayette, IN) using a glassy carbon working electrode, and an amperometric detector AMEL 559 HPLC detector (AMEL, Milan, Italy) linked to a chart recorder RC 102 (Pharmacia, Sweden). Injections of three extract samples were performed in the potential interval 0−300 mV versus Ag/AgCl, the increasing potential step was 25 mV, and the flow rate was 150 μL/min. The current produced in the electrochemical oxidation of the phenolic compounds was recorded. Once the three oxidation potentials (0, 125, and 250 mV vs Ag/AgCl) were selected, two standard molecules, quercetin (0 and 125 mV vs Ag/AgCl) and gallic acid (250 mV vs Ag/AgCl), were tested in the linear concentration interval 0.1−25.0 μM.

Samples were appropriately diluted to obtain current signals within the linear range of the applicable standard molecule.

Radical Scavenging Activity. The radical scavenging activity (RSA) was measured following the methodology described by Brand-Williams et al. (*20*). The bleaching rate of a stable free radical, 2,2-diphenyl-1-picrilhydrazyl (DPPH$^\bullet$) was monitored in the presence of the sample using a Perkin-Elmer Lambda Bio 20 spectrophotometer. In its radical form, DPPH$^\bullet$ absorbs at 515 nm, but upon reduction by an antioxidant or a radical species, its absoption decreases. A volume of 1.85 mL of 6.1 10^{-5} M DPPH$^\bullet$ methanol solution was used. The reaction was started by the addition of 75 μL of phenolic extract. DPPH$^\bullet$ bleaching was monitored at 25 °C for at least 60 min. In all cases, the DPPH$^\bullet$ bleaching rate was proportional to the sample concentration added to the medium. The following equation was chosen to obtain the reaction rate of DPPH$^\bullet$ bleaching (*21*)

$$1/A_t^3 - 1/A_0^3 = 3kt \qquad (1)$$

where k is the DPPH$^\bullet$ bleaching rate, A_0 is the initial absorbance, A_t is the absorbance at increasing time, t. The radical scavenging activity was expressed as the slope obtained from eq 1 per milliliter of phenolic extract (−O. D.$^{-3}$ min^{-1} mL^{-1}).

Polyphenols Gas Chromatographic Analysis. High-resolution gas chromatography (HRGC) was carried out on a Carlo Erba (Milano, Italy) Mega Series 5300, equipped with an on-column injection system and a FID, on a 30 m long SPB-5 column from Supelco (Milano, Italy); 0.32 i.d., 0.10-μm film thickness. The oven temperature was as follows: from 70 to 135 °C at 2 °C/min, 10 min at 135 °C, from 135 to 220 °C at 4 °C/min, 10 min at 220 °C, from 220 to 270 °C at 4 °C/min, 20 min at 270 °C. The temperature of the detector was held at 280 °C, and the carrier gas was He at 2 mL/min. Quantification of phenolics was done by peak area integration with Carlo Erba Mega Series Integrator (*17*, *22*).

Oxidative Stability. The Rancimat apparatus (Methrom Ltd. Herisau, Switzerland) was used to evaluate accelerated oxidation at high temperature, for example, 100 °C with an air flow of 10 L/h. Results were expressed as induction period (IP), in hours (*23*).

Analytical Indices. Acidity, peroxide value, and spectroscopic indices K_{232}, K_{270}, and ΔK in the UV region were determined according to EU official method (*24*). *p*-Anisidine value was determined according to the NGD C 36−1976 method (*25*). The totox index was derived from the peroxide value (PV)

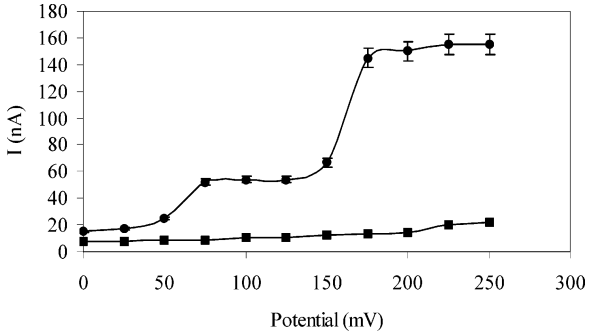

Figure 1. Typical hydrodynamic voltammogram obtained for the EVOO extracts (●) and buffer (■). The voltammogram shows three plateau regions; oxidation potentials were accordingly chosen.

and the p-anisidine value (AV), according to the following equation: Totox = (2PV) + AV (*26*).

Statistical Analysis. Principal components analysis (PCA) and stepwise general least-squares analysis (SGLSA) were performed using the Statistica for Windows software package (Statsoft, Tulsa, OK). PCA was applied to describe the data set and to detect the relative importance of individual variables for determining the data structure. SGLSA was applied to select the chemical variables that better explain both the antioxidant activity of phenolic extract (as measured by RSA and AOP) and the olive oil oxidative stability.

RESULTS AND DISCUSSION

Evaluation of Total Polyphenols Influence. To evaluate the AOP of the EVOOs, an electrochemical characterization was carried out. The hydrodynamic voltammetry of the EVOO extracts showed three relevant potential zones ($E_1 = 0$ mV vs Ag/AgCl, $E_2 < 150$ mV vs Ag/AgCl, $E_3 < 280$ mV Ag/AgCl) where the electrochemical oxidation of the samples resulted independent from the applied potential, being under the diffusion control, hence representing the electrochemical oxidizable moieties of extracted phenolic compounds **Figure 1**. Since the oxidation potential of a compound depends on the energy required to donate an electron, the lower the oxidization potential, the more easily the compound will donate an electron, and the higher its expected antioxidant activity.

The standard molecules used to calibrate the method were quercetin for 0 and 125 mV and gallic acid for 250 mV. They were chosen on the basis of cyclic voltammetry experiments (data not shown).

The quercetin and gallic acid calibrations at 0, 125, and 250 mV vs Ag/AgCl were linear in the concentration range 0.1−25.0 μM, the equations were: $y_{(0mV)} = 5.3$[quercetin] − 0.2 ($R^2 = 0.998$); $y_{(125mV)} = 10.1$[quercetin] + 0.2 ($R^2 = 0.999$); $y_{(250mV)} = 4.4$[gallic acid] + 1.0 ($R^2 = 0.999$), where y is the recorded current in nA. These were used to quantify the μg/mL quercetin equiv (QE) at potentials 0 and 125 mV versus Ag/AgCl (QE_0 and QE_{125}), and μg/mL gallic acid equivalent (GAE) at 250 mV vs Ag/AgCl (GAE_{250}).

The correlations of QE_0, QE_{125}, and GAE_{250} with RSA and total polyphenols (TPP) were investigated and resulted linear in the working range. TPP was better correlated with GAE_{250}, whereas, as expected, the RSA correlated best with QE_0, which represents the most readily oxidizable compounds **Table 2**. Therefore, QE_0 was used as a measure of AOP.

TPP content, RSA, AOP, and oxidative spoilage indexes were determined; raw data are reported in **Table 3**.

All samples except S22 did not exceed the legal limits for acidity, peroxide value, K_{232}, K_{270}, and ΔK fixed by EU Regulation 2568/91. Sample S22 exceeded the limit for peroxide value, thus showing an initial degradation stage. Despite the degradation indices of almost all the samples not exceeding the legal limits, it is possible to notice a high variation of some stability indices among the samples: peroxide values varied 4-fold, and induction time as measured by Rancimat varied almost 5-fold.

Table 2. Correlation between Total Polyphenols, Radical Scavenging Activity (RSA), and Oxidative Stability with Electrochemical Parameters

electrochemical parameter	total polyphenols	RSA	oxidative stability
QE_0	$r = 0.876$	$r = 0.884$	$r = 0.808$
	$p = 0.0005$	$p = 0.0003$	$p = 0.0007$
QE_{125}	$r = 0.882$	$r = 0.806$	$r = 0.843$
	$p = 0.0001$	$p = 0.0006$	$p = 0.0002$
GAE_{250}	$r = 0.896$	$r = 0.721$	$r = 0.879$
	$p = 0.0005$	$p = 0.0001$	$p = 0.0009$

It is noteworthy that oil samples from the same variety, at the same ripening stage, extracted with the same technology, and taken in the same region but different location (e.g., S1−S10 and S2−S8), showed different polyphenols content, antioxidant activity, and oil stability. Cultivar, degree of maturation, climate and type of extraction method are the factors affecting the phenolic content of EVOO (*9*, *27*, *28*). Some authors reported that the provenience of sample from different geographical areas in the same region does influence TPP content, despite differences in climate and orography (*29*−*31*), but there are also reported differences in TPP content up to 45% between oils differing exclusively for their provenience from different areas of the same region (*22*). The influence of sample provenience on TPP content, and thus on antioxidant activity of polyphenols and oil stability, could either depend on the cultivar or could be affected by other factors (climate, orography, and physiological conditions of the plant).

To finding out the simplest model able to describe the data reported in **Table 3** satisfactorily, principal component analysis (PCA) was applied to the data set. Final data processing by PCA was obtained by subtracting three non significant variables (i.e., K_{270}, ΔK, *p*-anisidine value) from the initial matrix. Results are reported in **Figure 2** (loading plot) and **Figure 3** (score plot) with an explained variance of 85.4%, of which 58.6% was along the first principal component (PC 1) and 26.8% was along PC 2. On the basis of the distribution of the virgin oil samples, it is possible to assume that product with high TPP content and characterized by polyphenols with high antioxidant activity (higher QE_0) generally showed low peroxide and Totox values. Moreover, the antioxidant activity of polyphenol extracts showed to be positively correlated with the oxidative stability as measured with Rancimat ($r = 0.810$, $p < 0.001$; $r = 0.808$, $p < 0.001$; for RSA and AOP, respectively). These results, which are in agreement with those of Mannino et al. (*14*), confirm the importance of polyphenols content in the improvement of oxidative stability of olive oils (*6*, *8*, *9*). With regard to this subject, it is noteworthy that TPP content showed a higher correlation coefficient than antioxidant activity indices with oxidative stability of oil ($r = 0.904$, $p < 0.001$).

Evaluation of Single Polyphenols Influence. Because the polyphenol content is so important for olive oil quality, an accurate methodology of quantification is needed. It is known that responses of single phenols to the Folin reagent are significantly different for each other, thus making the method unsuitable for the accurate measurement of the phenolic content. Different analytical techniques, GC, HPLC, GC-MS, and HPLC-

Table 3. Antioxidant Activity, Oxidative Spoilage Indexes, and Oxidative Stability of 22 EVOO Samples (Mean Values ($n = 3$))

sample	total polyphenols GAE (mg kg^{-1})	RSA (–O. D.$^{-3}$ min^{-1} mL^{-1})	AOP (QE_0) (mg kg^{-1})	QE_{125} (mg kg^{-1})	GAE_{250} (mg kg^{-1})	peroxide value (meq O^2 kg^{-1})	K_{232}	K_{270}	ΔK	*p*-anisidine no.	totox	oxidative stability (h)
S1	259[a]	0.582[b]	89[b]	220[b]	557[b]	5.3[a]	1.8[a]	0.15[a]	–0.002[a]	3.6[a]	14.2[a]	14.9[a]
S2	57	0.168	34	32	74.9	11.5	1.98	0.128	–0.002	4.08	26.1	5.75
S3	336	0.449	115	389	916	7.8	1.78	0.106	–0.002	3.72	19.3	12.6
S4	369	0.518	65	310	937	9.7	2.09	0.15	–0.001	3.12	22.5	16
S5	390	0.671	165	622	1412	7.2	1.72	0.093	–0.002	4.44	18.9	18.4
S6	396	0.569	145	450	1380	8.2	2.01	0.155	–0.002	3.78	20.2	17.5
S7	189	0.256	33	58	140	8.8	1.76	0.123	0	3.73	18.7	5
S8	115	0.189	25	51	74.3	8.5	1.96	0.113	0	2.4	19.4	7.65
S9	169	0.298	60	136	235	7.7	1.67	0.092	–0.003	3.84	19.2	6.32
S10	131	0.306	57	67	127	7	1.47	0.142	–0.002	3.96	18	8.53
S11	239	0.469	91	212	351	7	1.84	0.109	–0.001	3.24	17.2	6.2
S12	127	0.367	34	75	148	6.3	1.5	0.152	–0.001	3.36	16	9.17
S13	232	0.298	74	267	573	14.5	2.36	0.139	0	3.54	31.5	9.15
S14	197	0.330	100	271	435	8.9	1.63	0.14	–0.002	4.26	22.1	7.48
S15	296	0.871	169	463	778	7.3	1.77	0.104	–0.003	3.42	18.1	11.4
S16	191	0.342	62	174	269	7.5	1.93	0.148	–0.003	4.32	19.3	8.67
S17	305	0.548	136	522	810	6	1.88	0.15	–0.003	2.76	14.8	14.8
S18	208	0.389	87	267	456	5.7	1.73	0.111	–0.003	4.26	15.7	13
S19	311	0.689	122	475	847	6	1.96	0.153	–0.002	3.78	17.8	13.6
S20	633	1.235	220	625	1222	5.5	1.99	0.158	–0.002	3.18	14.2	24.4
S21	212	0.554	84	212	383	7.5	1.76	0.123	–0.001	3.6	18.7	10.6
S22	60	0.390	33	56	84	24	1.92	0.143	0.002	4.2	52.2	5.52

[a] Coefficient of variation below 5%. [b] Coefficient of variation below 10%.

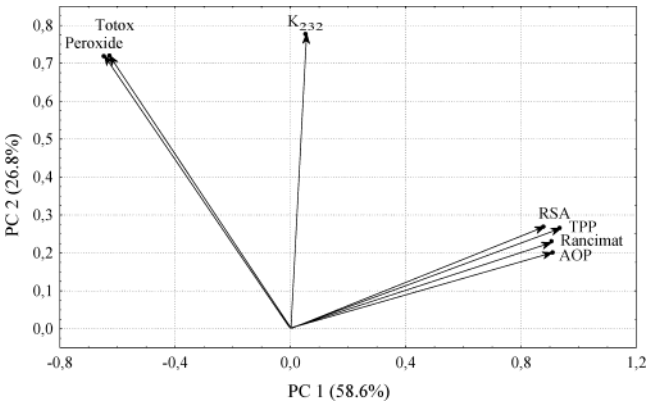

Figure 2. Principal component loading plot from antioxidant activity, oxidative stability, and oxidative spoilage indexes of 22 EVOO. Axes: *x*, PC1; *y*, PC2.

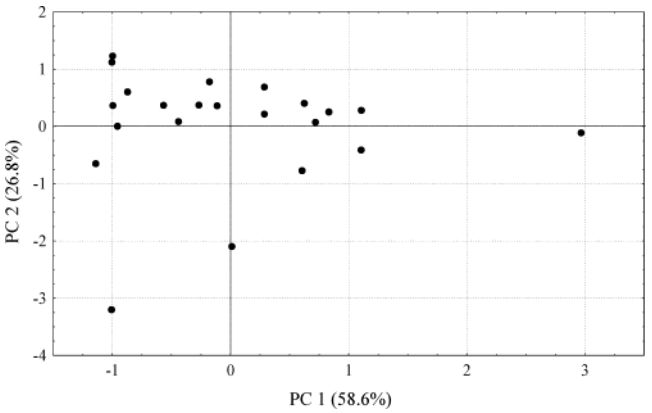

Figure 3. Principal component scores from antioxidant activity, oxidative stability, and oxidative spoilage indexes of 22 EVOO. Axes: *x*, PC1; *y*, PC2.

MS, have been used for the detection and quantitation of the individual PPs of olive oil, but this analyses did not quantify all the phenolic compounds.

To investigate the importance of each single phenolic compound (PP) in the determination of the RSA of the phenolic extract, the quantification of simple PPs and the main aglycons of glucoside was carried out by HRGC (data are reported in **Table 4**).

The content of tyrosol, hydroxytyrosol, caffeic acid, *p*-coumaric acid, ferulic acid, *p*-hydroxybenzoic acid, protocatechuic acid, syringic acid, vanillic acid, ligstroside aglycon, and 3′4′-DHPEA-EA (an isomer of oleuropein aglycon) was determined, and each compound was considered as a parameter for further statistical analysis.

A matrix containing all the 22 oil samples, each with the 11 above-mentioned parameters, was used to obtain an explanation of the correlation, if any, between the polyphenolic composition of olive oil, the RSA, and AOP of phenolic extract and the stability of olive oils as measured by Rancimat. Data processing was developed in two steps as follows: Data relative to PPs composition as determined by HRGC were processed by PCA, and four principal components were extracted with an explained variance of 66.7% (23.9 along PC 1, 16.7 along PC 2, 15.1 along PC 3, and 11.0 along PC 4). The loading of each PP on the principal components is reported in **Table 5**. To detect the importance of derived variables (PCs) on the determination of radical scavenging activity, antioxidant power and stability to oxidation, the PCs were processed by stepwise general least-squares (SGLSA) multiple regression analysis, SGLSA being aimed at detecting cause-effect relationships. Other authors used SGLSA to study the effect of antioxidants on virgin oil stability (*5*). To the purpose of this study SGLSA was preferred to PLS, as recommended by other authors for similar investigation (*32*), because the variables considered in this study were not correlated (principal components are orthogonal for construction). Results of the SGLSA are reported in **Table 6**. The most significant variables were found to be PC 2 and PC 3, which are positively correlated with RSA. 3′4′-DHPEA-EA weighed on PC 2, while syryingic acid and *p*-cumaric acid weighed on PC 3. By the results of SGLSA, it is possible to hypothesize that 3′4′-DHPEA-EA, syryngic acid, and *p*-cumaric acid are the most important

Table 4. Amounts of Simple Phenols and Aglycons Detected by HRGC[a]

sample	ferulic acid	syringic acid	caffeic acid	*p*-coumaric acid	*p*-OH benzoic acid	vanillic acid	protocatecuic acid	tyrosol	OH-tyrosol	3′4′-DHPEA-EA	ligstroside aglycon
S1	0.48 ± 0.09	0.67 ± 0.04	0.12 ± 0.01	1.11 ± 0.06	nd	0.31 ± 0.05	0.56 ± 0.04	10.85 ± 0.98	9.44 ± 1.54	21.6 ± 3.0	23.9 ± 2.6
S2	1.12 ± 0.11	0.95 ± 0.04	0.56 ± 0.03	1.18 ± 0.12	0.45 ± 0.05	0.91 ± 0.12	1.56 ± 0.24	4.42 ± 0.38	3.77 ± 1.12	11.5 ± 2.8	22.5 ± 4.2
S3	0.77 ± 0.21	nd	0.96 ± 0.11	1.22 ± 0.07	0.67 ± 0.31	1.32 ± 0.35	1.01 ± 0.16	3.46 ± 0.36	2.84 ± 0.77	28.3 ± 4.7	37.2 ± 4.1
S4	1.26 ± 0.34	1.15 ± 0.21	0.88 ± 0.12	0.76 ± 0.31	nd	0.76 ± 0.14	0.58 ± 0.13	33.76 ± 3.85	28.44 ± 4.67	9.8 ± 2.6	10.4 ± 2.3
S5	1.34 ± 0.17	2.12 ± 0.76	nd	0.49 ± 0.01	0.12 ± 0.01	0.78 ± 0.19	1.04±0.11	11.89 ± 1.89	18.65 ± 2.86	7.4 ± 2.3	11.2 ± 1.1
S6	0.15 ± 0.06	1.21 ± 0.33	0.31 ± 0.05	0.86 ± 0.04	0.44 ± 0.23	0.55±0.07	0.87±0.07	9.42 ± 0.92	8.64 ± 1.65	47.3±12.4	16.3 ± 2.4
S7	0.65 ± 0.12	0.89 ± 0.18	0.82 ± 0.07	0.76 ± 0.10	0.22 ± 0.01	0.45±0.04	0.67±0.03	4.91 ± 0.40	2.67±0.99	23.6 ± 2.3	25.2 ± 2.8
S8	1.07 ± 0.21	1.76 ± 0.12	0.32 ± 0.04	1.25 ± 0.81	0.28 ± 0.01	0.61 ± 0.21	1.21 ± 0.23	38.38 ± 4.02	21.62 ± 3.54	8.1±1.8	12.0 ± 3.4
S9	1.32 ± 0.13	0.94 ± 0.20	0.43 ± 0.10	0.51 ± 0.03	0.60 ± 0.05	0.87 ± 0.25	1.32 ± 0.24	4.68 ± 0.45	3.54 ± 1.25	13.4 ± 2.5	18.5 ± 1.9
S10	2.12 ± 0.42	1.67 ± 0.35	1.14 ± 0.88	0.77 ± 0.08	nd	1.14 ± 0.15	nd	6.44 ± 0.56	1.98 ± 0.65	11.9 ± 1.1	15.4 ± 2.1
S11	1.87 ± 0.19	1.46 ± 0.55	nd	1.02 ± 0.08	0.23 ± 0.03	0.45 ± 0.02	0.78 ± 0.05	9.91 ± 0.98	2.51 ± 0.98	18.9 ± 1.9	35.2 ± 3.6
S12	1.17 ± 0.25	0.87 ± 0.05	0.65 ± 0.05	1.24 ± 0.48	0.23 ± 0.01	1.32 ± 0.67	0.96 ± 0.06	5.28 ± 0.41	2.55 ± 0.76	10.7 ± 2.1	29.0 ± 3.8
S13	0.83 ± 0.17	0.76 ± 0.07	0.22 ± 0.02	0.73 ± 0.07	0.45 ± 0.08	0.69 ± 0.21	1.07 ± 0.07	12.14 ± 1.23	10.43 ± 1.21	6.2 ± 1.4	26.1 ± 3.5
S14	0.67 ± 0.10	0.58 ± 0.07	0.25 ± 0.03	0.54 ± 0.03	0.31 ± 0.04	0.98 ± 0.20	0.99 ± 0.07	20.63 ± 2.02	24.72 ± 3.21	5.3 ± 1.6	10.9 ± 1.4
S15	0.43 ± 0.04	0.95 ± 0.07	0.44 ± 0.23	0.87 ± 0.14	0.72 ± 0.14	nd	0.93 ± 0.18	8.72 ± 0.88	4.43 ± 1.55	14.5 ± 3.1	45.0 ± 7.4
S16	1.97 ± 0.56	1.12 ± 0.12	0.38 ± 0.12	0.79 ± 0.09	0.20 ± 0.08	0.89 ± 0.18	0.58 ± 0.14	6.48 ± 0.62	14.44 ± 2.89	6.66 ± 1.6	20.8 ± 1.8
S17	1.04 ± 0.32	2.04 ± 0.87	0.62 ± 0.08	0.55 ± 0.20	0.34 ± 0.02	0.65 ± 0.07	0.83 ± 0.17	8.63 ± 0.76	9.87 ± 1.43	24.3 ± 4.3	38.7 ± 6.5
S18	2.36 ± 0.39	1.87 ± 0.56	0.76 ± 0.22	1.87 ± 0.18	0.82 ± 0.12	0.67 ± 0.09	1.21 ± 0.18	6.75 ± 0.68	7.21 ± 1.43	15.5±1.5	22.3 ± 3.3
S19	0.79 ± 0.09	2.44 ± 0.41	1.03 ± 0.40	2.22 ± 0.90	0.38 ± 0.08	1.54 ± 0.55	1.12 ± 0.16	2.74 ± 0.24	1.03 ± 0.52	18.4 ± 2.6	33.5 ± 2.4
S20	0.98 ± 0.15	3.27 ± 0.88	0.29 ± 0.09	1.76 ± 0.11	0.44 ± 0.18	0.49 ± 0.04	0.85 ± 0.09	12.06 ± 1.67	17.41 ± 2.31	31.5 ± 5.5	24.1 ± 2.2
S21	0.99 ± 0.13	1.39 ± 0.18	0.68 ± 0.06	1.43 ± 0.41	0.86 ± 0.07	0.53 ± 0.08	0.98 ± 0.11	45.33 ± 8.54	8.55 ± 2.21	21.5 ± 3.6	8.2 ± 1.6
S22	0.74 ± 0.10	1.54 ± 0.22	0.97 ± 0.11	1.34 ± 0.65	nd	0.33 ± 0.11	2.23 ± 0.67	20.64 ± 2.04	1.55 ± 0.66	16.2 ± 3.4	31.5 ± 4.3

[a] Results are expressed as μg/mL of resorcin.

Table 5. Results of PCA Analysis Applied on HRGC Data (Loadings of Each PP on Four Extracted PCs)

PP	PC 1	PC 2	PC 3	PC 4
caffeic acid	–0.29577	–0.56302	0.167044	0.324789
p-coumaric acid	–0.17281	–0.16525	0.715798	0.496039
ferulic acid	0.090985	–0.64052	0.267777	–0.35
p-hydroxybenzoic acid	–0.17291	0.136875	0.132884	0.556406
protocatecuic acid	0.039448	0.055605	–0.04612	0.787598
syringic acid	0.14444	0.028259	0.885942	–0.14447
vanillic acid	–0.16794	–0.77854	–0.00917	0.004269
tyrosol	0.831451	0.1319	0.072353	0.285727
hydroxytyrosol	0.838055	0.11459	–0.01355	–0.2471
3′,4′-DHPEA-EA	–0.42145	0.574838	0.374865	0.055713
ligstroside aglycon	–0.79457	0.171917	–0.00814	0.143859
variance explained	0.218978	0.161257	0.142068	0.144666

phenolics in the determination of olive oil radical scavenging activity. Similar results were obtained using SGLSA to study the effect of single polyphenols on antioxidant power as measured by electrochemical detection (**Table 7**) and on oil stability to oxidation (**Table 8**). In this study, hydroxytyrosol, which was weighed on the PC 1, was shown not to significantly affect the RSA or AOP of phenolic extracts and the stability of virgin olive oils. These results were quite unexpected; in fact, literature data indicate that, among the olive oil polyphenols (not including 3′4′-DHPEA-EA and the dialdeidic form of elenolic acid linked to hydroxytyrosol, 3′4′-DHPEA-EDA), hydroxytyrosol shows the highest antioxidant activity either in model systems or as pure standard (*14*, *33*−*38*), followed by caffeic acid and oleuropein. Despite this evidence, other studies that investigated the correlation between hydroxytyrosol content and oxidative stability of olive oils did not show a positive correlation between the content of this compound, Rancimat induction time, and antioxidant activity (*39*, *40*). To our knowledge, the only study conduced on EVOOs that stressed out a positive correlation between hydroxytyrosol content and both oxidative stability and antioxidant activity is that of Pizzale et al. (*13*). The lack of a positive correlation between hydroxytyrosol, oxidative stability index, and antioxidant capacity could be ascribed to the fact that this compound showed an increase along storage time that is explained by hydrolysis of complex polyphenols (*31*, *41*−*43*). In other studies (*39*), this fact implied that samples which underwent aging reactions such as hydrolysis and oxidation, thus showing low oxidative stability as measured by Rancimat, also showed the highest hydroxytyrosol content. In light of these findings, it is possible to hypothesize that the potential positive contribution of hydroxytyrosol content to antioxidant activity could have been masked by the variability of this compound due to hydrolytic reaction that were occurred simultaneously to oxidative spoilage or not.

3′4′-DHPEA-EA showed a positive contribution to antioxidant activity (RSA or AOP), this could be due to the antioxidant activity of this compound that according to Gordon et al. (*44*) is higher than that of hydroxytyrosol and according to other authors shows a protective effect on oil oxidation that is similar to that of hydoxytyrosol (*9*, *44*, *45*). Other than being an effective antioxidant 3′4′-DHPEA-EA is present in high amounts in the phenolic fraction of olive oils (*9*, *40*, *43*, *46*, *47*) and is the main polyphenols in some oil varieties (*31*).

The positive correlation between PC 3, on which loaded syryngic and *p*-coumaric acid, with RSA and AOP of polyphenol extracts and oil stability is another unexpected result. Syryngic and *p*-coumaric acids are generally considered weaker antioxidants with respect to other phenolic acids such as caffeic acid (*14*, *48*, *49*). Also, they could show higher antioxidant activity than caffeic acid under certain test conditions (*7*, *50*). It seems unprobable that syryngic and *p*-coumaric acids could significantly influence the RSA and AOP of phenolic extracts as well as the stability of oil because of their relatively low antioxidant activity and their low concentration in the tested olive oils (**Table 4**). Thus, it is possible to hypothesize that these compounds show a covariance with other components of olive oil not detected by the adopted HRGC analysis. On the basis of literature data, a possibly active compound could be 3′4′-DHPEA-EDA, which shows an high antioxidant activity (*9*) and is present in EVOOs in high concentration (*9*, *40*, *43*, *46*, *47*). By an elaboration of the data presented by Lavelli (*40*), is it possible to state that 3′4′-DHPEA-EDA shows a positive linear correlation with the radical scavenging activity tested by DPPH method and expressed as ARP ($1/EC_{50}$) as suggested by

Table 6. SGLSA Results for RSA (Model Significance, Univariate Results for Each Variable, and Parameters Estimation)

	d. f.	ss	ms	*F*	*p*	R^2 adj	param	std err	*t*
model	4	0.689203	0.172301	5.381679	0.005489	0.454924			
intercept	1	5.000557	5.000557	156.1884	5.39E–10		0.476758	0.038148	12.49754
PC 1	1	0.009877	0.009877	0.308511	0.585831		0.021688	0.039046	0.555438
PC 2	1	0.280009	0.280009	8.745849	0.0088207[a]		0.115472	0.039046	2.957338
PC 3	1	0.386956	0.386956	12.08625	0.0028873[a]		0.135744	0.039046	3.476529
PC 4	1	0.012362	0.012362	0.386106	0.542596		–0.02426	0.039046	–0.62137
residual	17	0.544275	0.032016						
total	21	1.233478							

[a] Correlation significant at $p < 0.05$ level.

Table 7. SGLSA Results for AOP (Model Significance, Univariate Results for Each Variable, and Parameters Estimation)

	d.f.	ss	ms	*F*	*p*	R^2 adj	param	std err	*t*
model		26292.01	6573.004	3.712264	0.023891	0.34064			
intercept	1	182506.7	182506.7	103.0751	1.24E–08		91.08107	8.971213	10.15259
PC 1	1	989.8861	989.8861	0.559062	0.464856		6.865671	9.182329	0.747705
PC 2	1	14036.12	14036.12	7.927239	0.011909[a]		25.85317	9.182329	2.815535
PC 3	1	9438.377	9438.377	5.330553	0.033788[a]		21.20015	9.182329	2.308799
PC 4	1	1827.634	1827.634	1.032201	0.323885		–9.329	9.182329	–1.01597
residual	17	30100.52	1770.619						
total	21	56392.53							

[a] Correlation significant at $p < 0.05$ level.

Table 8. SGLSA Results for Oxidative Stability (IP) (Model Significance, Univariate Results for Each Variable, and Parameters Estimation)

	d.f.	ss	ms	*F*	*p*	R^2 adj	param	std err	*t*
model		250.0239	62.50597	3.873769	0.020508	0.353748			
intercept	1	2765.059	2765.059	171.3628	2.63E–10		11.21091	0.856412	13.09056
PC 1	1	3.492505	3.492505	0.216446	0.647666		–0.40781	0.876565	–0.46524
PC 2	1	62.2955	62.2955	3.860725	0.065988		1.722339	0.876565	1.964873
PC 3	1	154.9926	154.9926	9.605571	0.006515[a]		2.716726	0.876565	3.099286
PC 4	1	29.24327	29.24327	1.812333	0.195906		–1.18006	0.876565	–1.34623
residual	17	274.3069	16.1357						
total	21	524.3308							

[a] Correlation significant at $p < 0.05$ level.

Brand-Williams et al. (*20*). The equation is ARP $= 4.9 \times 10^{-5}$ [3′4′-DHPEA-EDA] + 0.011 ($r = 0.94$, $p < 0.001$).

Other authors investigating the oxidative stability and antioxidant activity of EVOOs (*6*, *40*, *51*) found a positive linear relationship with *o*-diphenols content (tyrosol, hydroxytyrosol, 3′4′-DHPEA-EA, and 3′4′-DHPEA-EDA). On the basis of these results, linear correlation analysis was carried out to investigate if the sum of tyrosol, hydroxytyrosol, and 3′4′-DHPEA-EA was better correlated to the RSA and AOP of polyphenols and to oil stability than each single compound. The results of the analyses are reported in **Table 9**. The sum of *o*-diphenols is less related to all the stability parameters than each single compound and resulted not significantly related to antioxidant activity indexes. This result could be explained by the fact that not all of the *o*-diphenols were determined by the HRGC, and compounds with antioxidant activity were not considered in the analysis.

The *o*-diphenols undoubtedly play an important role on EVOOs stability. In fact, these compounds are more readily oxidized than total polyphenols when EVOOs undergo oxidation (*52*), and hence, they could be considered as more effective antioxidants. On the other hand, some literature results attest that *o*-diphenols show a lower contribution to oil stability than does TPP (*5*). In light of these findings, it is still not clear which compound provides a higher relative contribution to antioxidant activity and stability of olive oils when these compounds are present together in the food matrix.

Table 9. Univariate Linear Correlation among Polyphenols, Antioxidant Activity, and Oxidative Stability

	RSA	AOP (QE_0)	oxidative stability
tyrosol	$r = –0.02716$ $p = 0.9057$	$r = –0.16981$ $p = 0.4509$	$r = 0.004908$ $p = 0.9834$
hydroxytyrosol	$r = 0.13531$ $p = 0.5485$	$r = 0.178245$ $p = 0.4278$	$r = 0.381511$ $p = 0.0801$
3′,4′-DHPEA-EA	$r = 0.435972$[a] $p = 0.0435$	$r = 0.456757$[a] $p = 0.0336$	$r = 0.463655$[a] $p = 0.0303$
o-diphenols[b]	$r = 0.28344$ $p = 0.2013$	$r = 0.221869$ $p = 0.3211$	$r = 0.4298$[a] $p = 0.0469$

[a] Correlation significant at $p < 0.05$ level. [b] 3′,4′-DHPEA-EDA not included.

Literature data attests that hydroxytyrosol shows a high antioxidant activity, but the results of this study and of other studies (*39*, *40*) did not permit us to find a positive correlation between hydroxytyrosol content and the antioxidant activity of the phenolic fraction, as well as between hydroxytyrosol content and oil stability as measured with Rancimat. Moreover, on the basis of SGLSA results, 3′4′-DHPEA-EA content was shown to positively affect antioxidant activity of phenolic fraction.

No individual compound was identified as the main cause of the overall antioxidant activity because of the highly complex

polyphenolic set of EVOOs, and the total polyphenol determination by the Folin reagent was better correlated to antioxidant activity and oxidative stability than each tested PP or PPs groups such as *o*-diphenols. Analogous considerations were also drawn on the basis of similar analysis applied to other substrates (*32*).

It is worth noting that SGLSA considers the individual effect of single compounds, but synergistic effects among individual molecules and the presence of ions could affect the antioxidant activity of the phenolic extract of EVOO (*40*, *53*), further affecting the model variance. As regards the oxidative stability of EVOOs, the model variance could be further influenced by the presence of other natural antioxidants or by the oleic/linoleic ratio (*5*).

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