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ARTICLE

Prediction of Extra Virgin Olive Oil Varieties through Their Phenolic Profile. Potential Cytotoxic Activity against Human Breast Cancer Cells

Jesús Lozano-Sánchez,[†,‡] Antonio Segura-Carretero,*[,†,‡] Javier A. Menendez,[§] Cristina Oliveras-Ferraros,[§] Lorenzo Cerretani,[∥] and Alberto Fernández- Gutiérrez[†,‡]

[†]Department of Analytical Chemistry, Faculty of Sciences, University of Granada, Fuentenueva s/n, E 18071 Granada, Spain, [‡]Functional Food Research and Development Center, Health Science Technological Park, Avenida del Conocimiento s/n, E-18100 Granada, Spain, [§]Catalan Institute of Oncology, Girona Biomedical Research Institute (IdIBGi), Avenida de Francia s/n, E-17007 Girona, Spain, and [∥]Dipartimento di Scienze degli Alimenti, Alma Mater Studiorum, Università di Bologna, Piazza Goidanich 60, I-47521 Cesena (FC), Italy

The aim of this work was to develop a rapid resolution liquid chromatography coupled to electrospray ionization time-of-flight mass spectrometry (RRLC-ESI-TOF-MS) method followed by tetrazolium salt (MTT)-based cell viability assays for qualitative and quantitative classification of extra virgin olive oil (EVOO) varieties by phenolic and other polar compound contents as well as for rapid characterization of putative cytotoxic activities against human cancer cells. Five different Spanish EVOO varieties were analyzed, and RRLC-ESI-TOF-MS method was applied for qualitative and quantitative identification of most important phenolic compounds. We finally employed MTT-based cell viability protocol to assess the effects of crude EVOO phenolic extracts (PEs) on the metabolic status of cultured SKBR3 human breast cancer cells. MTT-based cell viability assays revealed a wide range of breast cancer cytotoxic potencies among individual crude PE obtained from EVOO monovarietals. Remarkably, breast cancer cell sensitivity to crude EVOO-PEs was up to 12 times higher in secoiridoids enriched-PE than in secoiridoids-low/null EVOO-PE.

KEYWORDS: Breast cancer; HER2; RRLC-MS; olive oil; phenolic compounds

INTRODUCTION

Food can be regarded as *functional* if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutrition, in a way that improves health and well-being or reduces the risk of disease. Extra virgin olive oil (EVOO) could be considered as functional food. Its health properties have been discussed extensively in literature. Olive oil has compounds that provide health benefits, including the prevention and treatment of diseases. Among olive oil compounds, the phenolic fraction has received considerable attention in recent years. Evidence from several studies have revealed that the protective effects of EVOO against chronic diseases such as atherosclerosis, cancer, obesity, diabetes, and coronary diseases are related to the phenolic compounds (*1*−*4*). The pharmaceutical interest in olive oil phenolic compounds due to their bioactivity on different cancer cells is also well-known (*1*, *5*−*7*) and has stimulated multidisciplinary research on the composition of olive biophenols. The bioactivity of the phenolic compounds in these chronic diseases could be related to different properties such as antioxidant and anti-inflamatory, although the molecular mechanism of these compounds in relation to many diseases could have different cellular targets.

The most important phenolic compounds that have been identified on EVOO may be divided into different groups such as phenolic acids, phenolic alcohols, secoiridoids, lignans, and flavones. Among these compounds, hydroxytyrosol and oleuropein aglycon have been related to different anticancer activities (*7*−*9*). In our hands, individual EVOO-derived complex phenolic compounds such as oleuropein aglycone efficiently inhibited proliferation and induced apoptotic cell death in human-derived breast cancer cell lines bearing high levels of the tyrosine kinase receptor HER2 (*erb*B-2), an oncoprotein which is found overexpressed in ∼15−30% of human breast carcinomas(*10*, *11*). Moreover, we established that isolated individual complex phenolic compounds and phenolic fractions mainly containing a sole phenolic component were not equivalent in their abilities to inhibit HER2-driven cell growth and to down-regulate the activity and expression of the HER2 protein itself. It is necessary to consider that because the biological effects of phenolic compounds, including breast cancer cytotoxic actions, are varied and compound specific, combinatorial effects (i.e., addition, antagonism or synergism) can occur in EVOO naturally exhibiting enriched or low levels of specific phenolic compounds.

*To whom correspondence should be addressed. Phone: +34 958 249510. Fax: +34958249510. E-mail: ansegura@ugr.es.

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These reported health properties of virgin olive oil phenolic fraction have promoted active research on methods to identify and quantify these compounds. A large quantity of papers related to the evaluation of EVOO polyphenols, published before the 1990, reported colorimetric methods, such as UV spectroscopy, that generally use the Folin−Ciocalteau reagent (*8*, *12*). The need to carry out an individual identification of each phenolic compound leads to the replacement of the conventional nonspecific methods by other more specific ones. Capillary electrophoresis (CE) and gas and liquid chromatography have been used (*13*, *14*). Gas chromatography (GC) is less common because a derivatization step is necessary (*15*). The results obtained by CE are very useful, with short analysis times and high efficiency peak separation, but the downside of this technique is the low concentration sensitivity (*16*−*18*). The usual technique to analyze the phenolic fraction is high performance liquid chromatography (HPLC) (*7*, *13*, *19*−*22*). Recently, an improvement in chromatographic performance has been achieved by the introduction of rapid-resolution LC (RRLC) and ultraperformance LC (UPLC). These approaches use narrow-bore columns packed with very small particles (1.8 μm) and high flow rate with delivery systems operating at high backpressures. The major advantages of RRLC over conventional HPLC are improved resolution, shorter retention times, higher sensitivity, and better performance. Coupling RRLC with mass spectrometry (MS) further offers a potent analytical alternative, which has been applied in recent publications characterizing food products.

Because the anticancer activity as well as many other biological effects of EVOO-derived phenolics appears to have compound-specific properties, the aim of this study was to characterize and examine different EVOO to test the hypothesis that a naturally occurring family of phenolic compounds present in dietary EVOO might have synergistic properties to develop an efficient EVOO-based cancer preventive or intervention clinical strategy. First, we have developed a rapid and sensitive RRLC/MS method to identify and quantify the olive oil phenolic compounds with a high efficiency on the chromatographic separation together with the classification of extra virgin olive oil by phenolic profile. Moreover, the validation of the proposed method has been carried out with the sensitivity, linearity, and precision parameters. Second, we have employed whole crude phenolic extracts (PEs) directly obtained from 14 different monovarieties of EVOOs produced in Spain to preliminary delineate both the biological actions (in terms of cytotoxicity) and the clinical value (in terms of physiologically relevant concentration ranges) of complex multicomponent PEs against *HER2* gene-amplified SKBR3 breast cancer cells.

MATERIALS AND METHODS

Olive Oil. The olive oils used in this study were from five different monovarietal EVOOs obtained from different geographic zones in Spain: two Hojiblanca olive oils produced in Málaga (EVOO 1) and Sevilla (EVOO 9), seven Picual olive oils produced in Málaga (EVOO 2), Jaén (EVOOs 4, 10 and 11), Granada (EVOOs 5, and 6), and Córdoba (7), one Cornezuelo (EVOO 3), one Manzanilla (EVOO 8), and three Arbequina olive oils (EVOOs 12, 13, and 14). The EVOOs were produced in the same year (September 2008). Olives were processed by continuous industrial plants equipped with a hammer crusher, a horizontal malaxator, and a two-phase decanter. Samples were stored in bottles without headspace at room temperature and darkness before analysis. To isolate the phenolic fraction of olive oils from all varieties, solid phase extraction (SPE) with Diol-cartridges was used. EVOO (60 g) was dissolved and loaded onto the column. The cartridge was washed with 15 mL of hexane, which were then discarded in order to remove the nonpolar fraction of the oil. Finally, the sample was recovered by passing through 40 mL of methanol and the solvent was evaporated under vacuum. The residue was dissolved with 2 mL of methanol and filtered through a 0.25 μm filter before the RRLC analysis. The extracts of olive oils were diluted (1:10, v:v) with methanol.

Breast Cancer Cell Lines and Culture Conditions. SKBR3 breast cancer cells were obtained from the American Type Culture Collection (ATCC) and were routinely grown in Improved MEM (IMEM, Biosource International, Invitrogen SA, Barcelona, Spain) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine. Cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. SKBR3 cells were used between passages 12 and 18. The cultures were screened periodically to detect possible contamination of *Mycoplasma*.

Chemicals. All chemicals were of analytical reagent grade and used as received. Methanol and *n*-hexane, reagents used for the extraction of the phenolic compounds from the olive oil samples, were purchased from Panreac (Barcelona, Spain). Acetonitrile from Lab-Scan (Dublin, Ireland), acetic acid from Fluka and Sigma-Aldrich (Steinheim, Germany), and methanol were used for preparing mobile phase. Solvents were filtered using a solvent filtration apparatus model 58061 (Supelco, Bellefonte, PA). Double-deionized water with conductivity lower than 18.2 MΩ was obtained with a Milli-Q system (Millipore, Bedford, MA). Standards of hydroxytyrosol (HYTY), tyrosol (TY), vanillin, luteolin (Lut), apigenin (Apig), *p*-coumaric acid, ferulic acid, vanillic acid, and quinic acid were purchased from Sigma-Aldrich (St. Louis, MO), and (+)-pinoresinol was acquired from Arbo Nova (Turku, Finland). Oleuropein (Ole) was purchased from Extrasynthèse (Lyon, France). Stock solutions at concentration of 1000 mg/L for each phenol were first prepared by dissolving the appropriate amount of the compound in methanol and then serially diluted to working concentrations. MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was purchased from Sigma-Aldrich (St. Louis, MO).

RRLC-MS Analysis. The development of a rapid resolution liquid chromatography (RRLC) coupled to electrospray time-of-flight mass spectrometry (ESI-TOF-MS) method to characterize the phenolic profile in EVOOs was performed in an Agilent 1200-RRLC system (Agilent Technologies, Waldbronn, Germany) of the Series Rapid Resolution equipped with a vacuum degasser, autosampler, a binary pump, and a UV−vis detector. The chromatographic separation was carried out on a Zorbax Eclipse Plus C_{18} analytical column (4.6 mm × 150 mm, 1.8 μm particle size). The flow rate was 0.80 mL/min, and the temperature of the column was maintained at 25 °C. The mobile phases used were water with 0.25% acetic acid as eluent A and methanol as eluent B. The optimal chromatographic method consisted in the following multistep linear gradient: 0 min, 5% B; 7 min, 35% B; 12 min, 45% B; 17 min, 50% B; 22 min, 60% B; 25 min, 95% B, 27 min, 5% B, and finally a conditioning cycle of 5 min with the same conditions for the next analysis. The injection volume in the RRLC was 10 μL. The compounds separated were monitored in sequence first with DAD (240 and 280 nm) and then with a mass spectrometry detector.

MS was performed using the microTOF (Bruker Daltonik, Bremen, Germany) which was coupled to the RRLC system. At this stage, the use of a splitter was required to the coupling with the MS detector as the flow which arrived to the TOF detector had to be 0.2 mL/min in order to obtain reproducible results and stable spray. The TOF mass spectrometer was equipped with an ESI interface (model G1607A from Agilent Technologies, Palo Alto, CA) operating in negative ion mode. External mass spectrometer calibration was performed with sodium formiate clusters (5 mM sodium hydroxide in water/2-propanol 1/1 (v/v), with 0.2% of formic) in quadratic + high precision calibration (HPC) regression mode. The calibration solution was injected at the beginning of the run, and all the spectra were calibrated prior to polyphenol identification. The optimum values of source parameters were: capillary voltage of +4 kV; drying gas temperature, 190 °C; drying gas flow, 9 L/min; nebulizing gas pressure, 2 bar, and end plate offset, −0.5 kV. The values of transfer parameters were: capillary exit, −120 V; skimmer 1, −40 V; hexapole 1, −23 V, RF hexapole, 50 Vpp, and skimmer 2, −22.5 V. The source and transfer parameters were get for a good sensitivity and reasonable resolution of the mass range for compounds of interest (50−1000 m/z) in order to improve ionization performance.

The accurate mass data for the molecular ions were processed using the software Data Analysis 3.4 (Bruker Daltonik), which provided with a list of possible elemental formulas by using the Generate Molecular Formula Editor. The latter uses a CHNO algorithm providing standard functionalities such as minimum/maximum elemental range, electron configuration, and ring-plus double bonds equivalent, as well as a

sophisticated comparison of the theoretical with the measured isotopic pattern (Sigma-Value) for increased confidence in the suggested molecular formula. The widely accepted accuracy threshold for confirmation of elemental compositions has been established at 5 ppm for most of the compounds.

Metabolic Status Assessment (MTT-Based Cell Viability Assays). SKBR3 breast cancer cells were seeded at a density of ~3000 cells/200 μL per well in a 96-well plate. The next day, cells were treated with concentrations ranging from 0% to 0.1% (v/v) (i.e., 0%, 0.0001%, 0.001%, 0.01%, 0.05%, and 0.1% v/v) of the whole crude EVOO-PE dissolved in1 mL of ethanol (100% stock solution). Ethanolic dilutions were prepared immediately before starting each experiment by diluting 100% full strength EVOO-PE (i.e., 1 mL of the methanol extract was evaporated under vacuum to give the dried methanol extract; after a complete solvent removal, dried methanol extract was dissolved in 1 mL of 95% ethanol) in fresh culture medium.An appropriate amount of ethanol (v/v) was added to control cells. After 5 days of treatment (EVOO-PEs were not renewed during the entire period of culture treatment), cells were incubated with a solution of MTT at a concentration of 5 mg/mL for 3 h at 37 °C. The supernatants were then carefully aspirated, 100 μL of DMSO were added to each well, and the plates were agitated to dissolve the crystal product. Absorbances were read at 570 nm using a multiwell plate reader (model Anthos Labtec 2010 1.7 reader). Cell viability effects upon exposure to EVOO-PE were analyzed as percentages of the absorbance obtained in untreated control cells. For each treatment, cell viability was evaluated as a percentage using the following equation: (A_{570} of treated sample/A_{570} of untreated sample) × 100. Cell sensitivity to crude EVOO-PE was expressed in terms of the concentration of PE (v/v) needed to decrease by 50% cell viability (IC_{50} value). Because the percentage of control absorbance was considered to be the surviving fraction of cells, the IC_{50} values were defined as the concentration of EVOO-PE that produced 50% reduction in control absorbance.

Statistical Analysis. Statistical data treatment of the EVOO phenolic and other polar compounds profiles was performed using SPSS (v. 15.0, Statistical Package for the Social Sciences, Chicago, IL). Linear discriminant analysis (LDA) of the phenolic profiles was used to classify five different varieties of EVOO under study. LDA, a supervised classificatory technique, is widely recognized as an excellent tool to obtain vectors showing the maximal resolution between a set of previously defined categories. In LDA, vectors minimizing the Wilks' lambda, λ_w, were obtained. This parameter is calculated as the sum of squares of the distances between points belonging to the same category divided by the total sum of squares. Using the normalized variables, an LDA model capable of classifying the EVOO samples according to their respective olive variety was constructed. From the samples (descrived above), a matrix containing 51 injections was constructed and used for evaluation purposes. To construct the LDA training matrix, only the means of the replicates of the samples were included (14 objects); in this way, the internal dispersion of the categories was reduced, which was important to reduce the number of variables selected by the SPSS stepwise algorithm during model construction. A response column, containing the five categories corresponding to the five varieties of the EVOO, was added to both matrices. According to the SPSS stepwise algorithm, a predictor is selected when the reduction of λ_w produced after its inclusion in the model exceeds F_{in}, the entrance threshold of a test of comparison of variances or F-test. However, the entrance of a new predictor modifies the significance of those predictors which are already present in the model (described above). For this reason, after the inclusion of a new predictor, a rejection threshold, F_{out}, was used to decide if one of the other predictors should be removed from the model. The process terminates when there are no predictors entering or being eliminated from the model. The probability values of F_{in} and F_{out}, 0.05 and 0.10, respectively, were adopted.

As far as the cell viability data are concerned, statistical data treatment of the Two-group comparisons were performed by the Student's t test for paired and unpaired values. Comparisons of means of ≥3 groups were performed by ANOVA and the existence of individual differences, in case of significant F values at ANOVA, tested by Scheffé's multiple contrasts.

RESULTS AND DISCUSSION

Optimization of RRLC System and Quality Parameters. The mobile phases, gradient, injection volume, flow rate, and column temperature were optimized. First, different mobile phases A and B were tested in order to estimate the best organic ones. Regular methods to analyze the olive oil phenolic fraction by HPLC used mainly gradient elution with acetonitrile−aqueous acetic acid (*7*). Elution with methanol−aqueous acetic acid has also been used (*23*). An initial gradient time of 50 min was used. The flow-rate was 0.5 mL/min. First, the optimum solvent to be used as eluent B was selected among acetonitrile, methanol, and different mixtures of acetonitrile−methanol (70:30, 50:50, and 30:70; v/v), maintaining the composition of the eluent A (water with 0.5% acetic acid) based on the chromatographic conditions reported in a previous study by HPLC (*24*). The change of eluents affects the retention times, but also the selectivity. The differences in the selectivity are based on the different properties of solvation of acetonitrile and methanol that are especially remarkable with the polar compounds. The results obtained are shown on the **Figure 1a**. RRLC analysis showed great differences on the separation of the studied compounds using different mobile phases B. The separation of individual phenols, mainly related with secoiridoid derivates, was better when using methanol than acetonitrile or mixtures of acetonitrile/methanol as eluent B. Aglycones of oleuropein (Ol Agl), ligstroside (Lig Agl), and their decarboxymethyl forms (DOA and D-Lig Agl), the most important and abundant olive oil phenolic compounds, showed the best chromatographic resolution with methanol 100%. The importance of the separation of this fraction resides in their well-known pharmaceutical effects such as anticarcinogenic and antiatherogenic. Consequently, when using methanol as eluent B, these compounds were best separated, with a good resolution of the peaks for a better subsequent quantification. With the tested mobile phase B, the next step was to select the optimum solvent to be use as mobile phase A. Milli-Q water with different percentage of acetic acid was evaluated as mobile phase A. Their concentration was varied from 0.1 to 1%. Finally, the best separation, in terms of efficiency and resolution, was obtained with water with 0.25% acetic acid, and MeOH as mobile phases A and B, respectively.

Five different experimental gradients were tested, and among all of them, the best results were obtained with the multistep linear gradient detailed in the Materials and Methods. A good chromatographic resolution was obtained. To increase the resolution among the peaks, the injected volume was reduced from 20 to 10 μL.

In the next step, the effects of flow rate and temperature on resolution were also evaluated. RRLC system offers a unique opportunity to reduce analysis time dramatically by increasing flow rate and temperature. When the flow rate increases, the back pressure of the system rises until reaching the maximum value (80−90% of the pressure accepted by the chromatograph). However, if the temperature of the column also increases the viscosity of the mobile phase decreases and the system back pressure is reduced. Choosing a suitable temperature, the flow could be increased up to the maximum value. The flow rate from 0.5 to 1.5 mL/min (0.5, 0.8, 1, and 1.5 mL/min) was evaluated, and the temperature of the column was varied between 25 and 40 °C in 5 °C intervals. With a flow rate higher than 1.5 mL/min, in some percentages of methanol in the mobile phase, the pressure was higher than 600 bar, maximum value of the pressure accepted by the chromatograph. Finally, the optimum conditions were a flow rate 0.80 mL/min and the temperature of the column was maintained at 25 °C. The maximum pressure reached during this analysis was approximately 500 bar. At this stage, the use of a splitter 1:4 was required for obtaining reproducible results and stable spray. The detection was carried out UV at two wavelengths characteristic of the phenolic compounds of interest (280 and 240 nm) and mass spectrometry (TOF). A good

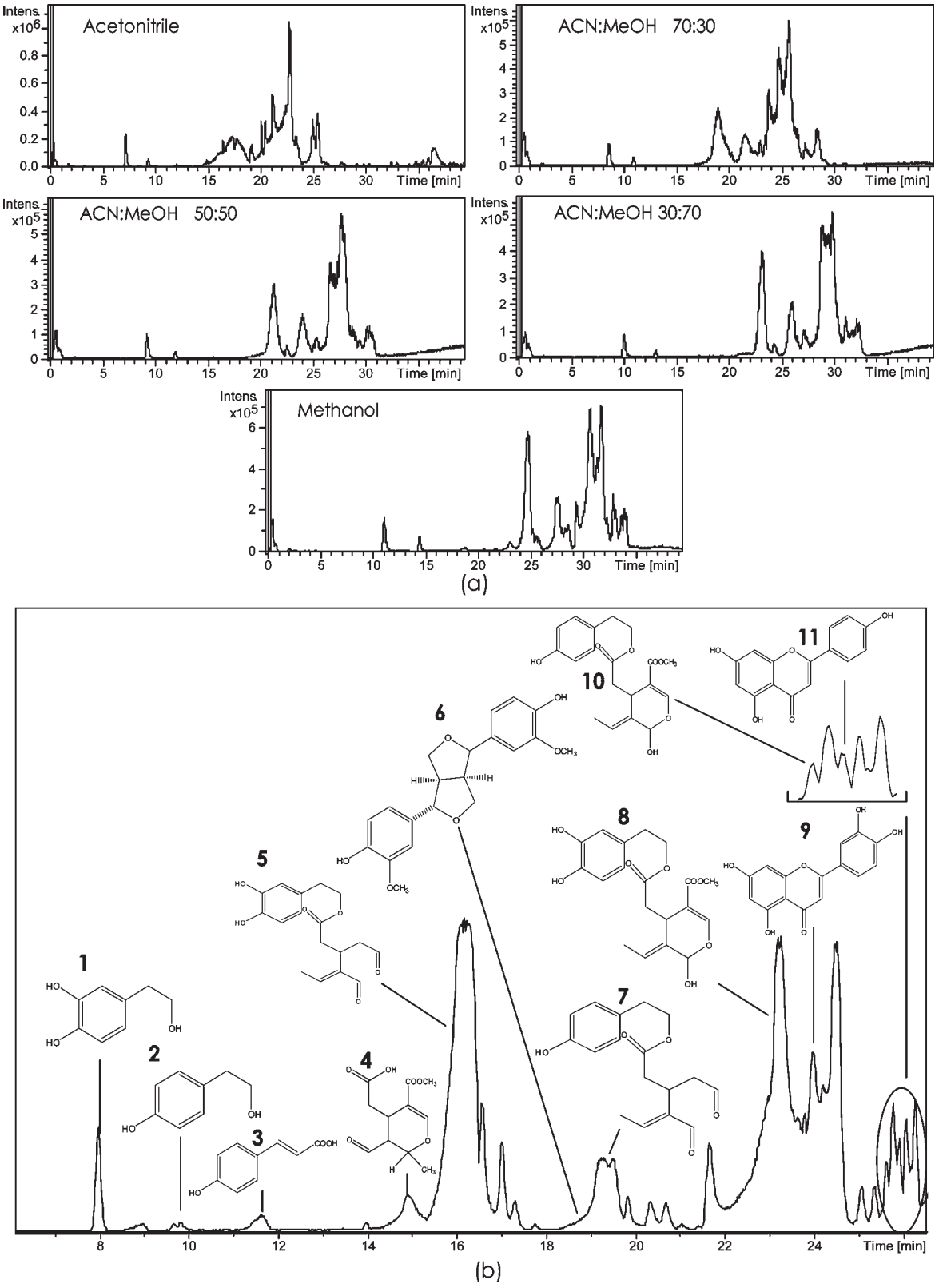

Figure 1. (**a**) Comparison of the base peak chromatograms (BPC) chromatograms obtained by RRLC-ESI-TOF using different mobile phase B. The mobile phase A was water with 0.5% acetic acid. (**b**) BPC base peak of the Picual EVOO-PE obtained by the proposed method. The main phenolic compounds are: 1, HYTY; 2, TY, 3, vanillin; 4, EA; 5, DOA; 6, Pin; 7, D-Lig Agl; 8, Ol Agl; 9, Lut; 10, Lig Agl; 11, Apig.

resolution of phenolic compounds was achieved in less than 27 min. **Figure 1b** shows the chromatogram of the Picual EVOO-PE obtained by the proposed method.

The validation of the proposed method was carried out with the linearity, sensitivity, and precision parameters. **Table 1** shows the following analytical parameters: relative standard deviation (RSD), limits of detection (LOD), and quantification (LOQ), calibration range, calibration curve equations, and regression coefficient (r^2).

The linearity range of the analytical method was established with standard solutions of phenolic and other polar compounds,

Table 1. Analytical Parameters of the Proposed Method

phenolic compds	RSD	LOD (μg/mL)	LOQ (μg/mL)	calibration range (μg/mL)	calibration equations	r^2
HYTY	0.20	0.042	0.141	LOQ−50	$y = 10070155x - 156121.750$	0.9956
TY	3.64	0.278	0.928	LOQ−50	$y = 2120772.229x - 185414.271$	0.9924
Lut	0.55	0.012	0.041	LOQ−25	$y = 37539998.992x + 296205.770$	0.9963
Apig	1.82	0.005	0.019	LOQ−25	$y = 83626939.058x + 332983.761$	0.9959
Pin	0.70	0.087	0.353	LOQ−50	$y = 3812047.291x - 44338.807$	0.9987
Ole	1.89	0.099	0.174	LOQ−350	$y = 4169427.500x + 104290.881$	0.9921
vanillic acid	2.58	0.078	0.249	LOQ−25	$y = 2964238.718x + 289237.551$	0.9928
vanillin	2.16	0.338	1.084	LOQ−50	$y = 1236826.8571x + 20524.3905$	0.9912
p-coumaric acid	1.74	0.066	0.223	LOQ−25	$y = 9352746.464x + 133498.367$	0.9907
ferulic acid	0.65	0.064	0.213	LOQ−25	$y = 13563857.601x + 68785.193$	0.9962
			Other Polar Compds			
quinic acid	1.16	0.055	0.179	LOQ−25	$y = 12771791.047x + 150060.98$	0.9918

Table 2. Main Phenolic Compounds Identified in a Representative Extract of Picual EVOO Variety by RRLC-ESI-TOF

compd	retention time (min)	*m*/*z* exptl	*m*/*z* calcd	molecular formula	error	σ
hydroxytyrosol	8	153.0556	153.0557	$C_8H_{10}O_3$	1.0	0.0064
tyrosol	9.9	137.0617	137.0608	$C_8H_{10}O_2$	1.4	0.0058
vanillin	11.7	151.0401	151.0401	$C_8H_8O_3$	0.3	0.0223
p-coumaric acid	13.5	163.0398	163.0401	$C_9H_8O_3$	1.8	0.0476
hydroxytyrosol acetate	14	195.0661	195.0663	$C_{10}H_{12}O_4$	0.8	0.0120
elenolic acid	15	241.0714	241.0718	$C_{11}H_{14}O_6$	1.7	0.0047
hydroxy elenolic acid	15.4	257.0667	257.0667	$C_{11}H_{14}O_7$	−0.5	0.0165
decarboxymethyl oleuropein aglycon	16.3	319.1193	319.1187	$C_{17}H_{20}O_6$	−1.0	0.0083
hydroxy D-oleuropein aglycon	16.6	335.1142	335.1136	$C_{17}H_{20}O_7$	−1.8	0.0134
syringaresinol	18.2	417.1562	417.1555	$C_{22}H_{26}O_8$	−1.7	0.0250
pinoresinol	18.8	357.1349	357.1344	$C_{20}H_{22}O_6$	−1.4	0.0050
decarboxymethyl ligstroside aglycon	19.2	303.1236	303.1229	$C_{17}H_{20}O_5$	0.7	0.0120
hydroxy D-ligstroside aglycon	19.9	319.1190	319.1187	$C_{17}H_{20}O_6$	−1.0	0.0151
10-hydroxy oleuropein aglycon	23	393.1200	393.1191	$C_{19}H_{22}O_9$	−2.3	0.0050
oleuropein aglycon	23.2	377.1247	377.1242	$C_{19}H_{22}O_8$	−1.2	0.0034
luteolin	23.7	285.0397	285.0405	$C_{15}H_{10}O_6$	2.5	0.0068
methyl D-oleuropein aglycon	25.4	333.1346	333.1344	$C_{18}H_{22}O_6$	−3.6	0.0135
ligstroside aglycon	25.6	361.1310	361.1293	$C_{19}H_{22}O_7$	3.1	0.0145
apigenin	25.8	269.0448	269.0451	$C_{15}H_{10}O_5$	2.9	0.0059
methyl oleuropein aglycon	26.2	391.1412	391.1398	$C_{20}H_{24}O_8$	−3.4	0.0069

such as HYTY, TY, Lut, Apig, Ole, Pin, vanillic acid, ferulic acid, *p*-coumaric acid, vanillin, and quinic acid. Calibration curves were prepared daily. All of them were obtained by plotting the standard concentration as a function of the peak area obtained from RRLC-ESI-TOF analyses. Calibration curves were calculated by using 10 points at different concentrations, estimated from the amounts of the analytes in samples, and were linear over the range of study (see **Table 1**). Furthermore, each different concentration was injected three times. The determination coefficients (r^2) were higher than 0.990 for all analytes.

The sensitivity of the method was studied by defining the LODs and LOQs for individual compounds in standard solutions. The LODs and LOQs were calculated using the signal-to noise ratio criterion of 3 and 10, respectively.

Repeatability of the method described was measured as relative standard deviation (RSD %) in terms of concentration. A methanolic extract was injected ($n = 6$) on the same day (intraday precision) and 3 times on the 2 consecutive days (interday precision, $n = 12$). Intraday repeatability of the developed method (for all the analytes) was from 0.07 to 3.92%, whereas the interday repeatability was from 0.10 to 3.23%, for MS detector.

Identification and Quantification of Phenolic and Other Polar Compounds in EVOOs. The identification of phenolic compounds was carried out by comparing both retention times and MS spectral data from olive oil samples and standards detailed in the Materials and Methods. Remaining compounds, for which no commercial standards were available, were identified by the interpretation of their mass spectral provided by the TOF-MS and the information previously reported (most of these compounds have been previously described in the olive oil samples). The analysis of the true isotopic pattern by ESI-TOF-MS in combination with excellent mass resolution and mass accuracy is the perfect choice for molecular formula determination using the Generate Molecular Formula Editor. To identify the phenolic compounds, a low tolerance of 0.05 and a low error (≤5 ppm) were chosen. The position of the molecular formula in the table of possible compounds was also considered. Most of the identified compounds are in position number 1. **Table 2** summarizes the main compounds identified in the Picual variety, including the information generated by TOF analyzer: retention time, experimental and calculated *m*/*z*, molecular formula, and error and σ value. Finally, 20 compounds from different families (simple phenols, flavonoids, lignans, and secoiridoids) were identified. To identify and quantify the phenolic compounds in EVOOs and their PEs used for MTT-based cell viability assays, the analysis of five different EVOO varieties (Hojiblanca, Picual, Cornezuelo, Manzanilla, and Arbequina) was carried out. **Figure 2** shows the resulting chromatograms of representative samples from the five varieties. Additional phenolic compounds were found in other olive oil extracts from different varieties such as vanillic acid (Hojiblanca, Arbequina, and Manzanilla varieties), ferulic acid

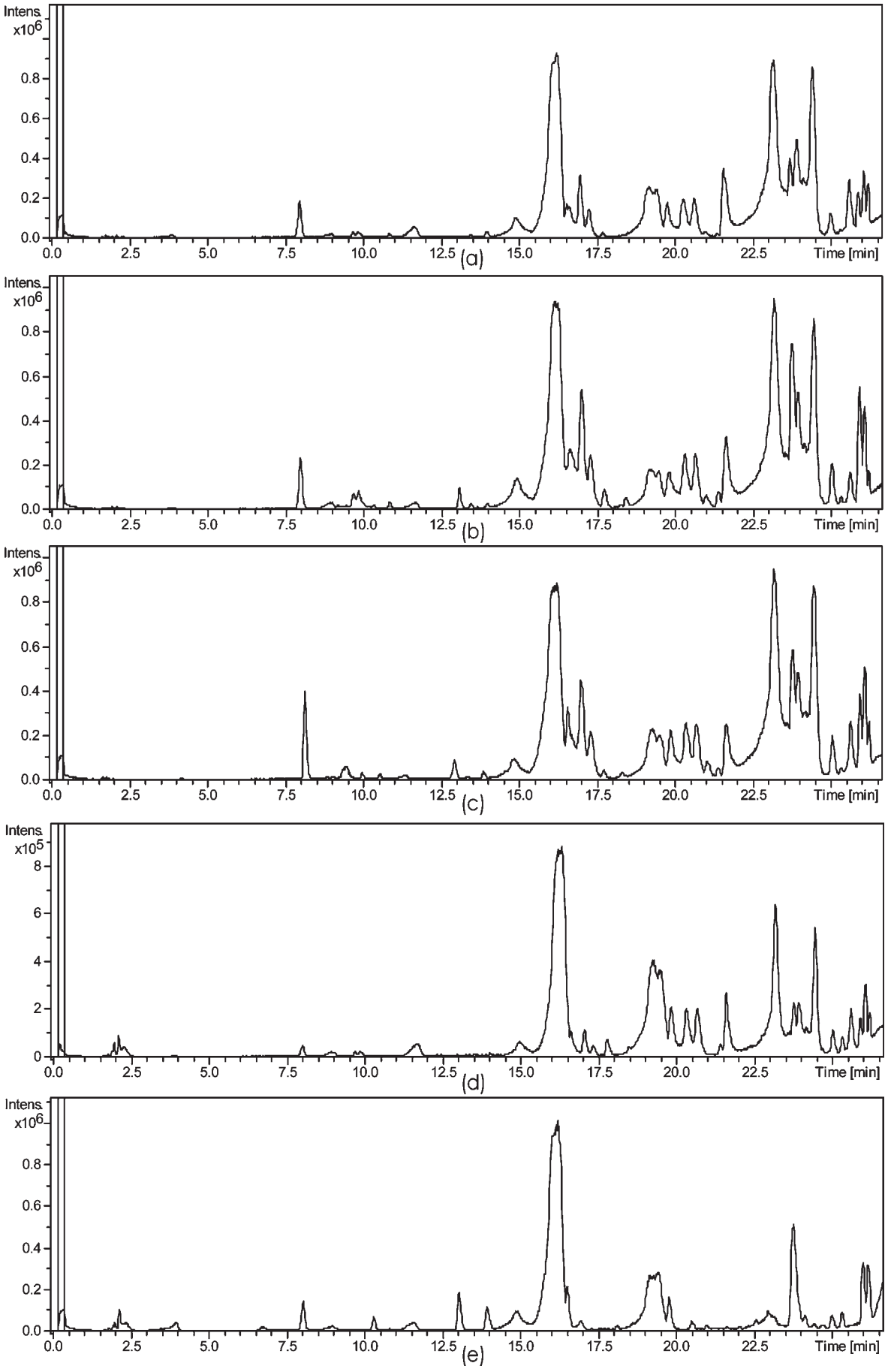

Figure 2. BPC of representative samples of the five EVOO varieties analyzed in this study by RRLC-ESI-TOF: (**a**) Picual; (**b**) EVOO Hojiblanca; (**c**) EVOO Manzanilla; (**d**) EVOO Cornezuelo; (**e**) EVOO Arbequina.

Table 3. Quantitative Results Expressed in mg Analyte/kg of EVOO; Value = $X \pm SD$

(a)

	Picual						
	EVOO 2 Málaga	EVOO 4 Jaén	EVOO 5 Granada	EVOO 6 Granada	EVOO 7 Córdoba	EVOO 10 Jaén	EVOO 11 Jaén
hydroxytyrosol	6.417 ± 0.073	11.314 ± 0.459	10.402 ± 0.039	10.122 ± 0.076	9.625 ± 0.208	4.903 ± 0.045	10.669 ± 0.083
tyrosol	4.712 ± 0.039	6.039 ± 0.059	4.991 ± 0.033	4.658 ± 0.011	4.162 ± 0.109	4.362 ± 0.075	4.487 ± 0.033
hydroxytyrosol acetate	0.699 ± 0.002	0.744 ± 0.008	0.683 ± 0.004	0.608 ± 0.004	0.680 ± 0.003	0.682 ± 0.001	0.692 ± 0.002
elenolic acid	7.157 ± 0.066	11.789 ± 0.084	11.940 ± 0.301	8.120 ± 0.039	13.123 ± 0.125	8.793 ± 0.353	9.086 ± 0.138
hydroxy elenolic acid	0.327 ± 0.004	0.233 ± 0.019	NQ[b]	1.181 ± 0.006	0.097 ± 0.002	NQ[b]	1.654 ± 0.037
oleuropein aglycon	146.005 ± 2.361	140.035 ± 1.254	143.330 ± 0.609	133.890 ± 0.124	157.917 ± 1.257	140.140 ± 4.141	125.778 ± 0.242
decarboxymethyl oleuropein aglycon	130.442 ± 1.987	133.794 ± 0.558	179.406 ± 2.328	185.778 ± 1.395	314.429 ± 2.189	212.442 ± 4.287	150.015 ± 0.188
hydroxy D-oleuropein aglycon	7.329 ± 0.133	10.156 ± 0.289	15.120 ± 0.140	30.795 ± 0.373	32.373 ± 1.584	11.225 ± 0.427	30.408 ± 0.315
10-hydroxy oleuropein aglycon	34.514 ± 0.228	61.794 ± 1.841	50.801 ± 0.477	59.782 ± 1.174	39.479 ± 0.806	16.731 ± 0.535	47.971 ± 1.669
methyl oleuropein aglycon	13.987 ± 0.381	27.316 ± 0.122	41.490 ± 0.404	10.417 ± 0.245	24.349 ± 0.516	3.022 ± 0.018	3.409 ± 0.110
methyl D-oleuropein aglycon	9.425 ± 0.304	11.929 ± 0.095	10.882 ± 0.107	14.147 ± 0.159	2.903 ± 0.079	2.523 ± 0.016	9.550 ± 0.184
ligstroside aglycon	30.603 ± 0.601	28.926 ± 0.461	25.646 ± 0.139	19.697 ± 0.301	16.261 ± 0.709	21.158 ± 0.156	20.931 ± 0.678
decarboxymethyl ligstroside aglycon	50.245 ± 0.051	51.788 ± 1.067	69.013 ± 0.184	49.606 ± 0.438	53.652 ± 0.354	55.432 ± 0.401	36.576 ± 0.280
hydroxy D-ligstroside aglycon	10.401 ± 0.107	16.172 ± 0.051	19.711 ± 0.055	19.936 ± 0.188	8.087 ± 0.353	6.306 ± 0.141	15.832 ± 0.557
pinoresinol	1.485 ± 0.003	1.810 ± 0.005	1.643 ± 0.023	1.550 ± 0.005	0.815 ± 0.032	1.907 ± 0.014	1.737 ± 0.006
hydroxy pinoresinol	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]
acetoxypinoresinol	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]
syringaresinol	0.870 ± 0.021	0.814 ± 0.011	0.807 ± 0.012	0.724 ± 0.012	0.772 ± 0.029	0.738 ± 0.007	0.797 ± 0.009
luteolin	1.531 ± 0.004	2.011 ± 0.034	2.728 ± 0.067	3.165 ± 0.025	4.041 ± 0.065	4.005 ± 0.120	4.071 ± 0.094
apigenin	0.271 ± 0.004	0.313 ± 0.001	0.588 ± 0.011	0.431 ± 0.021	0.811 ± 0.040	0.562 ± 0.014	0.728 ± 0.006
total phenolic contents	456.412 ± 3.838	516.977 ± 4.484	589.189 ± 3.141	554.606 ± 1.389	683.579 ± 4.169	494.933 ± 6.115	474. 392 ± 3.897
Other Polar Compounds							
quinic acid	ND[a]	1.238 ± 0.054	1.121 ± 0.004	0.872 ± 0.018	0.356 ± 0.012	ND[a]	ND[a]

(b)

	Hojiblanca		Manzanilla	Comezuelo	Arbequina		
	EVOO 1 Málaga	EVOO 9 Sevilla	EVOO 8 Sevilla	EVOO 3 Granada	EVOO 12 Reus	EVOO 13 Sevilla	EVOO 14 Sevilla
hydroxytyrosol	5.921 ± 0.310	5.868 ± 0.053	9.818 ± 0.427	1.367 ± 0.029	1.747 ± 0.038	2.621 ± 0.096	4.056 ± 0.072
tyrosol	4.238 ± 0.002	4.687 ± 0.138	5.452 ± 0.075	4.049 ± 0.147	3.298 ± 0.009	3.542 ± 0.006	3.594 ± 0.029
hydroxytyrosol acetate	0.687 ± 0.002	0.678 ± 0.001	0.799 ± 0.003	0.585 ± 0.002	1.360 ± 0.076	2.282 ± 0.074	3.039 ± 0.098
elenolic acid	14.942 ± 0.093	18.632 ± 0.192	12.084 ± 0.179	4.932 ± 0.092	4.522 ± 0.154	6.492 ± 0.269	8.697 ± 0.074
hydroxy elenolic acid	0.161 ± 0.003	NQ[b]	1.149 ± 0.018	ND[a]	ND[a]	0.511 ± 0.012	ND[a]
oleuropein aglycon	99.959 ± 0.730	146.695 ± 0.519	143.006 ± 1.980	75.507 ± 1.727	2.347 ± 0.061	10.148 ± 0.234	17.807 ± 0.103
decarboxymethyl oleuropein aglycon	145.746 ± 1.711	217.683 ± 1.870	229.706 ± 7.558	199.324 ± 0.864	93.423 ± 1.203	183.907 ± 1.865	238.406 ± 3.321
hydroxy D-oleuropein aglycon	10.961 ± 0.140	7.850 ± 0.243	23.090 ± 0.309	6.316 ± 0.011	17.103 ± 1.053	22.085 ± 0.433	12.679 ± 0.226
10-hydroxy oleuropein aglycon	17.970 ± 0.264	5.450 ± 0.077	17.467 ± 0.336	2.093 ± 0.090	0.457 ± 0.016	0.197 ± 0.005	NQ[b] b
methyl oleuropein aglycon	7.401 ± 0.018	0.474 ± 0.015	1.035 ± 0.028	1.835 ± 0.022	ND[a]	ND[a]	ND[a]
methyl D-oleuropein aglycon	3.488 ± 0.105	0.018 ± 0.0001	2.819 ± 0.002	0.559 ± 0.007	ND[a]	ND[a]	ND[a]
ligstroside aglycon	10.989 ± 0.393	12.780 ± 0.300	22.370 ± 0.135	13.695 ± 0.219	NQ[b] b	1.146 ± 0.016	1.294 ± 0.032
decarboxymethyl ligstroside aglycon	29.489 ± 0.676	32.986 ± 0.658	51.999 ± 1.843	102.139 ± 3.780	14.381 ± 0.495	54.774 ± 2.570	62.271 ± 1.179
hydroxy D-ligstroside aglycon	6.582 ± 0.082	1.182 ± 0.015	7.534 ± 0.051	11.233 ± 0.562	10.338 ± 0.265	17.713 ± 0.691	10.939 ± 0.343
pinoresinol	1.008 ± 0.002	0.867 ± 0.006	1.303 ± 0.019	0.869 ± 0.027	3.258 ± 0.023	2.634 ± 0.122	3.157 ± 0.019
hydroxy pinoresinol	ND[a]	ND[a]	ND[a]	ND[a]	1.160 ± 0.030	1.436 ± 0.015	1.551 ± 0.040
acetoxypinoresinol	2.700 ± 0.036	3.039 ± 0.032	2.252 ± 0.009	ND[a]	17.227 ± 0.230	11.009 ± 0.741	12.050 ± 0.045
syringaresinol	0.796 ± 0.013	0.885 ± 0.004	1.173 ± 0.029	0.687 ± 0.002	1.650 ± 0.042	1.796 ± 0.013	2.259 ± 0.018
luteolin	2.746 ± 0.056	8.691 ± 0.048	6.664 ± 0.247	1.996 ± 0.007	4.852 ± 0.061	4.697 ± 0.065	5.907 ± 0.086
apigenin	0.955 ± 0.033	1.757 ± 0.015	1.079 ± 0.013	0.362 ± 0.002	0.909 ± 0.006	0.881 ± 0.010	1.064 ± 0.027
total phenolic contents	366.741 ± 1.654	473.242 ± 6.346	537.475 ± 6.027	427.549 ± 6.155	178.013 ± 1.411	327.269 ± 9.104	388.774 ± 4.584
Other Polar Compounds							
quinic acid	NQ[b]	ND[a]	NQ[b]	1.969 ± 0.032	NQ[b]	ND[a]	2.361 ± 0.044

[a] Not detected. [b] Not quantified. Compound detected, but their concentration is between the detection and quantification limits.

(Manzanilla variety), acetoxypinoresinol (Hojiblanca, Manzanilla, and Arbequina varieties), and hydroxypinoresinol (Arbequina variety). Regarding the other polar compounds, quinic acid was also identified in the extracts deriving from all the analyzed varieties.

Eleven standard calibration graphs for the quantification of the principal compounds found in the samples were prepared using the 11 commercial standards detailed in Materials and Methods. All calibration curves showed good linearity between different

concentrations depending on the analytes studied (**Table 1**). The quantification was carried by RRLC-ESI-TOF.

The phenolic and other polar compound concentrations were determined using the area of each individual compound (three replicates) and by interpolation in the corresponding calibration curve. Phenolic compounds hydroxytyrosol, tyrosol, luteolin, apigenin, and (+)-pinoresinol such as quinic acid (other polar compound) were quantified by the calibration curves obtained from their respective commercial standards. The other phenolic compounds, which had no commercial standards, were tentatively quantified on the basis of other compounds having similar structures. Hydroxytyrosol acetate was quantified using a HYTY calibration curve, hydroxypinoresinol, (+)-1-acetoxypinoresinol, and syringaresinol using a (+)-pinoresinol calibration curve. Regarding secoiridoid group, all these compounds were quantified with oleuropein standard. It has to be taken into account that the response of the standards can be different from the one of the analytes present in the oil samples, and consequently the quantification of these compounds is only an estimation of their actual concentrations. **Table 3** summarizes the quantitative results obtained by RRLC-MS. Fourteen EVOOs from different varieties were quantified: two Hojiblanca varieties (EVOOs 1 and 9), seven Picual varieties (EVOOs 2, 4, 5, 6, 7, 10 and 11), one Cornezuelo (EVOO 3), one Manzanilla (EVOO 8), and three Arbequina (EVOOs 12, 13, and 14). The main components of the phenolic fraction were the derivates of hydroxytyrosol (3, 4-DHEPA) and tyrosol (*p*-HPEA) linked to the aldehydic and dialdehydic forms of elenolic acid: oleuropein aglycon (3,4-DHEPA-EA), ligstroside aglycon (*p*-HPEA-EA) and their hydroxylated, decarboxymethylated and methylated forms. Among the phenolic compounds, two secoiridoids, i.e. oleuropein aglycon and its decarboxymethyl derivative, were the most abundant compounds. In all varieties, the range of concentrations was from 76 to 158 mg/kg and from 93 to 314 mg/kg for oleuropein aglycon and decarboxymethyl oleuropein aglycon, respectively. Concerning oleuropein aglycon, in the Arbequina variety, the amount was considerably less (from 2 to 18 mg/kg). Similarly, the quantity of ligstroside aglycon in EVOO 13 and 14 was 10 times lower than in Hojiblanca and Cornezuelo and 20 times lower than in Picual and Manzanilla varieties. Regarding EVOO 12, this compound was not quantified because their concentration was between the detection and quantification limits.

The content of the decarboxymethyl ligstroside aglycon in the Cornezuelo variety was significantly higher than in the other ones.

On the other hand, significant amounts of lignans (+)-pinoresinol, (+)-1-acetoxypinorersinol, and hydroxypinoresinol were detected. Except (+)-pinoresinol, which was found in all oils, hydroxypinoresinol was found only in the Arbequina variety and acetoxypinoresinol only in the three varieties: Arbequina, Hojiblanca, and Manzanilla. The concentrations of three compounds in Arbequina variety were higher than in the other four varieties. The olive oils from this variety had also the highest amounts of syringaresinol: twice than that found in Manzanilla and three times more than in Picual, Hojiblanca, and Cornezuelo.

As far as the amounts of flavones and phenyl alcohols are concerned, luteolin and apigenin were more abundant in Hojiblanca, Arbequina, and Manzanilla, while the content of phenyl alcohols such as hydroxytyrosol and tyrosol were the highest in Picual and Manzanilla olive oils. Regarding phenolic acids, all the EVOOs analyzed contained low quantity of phenyl acids. Furthermore, vanillin, ferulic acid, vanillic acid, and *p*-coumaric acid were not quantified because their concentrations in different olive oils were between their detection and quantification limits (detailed previously). Total phenolic content from different EVOO varieties was tentatively calculated as sum of the individual phenolic compound concentrations.

Table 4. Predictors Selected and Their Corresponding Standardized Coefficients of the LDA Model Constructed to Predict the Variety of the EVOO Samples

predictors	*f*1	*f*2	*f*3	*f*4
hydroxytyrosol	1.86	0.74	−0.92	−6.23
tyrosol	−1.75	3.65	1.20	−3.18
hydroxy elenolic acid	3.56	0.80	−3.01	2.46
decarboxymethyl ligstroside aglycon	2.30	1.22	−4.46	0.12
pinoresinol	3.07	3.05	2.45	0.76
10-hydroxy oleuropein aglycon	−1.34	0.01	−7.52	10.19
hydroxy D-ligstroside aglycon	−3.83	−0.70	5.41	1.26
elenolic acid	2.91	−8.03	4.86	1.81
luteolin	−4.49	−0.70	−5.62	0.79
hydroxy D-oleuropein aglycon	0.14	6.43	6.43	−4.30
methyl oleuropein aglycon	−0.21	3.93	−2.60	0.23
methyl D-oleuropein aglycon	2.56	−4.63	2.81	−4.68
syringaresinol	−3.62	1.79	2.38	0.48
oleuropein aglycon	4.12	2.94	3.83	0.77

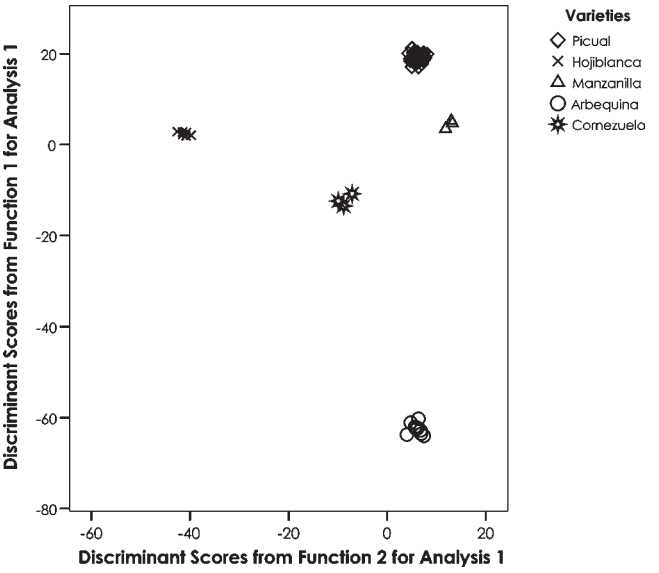

Figure 3. Score plot on the plane of the two LDA discriminant functions obtained to predict the olive varieties of EVOOs.

Classification of EVOOs by Phenolic Profile and other Polar Compounds. Using the normalized variables, an LDA model capable of classifying the EVOO samples according to their olive variety was constructed. When the LDA model was carried out, an excellent resolution between all the category pairs was achieved ($\lambda_w < 0.002$). The variables selected by the SPSS stepwise algorithm, and the corresponding standardized coefficients of this model, showing the predictors with large discriminant capabilities, are given in **Table 4**. For this model, and using leave-one-out validation, all the points of the training set were correctly classified (100%). The corresponding evaluation set, containing the 51 original data points, was then used to check the prediction capability of the model. Using a 95% probability, all the objects were correctly assigned. **Figure 3** shows the score plot on the plane of the two LDA discriminant functions obtained to predict the olive varieties of EVOOs.

Inhibitory Effects of Crude EVOO-PEs on Breast Cancer Cell Viability. To evaluate breast cancer cell sensitivity to crude EVOO-PE naturally bearing different amounts of complex polyphenols, SKBR3 cells were cultured in the absence or presence of a series of ethanolic dilutions in fresh culture medium detailed in

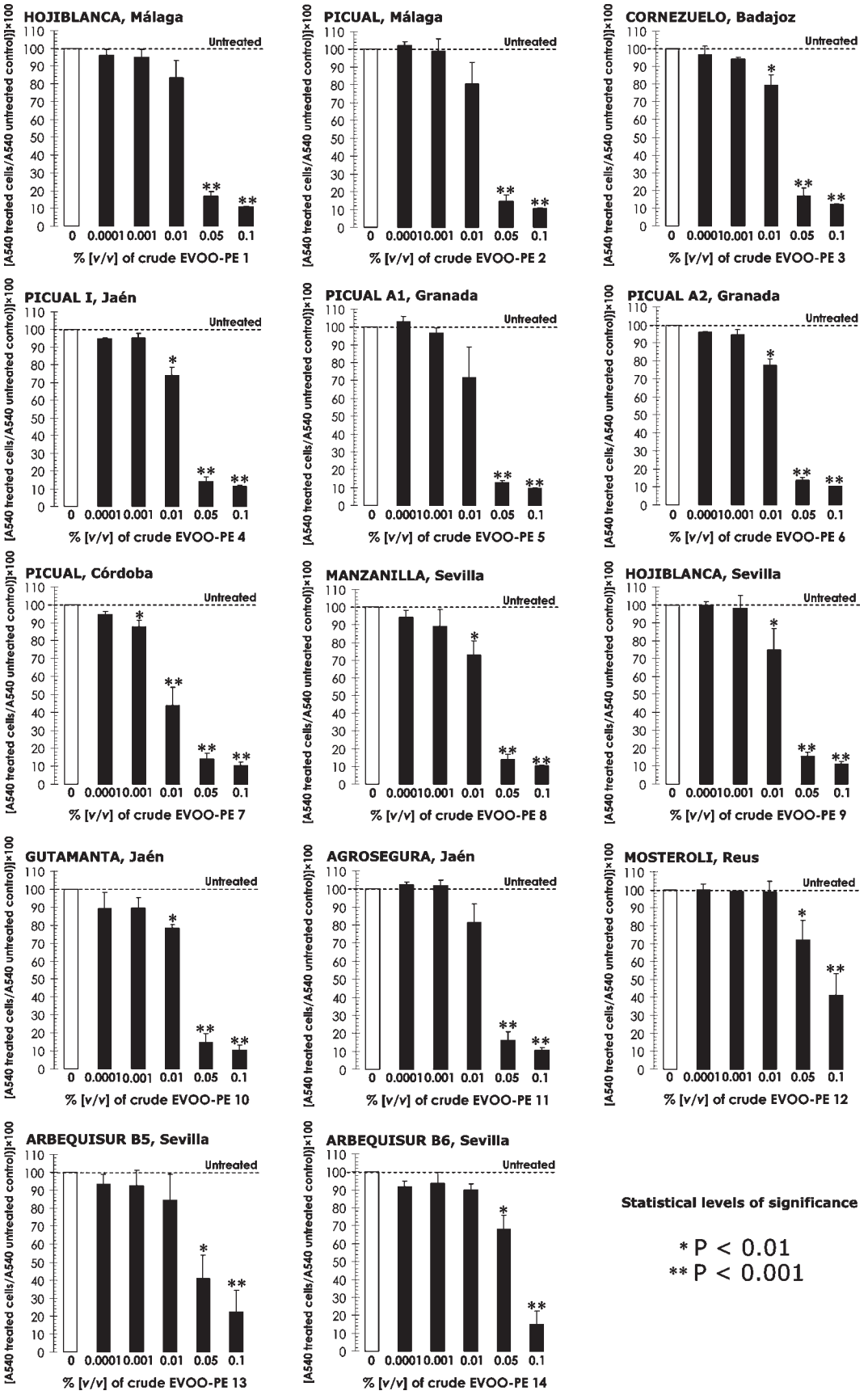

Figure 4. Effects of EVOO-PE on cell viability in HER2-overexpressing SKBR3 breast cancer cells. The metabolic status of SKBR3 cells treated with graded concentrations of individual EVOO-PE was evaluated using a MTT-based cell viability assays and constructing dose−response graphs as [A_{540} treated cells/A_{540} untreated control)] × 100. Results are means (columns) and 95% confidence intervals (bars) of three independent experiments made in triplicate.

Materials and Methods. The highest solvent concentration in culture media (0.1% v/v ethanol) had no significant effects on the metabolic status of SKBR3 cells (data not shown). SKBR3 cells represent a widely used tumor cell in vitro model characterized by

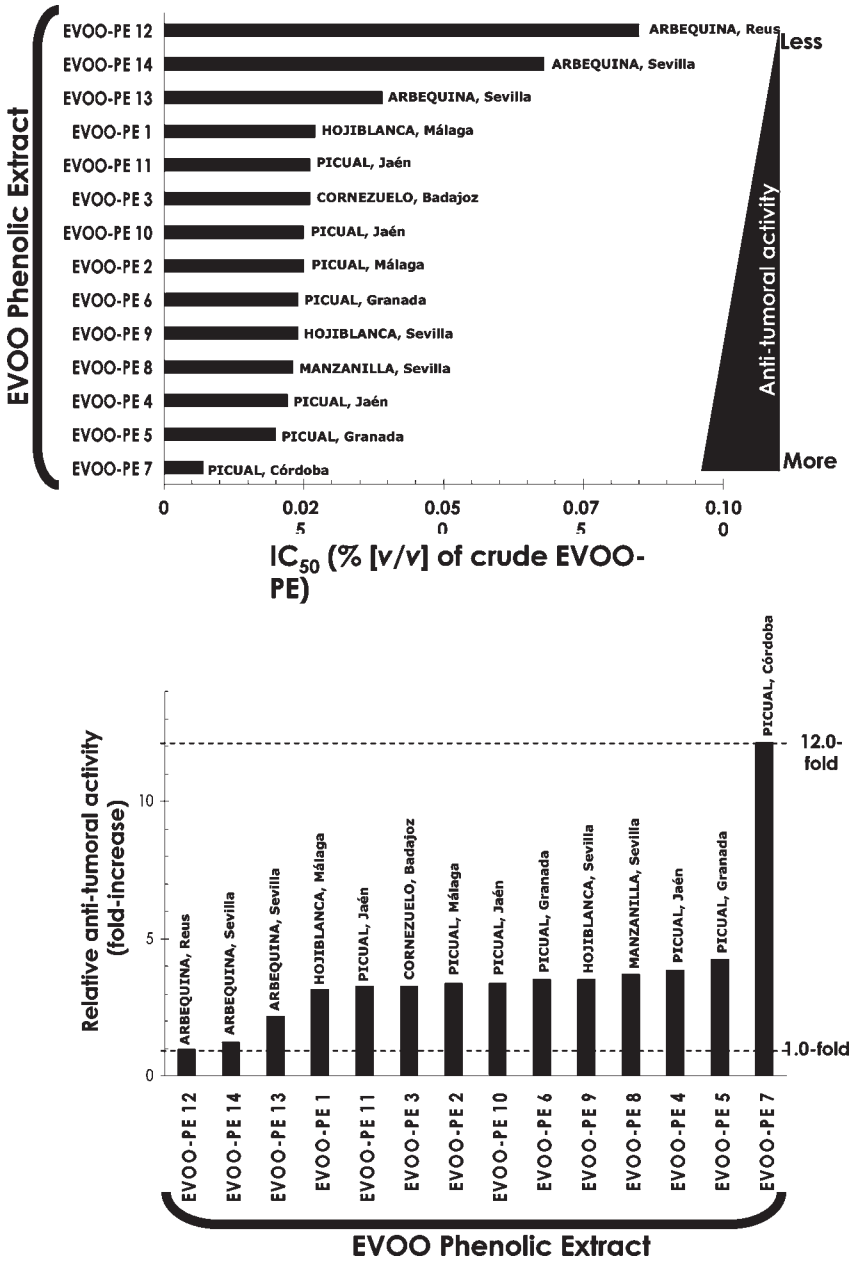

Figure 5. Differential antitumoral efficacy of EVOO-PE against SKBR3 cells. (top) Sensitivity of SKBR3 cells to individual EVOO-PE was expressed in terms of the concentration of PE (% [v/v]) required to decrease by 50% (IC_{50}) cell viability. Because the percentage of control absorbance in MTT-based cell viability assays (**Figure 4**) was considered to be the surviving fraction of cells, the EVOO-PE IC_{50} values were defined as the concentration of PE that produced 50% reduction in control absorbance (by interpolation upon construction of dose−response curves). (bottom) Comparative efficacy of EVOO-PE in SKBR3 cells was carried out by arbitrarily normalizing EVOO-PE IC_{50} values as fold-increases versus less-active EVOO-PE (= 1.0-fold).

exhibiting natural *HER2* oncogene amplification, HER2 oncoprotein overexpression, and HER2-dependency for cell proliferation and survival. After 5 days of treatment, SKBR3 cell numbers were measured using a tetrazolium salt-based (MTT) protocol. MTT-based cell viability assays revealed that all the crude EVOO-PE negatively affected metabolic status of SKBR3 cells in a concentration-dependent manner (**Figure 4**). However, we noted remarkable differences in the ability of individual EVOO-PEs to elicit cytotoxic responses in SKBR3 cells. Thus, concentrations as high as ~0.1% v/v were needed to significantly decrease cell viability when SKBR3 cells were cultured in the presence of the PE obtained from the monovariety EVOO 12. Conversely, concentrations lower than 0.01% v/v significantly decreased cell viability when SKBR3 cells were exposed to graded volumes of the PE obtained from the monovariety EVOO 7.

To accurately evaluate quantitative differences in the SKBR3 breast cancer cytotoxic activities among EVOO-PE, IC_{50} values (i.e., the concentration of each EVOO-PE needed to decrease cell viability by 50% relative to untreated control cells) were calculated by interpolation upon construction of dose−response curves. We obtained a wide series of IC_{50} values ranging from 0.007% v/v (EVOO-PE 7) to 0.085% v/v (EVOO-PE 12) (**Figure 5**, top). Upon this approach, crude EVOO-PE exhibited the following cytotoxic potencies: EVOO-PE 7 > EVOO-PE 5 > EVOO-PE 4 > EVOO 8-PE > EVOO-PE 9 > EVOO-PE 6 > EVOO-PE 2 > EVOO-PE 10 > EVOO-PE 3 > EVOO-PE 11 > EVOO-PE 1 > EVOO-PE 13 > EVOO-PE 14 > EVOO-PE 12 (**Figure 5**, bottom). Importantly, anti-SKBR3 cytotoxic activity was found to be up to 12-times higher when using EVOO-PE 7 than in the presence of EVOO-PE 12.

Table 5. Concentration of Phenolic Compounds in Crude EVOO-PE Stocks Used in Cultured SKBR3 Breast Cancer Cells. Value = X (μg analyte/mL ethanol) $\pm$ SD

(a)

	EVOO-PE 1	EVOO-PE 2	EVOO-PE 3	EVOO-PE 4	EVOO-PE 5	EVOO-PE 6	EVOO-PE 7
total phenyl alcohol contents	32.537 ± 0.894	35.485 ± 0.255	18.008 ± 0.388	54.290 ± 1.198	48.227 ± 0.217	46.165 ± 0.251	43.401 ± 0.856
hydroxytyrosol	17.763 ± 0.292	19.252 ± 0.219	4.103 ± 0.087	33.942 ± 1.377	31.205 ± 0.116	30.366 ± 0.227	28.875 ± 0.625
tyrosol	12.713 ± 0.008	14.137 ± 0.117	12.149 ± 0.043	18.116 ± 0.177	14.974 ± 0.099	13.975 ± 0.033	12.486 ± 0.329
hydroxytyrosol acetate	2.061 ± 0.006	2.096 ± 0.008	1.756 ± 0.007	2.231 ± 0.026	2.048 ± 0.012	1.825 ± 0.013	2.039 ± 0.009
total secoiridoid contents	1043.068 ± 4.607	1321.283 ± 11.519	1252.896 ± 10.131	1481.796 ± 12.865	1702.039 ± 9.031	1600.048 ± 3.849	1988.019 ± 14.345
elenolic acid	44.827 ± 0.279	21.471 ± 0.198	14.795 ± 0.276	35.363 ± 0.254	35.822 ± 0.904	24.358 ± 0.621	39.370 ± 0.375
hydroxy elenolic acid	0.483 ± 0.003	0.980 ± 0.014	ND[a]	0.698 ± 0.057	NQ[b]	3.545 ± 0.019	0.291 ± 0.001
oleuropein aglycon	299.881 ± 2.190	438.015 ± 7.084	226.521 ± 5.180	420.104 ± 3.762	429.991 ± 1.827	401.672 ± 0.374	473.752 ± 3.771
decarboxymethyl oleuropein aglycon	437.239 ± 5.132	391.326 ± 5.962	597.971 ± 2.591	401.384 ± 1.675	538.218 ± 6.983	557.335 ± 4.186	943.299 ± 6.569
hydroxy D-oleuropein aglycon	32.883 ± 0.420	21.987 ± 0.400	18.947 ± 0.003	30.467 ± 0.867	45.374 ± 0.431	92.384 ± 1.119	97.119 ± 4.751
10-hydroxy oleuropein aglycon	53.909 ± 0.792	103.541 ± 0.683	6.278 ± 0.271	185.384 ± 5.524	152.403 ± 1.433	179.345 ± 3.523	118.436 ± 2.419
methyl oleuropein aglycon	22.202 ± 0.054	41.936 ± 1.144	5.504 ± 0.067	81.949 ± 0.366	124.472 ± 1.212	31.251 ± 0.735	73.047 ± 1.549
methyl D-oleuropein aglycon	10.463 ± 0.315	28.277 ± 0.913	1.678 ± 0.021	35.787 ± 0.285	32.645 ± 0.320	42.442 ± 0.477	8.709 ± 0.239
ligstroside aglycon	32.967 ± 1.179	91.809 ± 1.804	41.085 ± 0.659	86.778 ± 1.383	76.940 ± 0.418	59.090 ± 0.904	48.785 ± 2.127
decarboxymethyl ligstroside aglycon	88.467 ± 2.026	150.736 ± 0.152	306.418 ± 11.340	155.365 ± 3.201	207.040 ± 0.553	148.817 ± 1.315	160.956 ± 1.062
hydroxy D-ligstroside aglycon	19.746 ± 0.246	31.203 ± 0.321	33.699 ± 1.687	48.516 ± 0.166	59.134 ± 1.340	59.808 ± 0.065	24.261 ± 1.060
total lignan contents	13.513 ± 0.041	7.065 ± 0.062	4.669 ± 0.101	7.874 ± 0.048	7.351 ± 0.059	6.820 ± 0.023	4.759 ± 0.184
pinoresinol	3.025 ± 0.005	4.454 ± 0.008	2.607 ± 0.082	5.431 ± 0.014	4.929 ± 0.068	4.648 ± 0.013	2.444 ± 0.096
hydroxy pinoresinol	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]
acetoxypinoresinol	8.099 ± 0.107	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]
syringaresinol	2.389 ± 0.037	2.610 ± 0.063	2.062 ± 0.026	2.433 ± 0.034	2.422 ± 0.024	2.171 ± 0.035	2.315 ± 0.087
total flavone contents	11.103 ± 0.230	5.403 ± 0.016	7.074 ± 0.019	6.969 ± 0.105	9.950 ± 0.235	10.785 ± 0.157	14.558 ± 0.252
luteolin	8.237 ± 0.167	4.593 ± 0.011	5.988 ± 0.022	6.031 ± 0.104	8.185 ± 0.202	9.494 ± 0.075	12.104 ± 0.196
apigenin	2.866 ± 0.099	0.810 ± 0.010	1.086 ± 0.015	0.939 ± 0.002	1.765 ± 0.033	1.291 ± 0.104	2.434 ± 0.121
total phenolic contents	1100.223 ± 3.559	1369.236 ± 1.515	1282.647 ± 9.985	1550.930 ± 4.808	1767.568 ± 4.638	1663.819 ± 1.389	2050.736 ± 6.073

(b)

	EVOO-PE 8	EVOO-PE 9	EVOO-PE 10	EVOO-PE 11	EVOO-PE 12	EVOO-PE 13	EVOO-PE 14
total phenyl alcohol contents	48.212 ± 0.546	33.701 ± 0.192	29.843 ± 0.094	47.544 ± 0.282	19.215 ± 0.145	25.335 ± 0.119	32.068 ± 0.407
hydroxytyrosol	29.561 ± 1.283	17.604 ± 0.159	14.710 ± 0.136	32.007 ± 0.250	5.241 ± 0.115	7.863 ± 0.289	12.169 ± 0.215
tyrosol	16.566 ± 0.225	14.062 ± 0.414	13.085 ± 0.224	13.462 ± 0.100	9.895 ± 0.027	10.627 ± 0.017	10.783 ± 0.089
hydroxytyrosol acetate	2.993 ± 0.010	2.035 ± 0.005	2.045 ± 0.005	2.075 ± 0.007	4.079 ± 0.022	6.845 ± 0.282	9.116 ± 0.292
total secoiridoid contents	1536.794 ± 24.781	1331.249 ± 1.503	1433.319 ± 18.55	1353.634 ± 11.136	427.711 ± 1.594	890.912 ± 29.574	1056.284 ± 5.305
elenolic acid	36.508 ± 0.538	55.898 ± 0.575	26.380 ± 1.059	27.257 ± 0.413	13.567 ± 0.461	19.475 ± 0.806	26.091 ± 0.222
hydroxy elenolic acid	3.379 ± 0.055	NQ[b]	NQ[b]	4.961 ± 0.111	ND[a]	1.533 ± 0.035	ND[a]
oleuropein aglycon	429.017 ± 5.941	440.086 ± 1.558	420.421 ± 12.424	377.334 ± 0.726	7.041 ± 0.182	30.443 ± 0.703	53.420 ± 0.308
decarboxymethyl oleuropein aglycon	689.119 ± 22.676	653.049 ± 5.610	637.325 ± 12.871	450.046 ± 2.364	280.268 ± 3.609	551.719 ± 11.963	715.219 ± 9.96
hydroxy D-oleuropein aglycon	69.292 ± 0.927	23.549 ± 0.727	33.674 ± 1.282	91.226 ± 0.944	51.307 ± 3.159	66.253 ± 1.297	38.038 ± 0.679
10-hydroxy oleuropein aglycon	52.401 ± 1.008	16.347 ± 0.231	50.193 ± 1.695	143.912 ± 5.008	1.371 ± 0.049	0.589 ± 0.002	NQ[b]
methyl oleuropein aglycon	3.105 ± 0.009	1.421 ± 0.045	9.066 ± 0.054	10.226 ± 0.330	ND[a]	ND[a]	ND[a]
methyl D-oleuropein aglycon	8.458 ± 0.004	0.054 ± 0.001	7.569 ± 0.048	28.650 ± 0.553	ND[a]	ND[a]	ND[a]
ligstroside aglycon	67.109 ± 0.405	38.339 ± 0.901	63.473 ± 0.468	62.793 ± 2.034	NQ[b]	3.438 ± 0.200	3.881 ± 0.098
decarboxymethyl ligstroside aglycon	155.999 ± 5.531	98.958 ± 1.975	166.297 ± 1.201	109.728 ± 0.840	43.143 ± 1.480	164.321 ± 7.710	186.814 ± 3.537
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hydroxy D-ligstroside aglycon	22.603 ± 0.152	3.546 ± 0.044	18.918 ± 0.424	47.498 ± 1.679	31.014 ± 0.795	53.138 ± 2.072	32.818 ± 1.029
total lignan contents	14.187 ± 0.071	14.374 ± 0.085	7.935 ± 0.063	7.602 ± 0.011	69.884 ± 0.607	50.627 ± 2.288	57.055 ± 0.043
pinoresinol	3.911 ± 0.057	2.601 ± 0.017	5.721 ± 0.042	5.211 ± 0.017	9.775 ± 0.069	7.904 ± 0.366	9.471 ± 0.059
hydroxy pinoresinol	ND[a]	ND[a]	ND[a]	ND[a]	3.479 ± 0.090	4.308 ± 0.004	4.653 ± 0.121
acetoxypinoresinol	6.756 ± 0.027	9.118 ± 0.095	ND[a]	ND[a]	51.681 ± 0.686	33.029 ± 1.225	36.152 ± 0.135
syringaresinol	3.519 ± 0.087	2.655 ± 0.001	2.213 ± 0.021	2.391 ± 0.028	4.949 ± 0.125	5.388 ± 0.039	6.779 ± 0.055
total flavone contents	23.232 ± 0.274	31.344 ± 0.047	13.702 ± 0.404	14.396 ± 0.298	17.283 ± 0.195	16.731 ± 0.081	20.916 ± 0.249
luteolin	19.992 ± 0.740	26.073 ± 0.174	12.016 ± 0.361	12.212 ± 0.281	14.555 ± 0.180	14.091 ± 0.196	17.721 ± 0.254
apigenin	3.239 ± 0.039	5.271 ± 0.046	1.685 ± 0.043	2.184 ± 0.019	2.728 ± 0.019	2.641 ± 0.031	3.195 ± 0.081
total phenolic contents	1622.425 ± 25.411	1410.669 ± 1.396	1484.798 ± 17.702	1423.176 ± 6.227	534.094 ± 2.011	983.606 ± 31.950	1166.323 ± 5.872

[a] Not detected. [b] Not quantified. Compound detected, but their concentration is between the detection and quantification limits.

Relationship Between Breast Cancer Cytotoxic Potencies and Phenolic Profiles of Crude EVOO-PEs. We wished to characterize and examine independently the notion that phenolic fractions directly obtained from different monovarieties of EVOO grown in Spain should exhibit different antibreast cancer cytotoxic activities. **Table 5** shows the content (in μg/mL) of each family of phenolics included in the 100% full strength stocks of individual EVOO-PE. As far as the total phenolic content is concerned, phenolic concentrations up to 500 mg/kg or even 1000 mg/kg have been described in VOOs from unripe olives of varieties

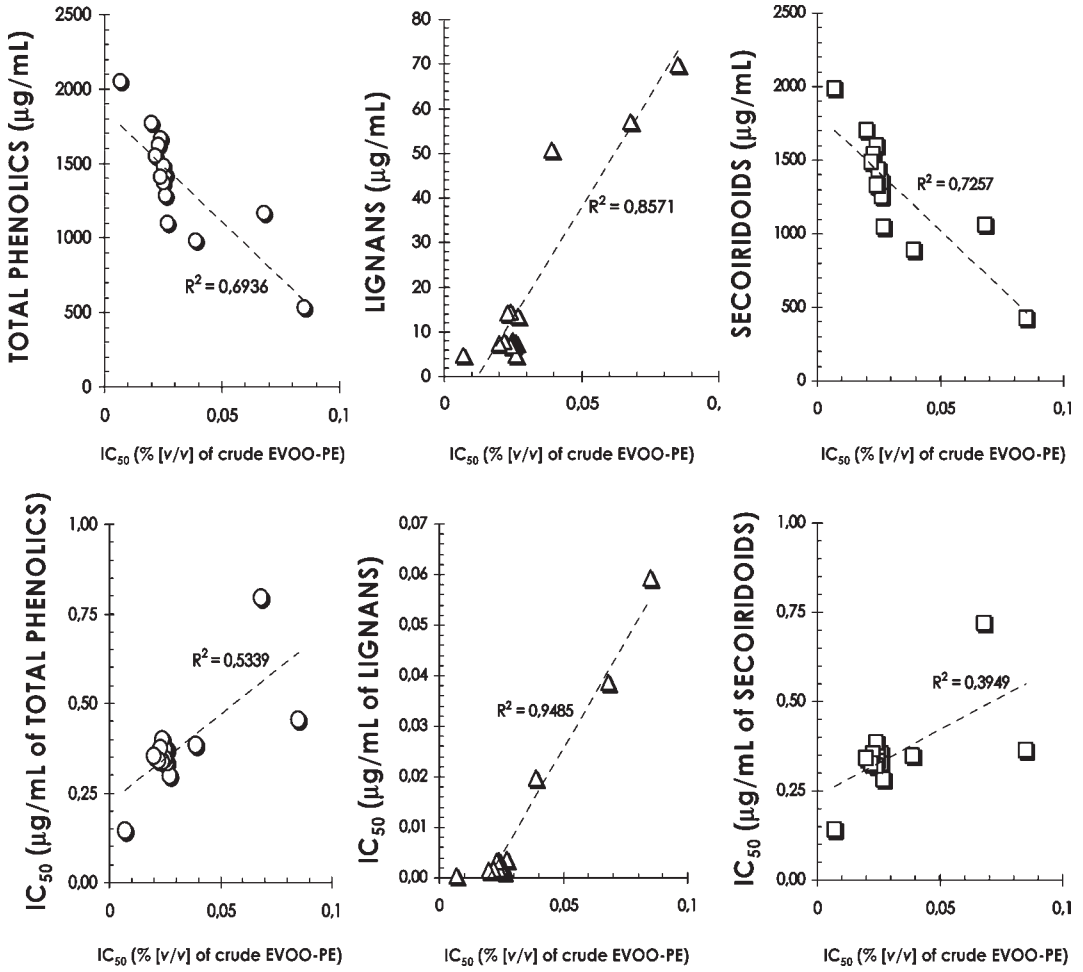

Figure 6. Correlations between phenolic composition and cytotoxic activity of crude EVOO-PE. Relationships between breast cancer cytotoxic activities (expressed as IC_{50} values in % v/v) and either concentrations of total phenolics, lignans, and secoiridoids in 100% full strength EVOO-PEs (top panels) or actual content of total phenolics, lignans, and secoiridoids in corresponding IC_{50} values in % v/v (bottom panels). Data are expressed on linear rather than log scales and adjusted linearly by plotting the regression line (R^2 values are shown).

grown in a hot environment (*25*). However, these oils are not appealing to most consumers due to their bitterness and pungency and they cannot be found on the market. In commercially available EVOOs, the concentration of phenolic compounds rather ranges between 100 mg/kg and 300 mg/kg (*26*). Because dietary EVOO intake has been reported to reach 50 g/day (*27*), an estimate of the daily intake of total phenols would range between 5 and 15 mg/day (up to 25 or 50 mg/day in EVOO-rich diets). Our stocks of crude phenolic extracts contained from ~500 to ~2000 μg/mL of total phenols. On the basis of these amounts, and because the IC_{50} values (as surrogates of the antibreast cancer cytotoxic activities of individual EVOO-PE) ranged from 0.14 to 0.8 μg/mL of total phenolics, all the breast cancer cytotoxic concentrations of EVOO-PE used in our studies can be easily achievable with the actual daily intake of EVOO.

Although the total content of phenolics varied up to 4 times when comparing the less active (i.e., EVOO-PE12) with the most active one (i.e., EVOO-PE7), most of the EVOO-PE differed little both in the total content and in the relative abundance of the main EVOO phenolic families (i.e., phenolic alcohols, flavones, lignans, and secoiridoids), thus suggesting that small variations in these parameters significantly impact the cytotoxic potency of multicomponent EVOO-PE. To validate this notion, we initially plotted IC_{50} values for each EVOO-PE as a function (on a linear−linear scale) of the total phenolic content (**Figure 6**, top panels). Linear regression analyses suggested a positive correlation between the cytotoxic potencies of EVOO-PE and the total phenolic content in their stocks (i.e., the higher content in total phenolic the lower the amount of EVOO-PE to decrease breast cancer cell viability by 50%). Because secoiridoids did account for more than 90% of phenolics in all the EVOO-PE, an almost equivalent correlation was observed when linear regression analyses were performed to assess a correlation between the EVOO-PE IC_{50} values and the concentration of secoiridoid in EVOO-PE stocks (i.e., lower IC_{50} values [v/v] positively related to higher concentrations of secoiridoids). Of note, a strong negative correlation was found between the absolute concentration of lignans in EVOO-PE stocks and EVOO-PE IC_{50} values. Indeed, the presence of lignans closely related with a loss of cytotoxic activity in EVOO-PE.

On the basis of the above-mentioned scenario, it could be reasonable to suggest that cytotoxic potencies of EVOO-PE, when calculated as % (v/v) of ethanolic EVOO-PE stocks, merely reflect a greater concentration of active polyphenols (i.e., secoiridoids) in any given % v/v experimental dilution rather than the occurrence of absence/presence of antagonistic/synergistic interactions between individual phenolic compounds in a given % v/v experimental dilution. To validate this notion, we converted IC_{50} values (in % v/v) into actual amounts of phenolics (in μg/mL) to assess a linear relationship between the two variables (**Figure 6**, bottom panels). We found a very strong positive correlation between the IC_{50} values in % v/v and their equivalents in μg/mL

of lignans. Thus, low IC_{50} values, which did correspond to highly active EVOO-PE, contained low to null amounts of lignans, whereas high IC_{50} values, which did correspond to poorly active EVOO-PE, were significantly enriched in their lignan contents. Remarkably, we failed to observe any significant correlation between IC_{50} values and their equivalent μg/mL contents in either total phenolics or secoiridoids (i.e., a lower IC_{50} value, and therefore, a higher cytotoxic activity, did not correspond to higher concentrations of secoiridoids). Most of the IC_{50} values, including those from poorly active EVOO-PE, contained ~0.3 μg/mL secoiridoids and, remarkably, the IC_{50} value from the most active PE (EVOO-PE) contained the lowest amount of total secoiridoids (~0.14 μg/mL).

As expected, the 14 EVOO varieties had significantly different phenolic compositions, in which secoiridoids were the major phenolic fraction (> 90% of total phenolics) in 11 EVOO monovarieties) and lignans were significantly enriched (5−10% of total phenolics) in three EVOO monovarieties (**Table 5**). When compared with EVOO PE containing low to undetectable amounts of lignans, our data clearly demonstrated that lignans-enriched EVOO varieties had a relatively weak ability to alter cell viability in the SKBR3 breast cancer model. Thus, the cytotoxic potency of the lignans-negative EVOO-PE 7 (Picual variety from Córdoba) was found to be 12 times higher than that observed in lignans-enriched EVOO-PE 12 (Arbequina variety from Reus). It should be noted, however, that PE exhibiting small differences in their secoiridoid content notably differed in their abilities to significantly decrease breast cancer cell viability. These findings, altogether, strongly suggest that quality rather than quantity of the entire battery of complex phenols present in individual EVOO-PE ultimately dictate their antibreast cancer cytotoxic effects. In this regard, because the cytotoxic effects of complex PE mixtures were not the algebraic sum of their main phenolic fractions, which were earlier reported to induced significant cytotoxic effects on their own (*10*, *11*), our current findings definitely support the notion that active phenolics may have not only additive but also synergic effects on physiological functions related to breast cancer cell survival. When considering that the presence of significant amounts of lignans directly related to a loss of breast cancer cytotoxic effects in EVOO-PE, we cannot exclude the possibility that antagonistic/protective cytotoxic interactions could take place also at the molecular level between EVOO complex phenols.

Although these experimental studies support the hypothesis of EVOO-derived complex phenols as breast cancer inhibiting compounds, forthcoming studies assessing the in vivo accessibility of EVOO phenolics to tumor tissues should be performed before suggesting that anticancer activity of EVOO-derived complex phenols should be expected from their direct local effects on the breast cancer tissues. In this regard, we should acknowledge that in vitro studies on biocompounds should always consider intestinal absorption and biotransformation. Unfortunately, the knowledge available on the metabolic fate of EVOO-derived complex phenols is still scarce. While absorption and bioavailability studies have revealed that tyrosol and hydroxyltyrosol can be retrieved in plasma and urine after olive oil consumption (*28*), there is an urgent need of data regarding the plasma/urine concentration of the free forms of various secoiridoid aglycones. Indeed, it is reasonable to suggest that limited bioavailability of EVOO-derived complex polyphenols and their conversion into less-active metabolites (e.g., glucuronidated or sulfated forms) could significantly affect their antibreast cancer potential in vivo. Conversely, it has been suggested that the unabsorbed fraction of EVOO-derived lignans such as pinoresinol can be used by intestinal flora to produce the mammalian lignans enterodiol and enterolactone, which have been shown to reduce invasion in breast cancer cell lines (*29*). Although enrichment with the lignans fraction closely related to lower breast cancer cytotoxic activities as assessed by MTT-based cell viability assays in vitro, caution must be applied when trying to extrapolate in vitro results into clinical practice because dietary lignans have been repeatedly related with reduction of breast cancer risk (*30*). Moreover, methylation by catechol-*O*-methyltransferase (COMT), which has been described in vitro and in animal studies regarding the polyphenol (−)-epigallocatechin-3-gallate [EGCG] (*31*), is a potential effect that could significantly alter the potent cytotoxic effects of secoiridoid aglycones in vitro against breast carcinomas in vivo. Experiments are currently underway in our laboratory to evaluate whether methylation of major EVOO-derived complex phenols may occur in breast cancer cells due to cytosolic COMT and whether cellular uptake and COMT-related metabolism may relate to the intrinsic responsiveness of breast cancer cells to EVOO phenolics. Because it has been recently established that methylation significantly decreases the anticarcinogenic activity of EGCG, thus providing a molecular explanation to epidemiological studies showing a significantly decrease in breast cancer risk only among those tea drinkers possessing at least one low-activity COMT allele (*32*), future EVOO-based intervention studies might benefit from the evaluation of interindividual variations in the methylation of EVOO-derived phenolics as well as from the notion that COMT inhibition may significantly increase the antibreast cancer properties of naturally occurring polyphenols (*33*).

In summary, the proposed RRLC-ESI-TOF-MS method, with the highest efficiency in the chromatographic separation of secoiridoids and their derivates, lignans, and flavones, followed by MTT-based cell viability protocol, might offer for the first time an easy, rapid, and objective manner not only to classify EVOO based on their phenolic profile but to identify further naturally occurring biophenols with potential antibreast cancer value.

ABBREVIATIONS USED

Apig, apigenin; D-Lig Agl, decarboxymethyl ligstroside aglycon; DOA, decarboxymethyl oleuropein aglycon; EA, elenolic acid; EVOO, extra virgin olive oil; EVOO-PE, extra virgin olive oil phenolic extract; HYTY, hydroxytyrosol; hydroxy D-ligstroside aglycon, hydroxy decarboxymethyl ligstroside aglycon; hydroxy D-oleuropein aglycon, hydroxy decarboxymethyl oleuropein aglycon; LDA, linear discriminant analysis; Lig Agl, ligstroside aglycon; Lut, luteolin; methyl D-oleuropein aglycon, methyl decarboxy oleuropein aglycon; MTT, metabolic status assessment; Ole, oleuropein; Ol Agl, oleuropein aglycon; Pin, (+)-pinoresinol; Ty, tyrosol.

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