Characterization and quantification of phenolic compounds of extra-virgin olive oils with anticancer properties by a rapid and resolutive LC-ESI-TOF MS method

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ABSTRACT

The characterization and quantification of extra-virgin olive oil (EVOO) phenolic compounds by a rapid resolution liquid chromatography (RRLC) method coupled to diode-array and time of flight mass spectrometry (TOF) detection systems was developed. The RRLC method transferred from a conventional HPLC one achieved better performance with shorter analysis times. The phenolic compounds were separated with a C18 column (150 mm × 4.6 mm, 1.8 μm) using water with 0.5% acetic acid and acetonitrile as mobile phases. Good peak resolution was obtained and 19 different phenols were identified in less than 20 min providing a new level of information about the samples in shorter time. The applicability of this analytical approach was confirmed by the successful analysis of three different EVOO varieties (Picual, Hojiblanca, and Arbequina) obtained from different trademarks. Besides identification of the most important phenolic compounds and their quantification in three different ways (RRLC-UV, RRLC-MS and a new approach using the total polyphenol content obtained with Folin Ciocalteau, the relative areas and the response factors), we also described the occurrence of correlations between the phenolic composition of EVOO-derived crude phenolic extracts and their anti-proliferative abilities toward human breast cancer-derived cell lines. When compared with lignans-rich EVOO varieties, secoiridoids-rich EVOO had a significantly strong ability to alter cell viability in four different types of human breast carcinoma cells.

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1. Introduction

For centuries, Mediterranean people have appreciated the nutritional, medical and cosmetics benefits of olive oil. Nowadays, a growing number of evidences point to the important role that extra-virgin olive oil (EVOO) plays as a crucial ingredient of the Mediterranean diet regarding their beneficial effects on health [1]. The hypothesis that minor components such as phenolic compounds could play a major role in the healthy effects of EVOO – including the prevention of chronic diseases such as cancer, obesity, diabetes, or coronary diseases – has gradually been increasing and several studies have attempted to elucidate the ultimate mechanisms through which EVOO-derived phenols might contribute to these healthy properties [2–8]. On the other hand, phenolic compounds also affect the organoleptic properties (flavour, astringency, . . .) [9–11] and oxidative stability of EVOO [12,13]. Considering the importance of this class of analytes, it would be very interesting to develop fast and powerful analytical methods for the characterization and quantification of this important family of EVOO compounds.

The development of methodologies for the determination of phenols in EVOO has been discussed extensively in literature. Due to the need to carry out an individual identification of each phenolic compound present in the extracts, the traditional methods

were replaced with separative techniques [*e.g.* gas chromatography (GC) [14], high-performance liquid chromatography (HPLC) [15,16], and capillary electrophoresis (CE) [17] coupled to different detectors [18]. However, most of the discussions have been focused on the optimization of high-performance liquid chromatography (HPLC) methods, mainly with reversed phase C18 columns and different mobile phases and gradients, followed by ultraviolet (UV) [19], electrochemical [20], fluorescence [21] or mass spectrometric (MS) detection [17,22]. Recently, an improvement in chromatographic performance has been achieved by the introduction of rapid-resolution LC (RRLC) and ultra-performance LC (UPLC) [23]. These approaches use narrow-bore columns packed with very small particles (1.8 μm) and high flow rate with delivery systems operating at high back-pressures. The major advantages of RRLC over conventional HPLC are improved resolution, shorter retention times, higher sensitivity, and better performance. Coupling RRLC with MS further offers a potent analytical alternative, which has been applied in recent publications characterizing food products [24–28].

Our first goal herein was to develop a RRLC-ESI-TOF MS-based alternative method for the rapid identification and quantitation of the most representative phenolic compounds present in different EVOOs. Quantitative or semi-quantitative information on olive oil phenols is of great interest to find the compounds responsible of the olive oil benefits and to distinguish EVOOs with different anti-cancer properties. Pure reference standards for each analyte are required to get an accurate quantitation based on the construction of calibration curves; however, due to the lack of commercial standards, only a few compounds have been quantified in this way and different approaches have been followed trying to quantify as many compounds as possible in this complex matrix: considering external standards with similar structure or even with structure completely different to the compound under study and using internal standards added to the extract that we wanted to analyze. One of the limitations of these approaches is that the relative response of the different compounds in mass spectrometry (and also in other detection systems) is very sensitive to the variations in chemical structure and the error in the quantification may be high. In the current work, we propose a new approach for direct and reliable quantitation of olive oil phenolic compounds taking into account the total polyphenol content determined by Folin Ciocalteau method and the response factors of the phenolic compounds in MS. The quantitative results were compared with those obtained using RRLC-UV and RRLC-ESI-TOF MS.

On the other hand, in previous studies we have demonstrated that individual phenolic fractions obtained from a 50/50 mixture of two commercial EVOO samples induced cytotoxic effects toward cultured human breast cancer cells [4,5,7]. Importantly, EVOO polyphenols (*i.e.* lignans and secoiridoids) – but not monophenols and phenolic acids – strongly suppressed the growth of breast cancer cells bearing high levels of HER2 (*erb*B2) – one of the most commonly analyzed oncogenes that plays a decisive role in malignant transformation, tumorigenesis and metastasis in a biologically aggressive subset of human breast carcinomas. However, it is necessary to consider that because of the biological effects of phenolic compounds – including tumoricidal 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 phenolics.

Therefore the second aim of this study was to investigate the anti-breast cancer effects of whole crude EVOO phenolic extracts containing significantly different phenolic compositions and to determine the relationship between the chemical nature and/or the concentration of individual phenolic compounds and the ability of each crude EVOO phenolic extract to decrease breast cancer cell viability.

2. Materials and methods

2.1. Chemicals and samples

Methanol and *n*-hexane of HPLC grade used for the extraction of the phenolic compounds from the olive oil samples were supplied from Panreac (Barcelona, Spain). Acetonitrile from Lab-Scan (Dublin, Ireland) and acetic acid from Panreac (Barcelona, Spain) were used for preparing mobile phase. Water was deionized by using a Milli-Q-system (Millipore, Bedford, MA, USA).

Standards of hydroxytyrosol, tyrosol, luteolin and apigenin were purchased by Sigma–Aldrich (St. Louis, MO, USA) and (+)-pinoresinol was acquired from Arbo Nova (Turku, Finland). Other phenolic compounds used as pure standard samples, elenolic acid and ligstroside aglycon, were isolated from EVOOs by semipreparative HPLC [29]. Stock solutions at concentration of 500 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.

Eight EVOO samples used for the study were acquired from a supermarket (Granada, Spain). EVOOs of three different olive fruit varieties so-called Picual, Hojiblanca and Arbequina and from different trademarks (Carbonell, Borges, Hojiblanca and Coosur) were chosen for the analysis. As it is considered in literature EVOOs are relatively constant in terms of lipid composition, but the micronutrient contents (*e.g.* α-tocopherol, carotenoids, sterols and phenolic compounds) significantly vary based upon the localization of cultivation, climate, olive variety and production techniques [30,31].

2.2. Breast cancer cell lines and culture conditions

MCF-7 and SKBR3 breast cancer cells were obtained from the American Type Culture Collection (ATCC) and they were routinely grown in Improved MEM supplemented with 5% fetal bovine serum (FBS) and 2 mM ʟ-glutamine. Construction of pBABE/HER2, retroviral infection of MCF-7 and stable selection of MCF-7/HER2 cells were performed as described elsewhere. JIMT-1 cells were established at Tampere University and are available from the German Collection of Microorganisms and Cell Cultures (http://www.dsmz.de/). JIMT-1 cells were grown in F-12/DEMEM (1:1) supplemented with 10% FBS and 2 mM ʟ-glutamine. Cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

2.3. Sample extraction

Specific solid phase extraction (SPE) method with Diol-cartridges, previously described elsewhere [32], was used with the aim of obtaining the major number of phenolic compounds at the highest concentration from each EVOO matrix. Briefly, the extraction consisted of passing through a column, previously conditioned with 10 mL of methanol and 10 mL of hexane, 60 g of EVOO dissolved in 60 mL of hexane. After removing the non-polar fraction with 15 ml of hexane, the phenolic compounds were recovered with methanol (40 ml). The final volume was dried in a rotary evaporator under reduced pressure at 35 °C and the residue was reconstituted in 2 mL of methanol.

2.4. Determination of total phenols

Total phenol content of different EVOO extracts was determined using Folin-Ciocalteu technique [33]. Briefly, 50 μL of the 1:10 diluted methanolic extracts of EVOOs was assayed with 250 μL of Folin reagent and 500 μL of saturated solution of sodium carbonate. The mixture was diluted with water to a final volume of
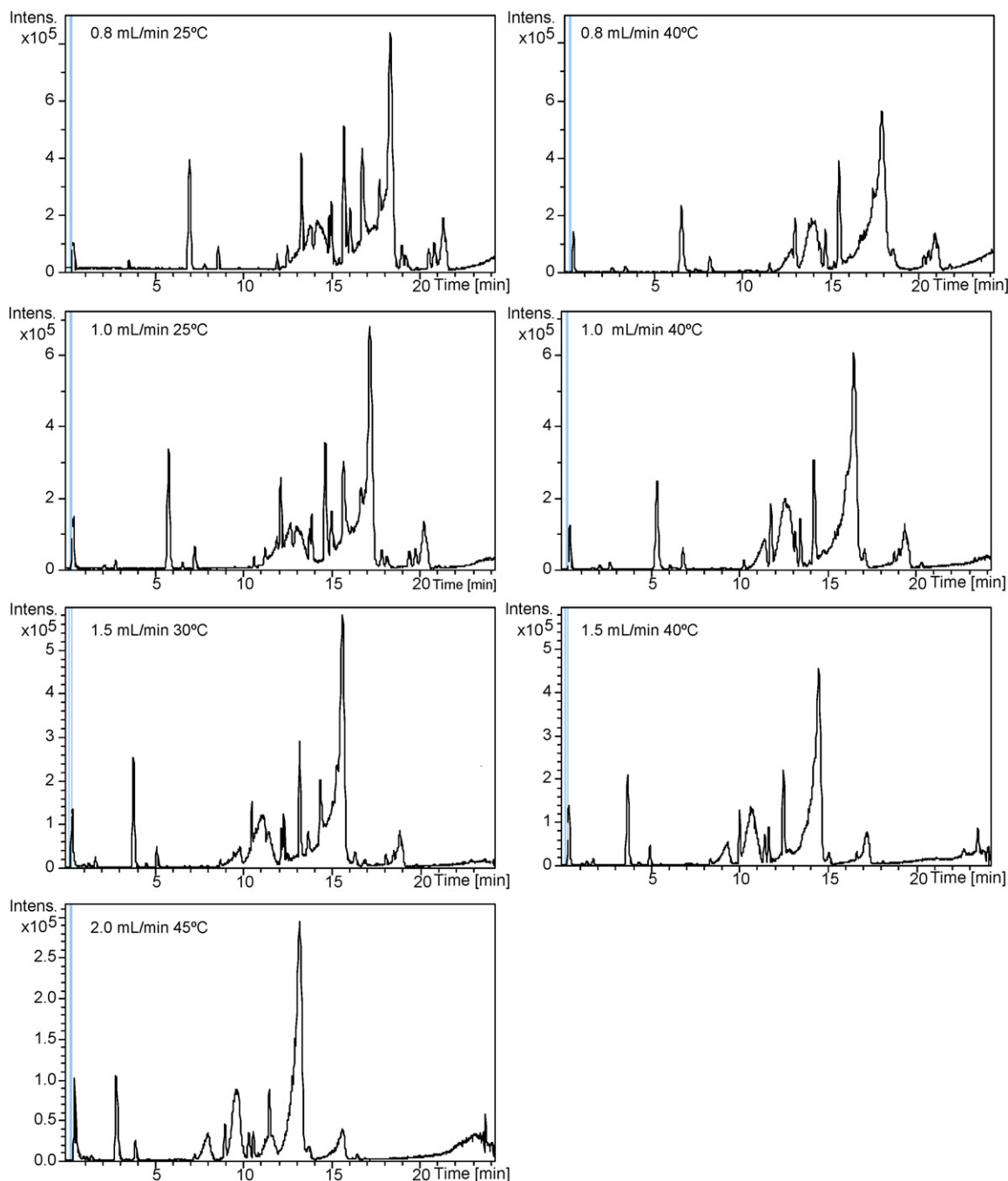
Fig. 1. RRLC-ESI-TOF Base peak chromatograms (BPC) of an olive oil extract (Picual Borges) using different mobile phase flow rates and temperatures. The rest of chromatographic conditions were those described in Section 2, the analytical column was a C_{18} Zorbax column, 1.8 μm i.d., 4.6 mm × 150 mm and the mobile phase: water + 0.5% acetic acid (A) and ACN (B).

5 mL. The absorbance relative to that of the blank was measured using Spectronic Genesys 5 (Rochester, NY, USA) at 725 nm after incubation for 1 h at room temperature. The total polyphenol content was expressed as mg/kg olive oil of caffeic acid. For the caffeic acid, the curve absorbance versus concentrations is described by the equation $y = 28.773x − 0.2104$ ($R^2 = 0.998$).

2.5. Rapid resolution liquid chromatography analyses

An Agilent 1200-RRLC system (Agilent Technologies, Waldbronn, Germany) equipped with a vacuum degasser, autosampler, a binary pump and a UV–vis detector was used for the chromatographic determination. Polyphenolic compounds were separated by using a Zorbax C18 analytical column (4.6 × 150 mm, 1.8 μm particle size) protected by a guard cartridge of the same packing, operating at 30 °C and a flow rate of 1.5 mL/min. The mobile phases used were water with acetic acid (0.5%) (Phase A) and acetonitrile (Phase B) and the solvent gradient changed according to the following conditions: 0–10 min, 5–30% B; 10–12 min, 30–33% B; 12–17 min, 33–38% B; 17–20 min, 38–50% B; 20–23 min, 50–95% B. Finally, the B content was decreased to the initial conditions (5%) in 2 min and the column re-equilibrated for 10 min. A volume of 10 μL of the 1:10 diluted methanolic extracts of olive oil was injected. The compounds separated were monitored in sequence first with DAD (240 and 280 nm) and then with a mass spectrometry detector.
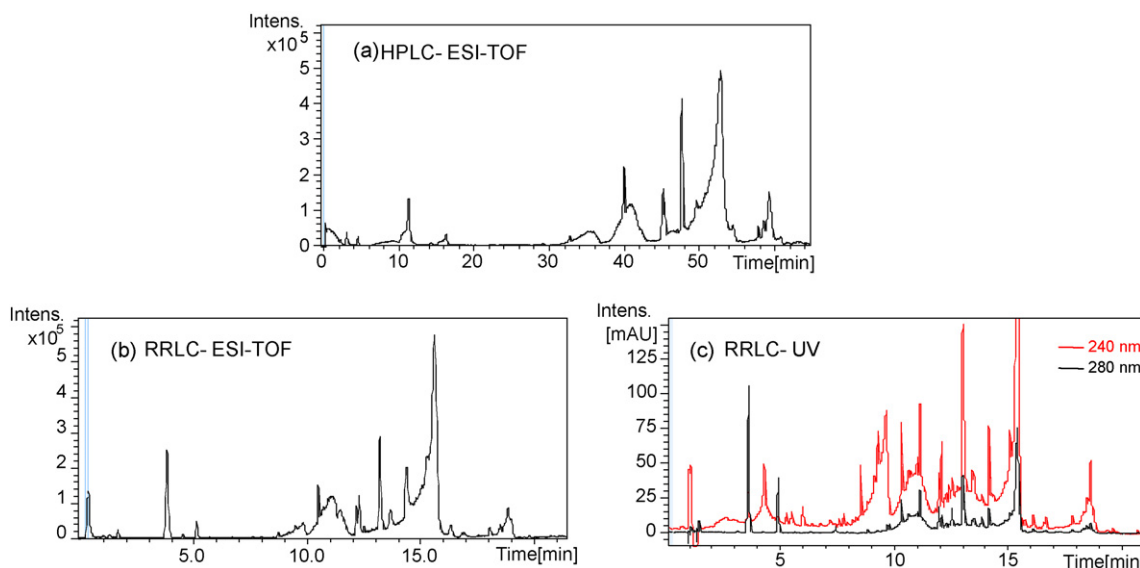
Fig. 2. Comparison of the chromatograms obtained with the previous HPLC-ESI-TOF method (a) and the new optimized RRLC method with both detectors: TOF (b) and UV (280 and 240 nm) (c). Chromatographic conditions were described in Section 2.

2.6. Mass spectrometry

The RRLC system was coupled to a Bruker Daltonik microTOF mass spectrometer (Bruker Daltonik, Bremen, Germany) using an orthogonal electrospray interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA). TOF analyzers provide greatly improved mass resolution (8000–10,000 at 250 m/z) and significantly higher sensitivity and accuracy when acquiring full-fragment spectra compared with traditional instruments. The parameters of the mass spectrometer were similar to those previously optimized in recent works with the same EVOO matrix [29] acquiring spectra in the range of 50–800 m/z in the negative mode. The flow rate used in the new RRLC method 1.5 ml/min was too high for achieving a stable electrospray ionization (ESI) (maximum flow rate is around 1 mL/min), therefore it was necessary to use a flow divisor 1:6. In that way, the flow delivered into the mass spectrometer was reduced to 0.21 mL/min, low enough to avoid the introduction of humidity in the system. According to this inflow the ESI parameters were chosen: nebulizer pressure was set at 2 bar, dry gas flow 9 L/min and dry gas temperature 190 °C.

SmartFormula™ tool within DataAnalysis was used for the calculation of elemental composition of compounds; it lists and rates possible molecular formulas consistent with the accurate mass measurement and the true isotopic pattern (TIP). If the given mass accuracy leads to multiple possible formulas, the TIP adds a second dimension to the analysis, using the masses and intensities of each isotope to do a sophisticated comparison of the theoretical with the measured isotope pattern (SigmaValue™). The smaller the sigma value and the error the better the fit, therefore for routine screening an error of 5 ppm and a threshold sigma value of 0.05 are generally considered appropriate.

2.7. Metabolic status assessment (MTT-based cell viability assays)

Breast cancer cells were seeded at a density of 3000 cells per well in a 96-well plate. The next day, cells were treated with concentrations ranging from 0% to 0.1% (v/v) of crude EVOO phenolic extracts dissolved in ethanol. An appropriate amount of ethanol (v/v) was also added to control cells. After 5 days of treatment (extracts were not renewed during the entire period of cell exposure), the cells

Table 1
Main phenolic compounds identified in an olive oil extract (Picual Borges) by RRLC-ESI-TOF including: retention time, ISCID (Internal source collision induced dissociation), fragments, m/z experimental and calculated, molecular formula, error and sigma.

Compounds	Retention time (min)	Fragments	m/z experimental	m/z calculated	Molecular formula	Error	Sigma
Hydroxytyrosol	3.9	123	153.0559	153.0557	$C_8H_{10}O_3$	−0.9	0.013
Tyrosol	5.2		137.0606	137.0608	$C_8H_9O_2$	1.7	0.059
Hydroxy elenolic acid	8.7	181, 137	257.0651	257.0667	$C_{11}H_{13}O_7$	6.0	0.026
Hydroxytyrosol acetate	9.0		195.0670	195.0662	$C_{10}H_{11}O_4$	−3.8	0.036
Elenolic acid	9.8	139	241.0713	241.0718	$C_{11}H_{13}O_6$	2.0	0.042
Hydroxy D-oleuropein aglycon	10.5	199	335.1150	335.1136	$C_{17}H_{19}O_7$	−4.2	0.053
Decarboxymethyl oleuropein aglycon	11.1	183	319.1190	319.1187	$C_{17}H_{19}O_6$	−1.0	0.031
Luteolin	12.1		285.0407	285.0405	$C_{15}H_9O_6$	−0.8	0.006
Syringaresinol	12.2		417.1537	417.1555	$C_{22}H_{25}O_8$	4.3	0.034
Hydroxy D-ligstroside aglycon	12.3	199	319.1202	319.1187	$C_{17}H_{19}O_6$	−4.6	0.045
Pinoresinol	12.7		357.1347	357.1344	$C_{20}H_{21}O_6$	−0.9	0.017
Acetoxypinoresinol	13.1		415.1389	415.1398	$C_{22}H_{24}O_8$	0.9	0.013
10-Hydroxy oleuropein aglycon	13.2		393.1205	393.1191	$C_{19}H_{21}O_9$	−3.5	0.044
Decarboxymethyl ligstroside aglycon	13.3	183	303.1245	303.1238	$C_{17}H_{19}O_5$	−2.4	0.014
Apigenin	14.0		269.0452	269.0455	$C_{15}H_9O_5$	1.4	0.030
Methyl D-oleuropein aglycon	15.2		333.1357	333.1344	$C_{18}H_{21}O_6$	−3.9	0.052
Oleuropein aglycon	15.6	345, 307, 275	377.1253	377.1242	$C_{19}H_{21}O_8$	−2.8	0.044
Methyl oleuropein aglycon	18.5	345, 275	391.1405	391.1398	$C_{20}H_{23}O_8$	−1.8	0.079
Ligstroside aglycon	18.7	291, 241	361.1303	361.1293	$C_{19}H_{21}O_7$	−2.8	0.046

Table 2
Analytical parameters for the RRLC-ESI-TOF and RRLC-UV methods: relative standard deviation (RSD%), limit of detection (LOD) and quantitation (LOQ), linearity, calibration curves and r^2.

Analytes	RSD%	LOD (μg/ml)	LQ (μg/ml)	Linearity (μg/ml)	Calibration curves	r^2
Hyty						
UV	1.4	0.15	0.50	LQ–100	$y = 6.533x + 11.177$	0.996
TOF	4.6	0.09	0.30	LQ–50	$y = 39934x + 42004$	0.993
Ty						
UV	1.5	0.35	0.66	LQ–100	$y = 4.197x + 2.417$	0.999
TOF	2.1	0.31	1.03	LQ–50	$y = 12596x + 26635$	0.991
EA						
UV	1.1	3.50	11.67	LQ–300	$y = 10.665x - 25.26$	0.998
TOF	3.4	1.44	4.80	LQ–300	$y = 6688x + 76261$	0.991
Pin						
UV	1.8	0.08	0.26	LQ–100	$y = 5.632x + 5.094$	0.999
TOF	3.3	0.06	0.20	LQ–50	$y = 37578x + 53556$	0.991
Lut						
UV	0.8	0.04	0.13	LQ–100	$y = 11.131x + 7.926$	0.999
TOF	2.8	0.02	0.06	LQ–25	$y = 114566x + 59826$	0.994
Apig						
UV	0.8	0.04	0.13	LQ–100	$y = 17.292x + 4.996$	0.999
TOF	2.0	0.02	0.06	LQ–25	$y = 150131x + 118916$	0.991
Lig Agl						
UV	1.6	1.50	5.00	LQ–100	$y = 0.853x + 3.851$	0.991
TOF	3.0	0.43	1.43	LQ–300	$y = 9019x + 59184$	0.993

Every compound was quantified in UV at 280 nm except EA at 240 nm.
Hyty: hydroxytyrosol; Ty: tyrosol; EA: elenolic acid; Pin: pinoresinol; Lut: luteolin; Apig: apigenin; Lig Agl: ligstroside aglycon.

Table 3
Quantitative results (mg/kg) achieved by RRLC-UV, RRLC-ESI-TOF MS and the approach using the results obtained with Folin Ciocalteau method, the relative areas and the response factors.

Olive oils	Hyty	Ty	EA	Lut	Pin	Apig	Lig Agl
P. Carbonell							
UV	11.23 (a)	10.98 (a)	62.73 (a)	2.94 (a)	5.26 (a)	0.93 (a)	45.54 (a)
TOF	10.67 (a)	9.24 (b)	48.15 (b)	3.28 (a)	1.54 (b)	1.00 (a)	48.30 (a)
RF	9.25 (b)	7.89 (c)	47.81 (b)	3.45 (a)	1.80 (b)	1.28 (a)	47.67 (a)
P. Borges							
UV	22.37 (a)	12.43 (a)	78.34 (a)	1.59 (a)	3.37 (a)	0.47 (a)	51.01 (a)
TOF	20.20 (a)	11.84 (a)	68.18 (b)	1.84 (b)	0.77 (b)	0.43 (a)	64.78 (b)
RF	17.90 (a)	10.31 (a)	67.48 (b)	2.11 (b)	1.22 (b)	0.74 (a)	65.83 (b)
A. Carbonell							
UV	4.32 (a)	3.84 (a)	27.17 (a)	4.27 (a)	5.61 (a)	1.34 (a)	6.08 (a)
TOF	3.64 (a)	2.74 (b)	8.61 (b)	4.17 (a)	2.56 (b)	1.56 (a)	6.98 (a)
RF	3.80 (a)	3.22 (b)	13.17 (b)	4.82 (a)	2.95 (b)	1.83 (a)	9.96 (b)
A. Borges							
UV	4.02 (a)	2.78 (a)	31.12 (a)	4.28 (a)	5.54 (a)	1.24 (a)	9.91(a)
TOF	3.37 (b)	2.33 (a)	10.47 (b)	4.41 (a)	2.24 (b)	1.22 (a)	17.38 (b)
RF	3.37 (b)	2.72 (a)	12.26 (b)	4.84 (a)	2.52 (b)	1.57 (a)	19.77 (b)
H. Borges							
UV	10.25 (a)	7.41 (a)	56.67 (a)	3.28 (a)	5.98 (a)	1.13 (a)	33.29 (a)
TOF	9.76 (a)	6.56 (a)	33.00 (b)	3.47 (a)	2.26 (b)	0.99 (a)	38.23 (a,b)
RF	9.85 (a)	6.76 (a)	38.85 (b)	3.73 (a)	2.41 (b)	1.26 (a)	44.37 (b)
H. Hojiblanca							
UV	5.87 (a)	6.28 (a)	67.6 (a)	4.17 (a)	3.31(a)	2.27 (a)	26.78 (a)
TOF	5.21 (a)	5.35 (a)	43.47 (b)	4.15 (a)	0.68 (b)	2.51 (a)	32.20 (a,b)
RF	5.50 (a)	5.71 (a)	46.92 (b)	5.03 (a)	1.02 (b)	3.12 (b)	38.03 (b)
H. Carbonell							
UV	10.30 (a)	6.25 (a)	67.74 (a)	5.64 (a)	5.34 (a)	2.07 (a)	38.82 (a)
TOF	9.51 (a)	5.62 (a)	50.46 (b)	5.75 (a)	1.13 (b)	1.57 (a)	50.73 (b)
RF	8.83 (a)	5.44 (a)	52.18 (b)	6.29 (a)	1.56 (b)	1.95 (a)	53.22 (b)
P. Coosur							
UV	7.73 (a)	7.15 (a)	50.18 (a)	1.77 (a)	6.42 (a)	0.51 (a)	58.04 (a)
TOF	7.08 (a)	6.76 (a)	31.62 (b)	1.82 (a)	1.57 (b)	0.49 (a)	48.86 (a)
RF	6.95 (a)	6.63 (a)	35.79 (b)	2.12 (a)	2.03 (b)	0.64 (a)	53.48 (a)

P: Picual; A: Arbequina; H: Hojiblanca.
Means in the same table cell with different letters are significantly different ($p \leq 0.05$).

were incubated with a solution of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma, St. Louis, MO, USA) at a concentration of 5 mg/mL for 3 h at 37 °C. The supernatants were then carefully aspirated, 100 μL of DMSO was added to each well, and the plates were agitated to dissolve the crystal product. Absorbances were read at 570 nm using a multi-well plate reader (Model Anthos Labtec 2010 1.7 reader). The cell viability effects from the exposure of cells to each crude EVOO phenolic extract were analyzed as percentages of the control cell absorbances. For each treatment, cell viability was evaluated as a percentage using the following equation: (A_{570} of treated sample/A_{570} of untreated samples) × 100. Breast cancer cell sensitivity to crude EVOO phenolic extracts was expressed in terms of the concentration of extract (v/v) required to decrease by 50% cell viability (IC_{50} value). Since the percentage of control absorbance was considered to be the surviving fraction of cells, the IC_{50} values were defined as the concentration of extracts that produced 50% reduction in control absorbance (by interpolation).
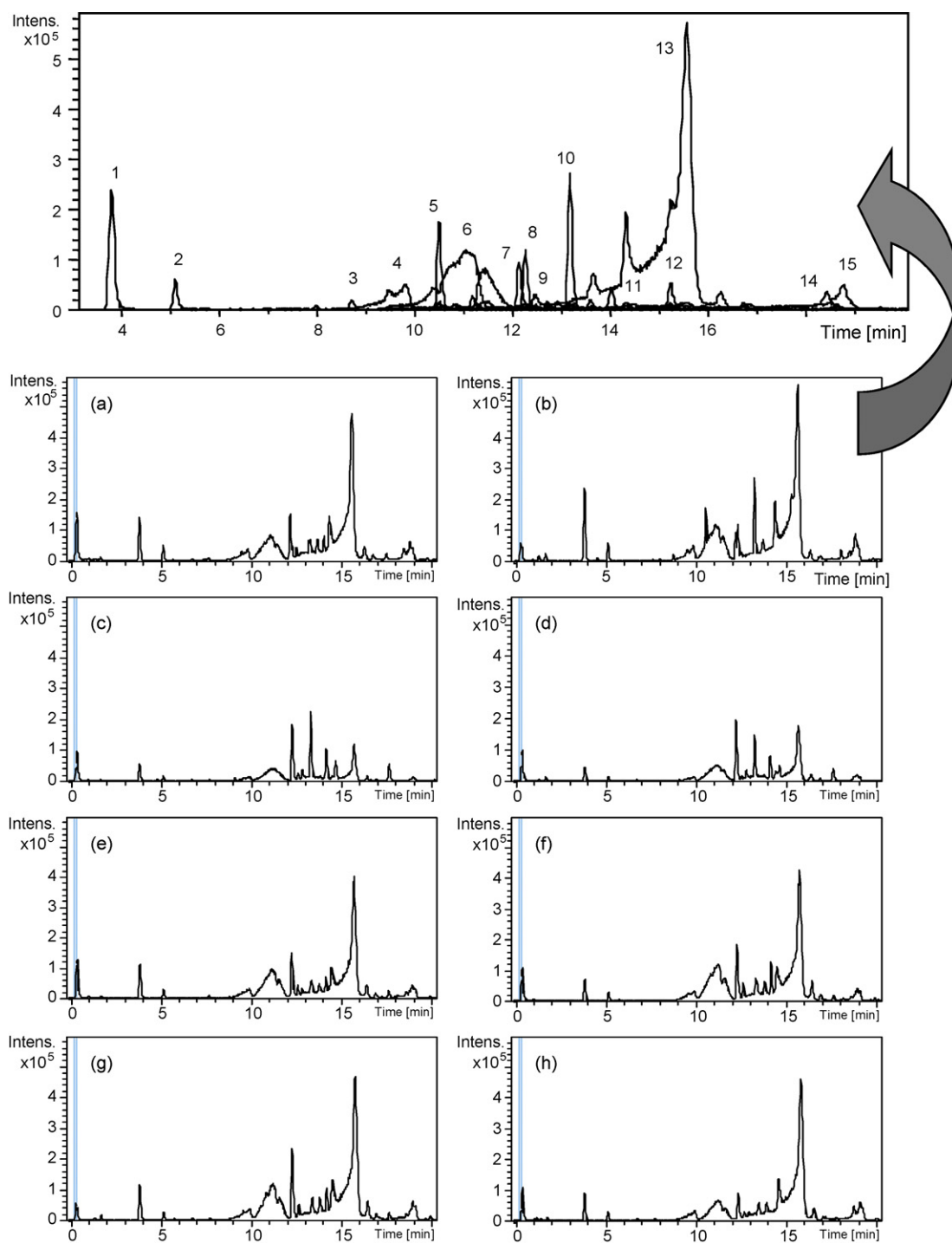
Fig. 3. (A) Extracted ion chromatograms (EICs) of the main phenolic compounds identified in the olive oil extract (Picual Borges) using the optimized RRLC-ESI-TOF method. Peak identification: 1, Hyty; 2, Ty; 3, H-EA; 4, EA; 5, H-DOA; 6, DOA; 7,Lut; 8, H-D-Lig Agl; 9, Pin; 10, H-Ol Agl; 11, Apig; 12, Methyl-DOA, 13, Ol Agl; 14, Methyl-Ol Agl; 15, Lig Agl. (B) Base peak chromatograms (BPCs) of the eight extra-virgin olive oils chosen for this study. (a) Picual Carbonell; (b) Picual Borges; (c) Arbequina Carbonell; (d) Arbequina Borges; (e) Hojiblanca Borges; (f) Hojiblanca Hojiblanca; (g) Hojiblanca Carbonell; (h) Picual Coosur.

Table 4
Response factors (RFs) of the seven phenols standards determined with ESI-TOF MS. RFs are expressed relative to Hyty, which is set to 1.00.

Compounds	Slope (a)	RFs
Hyty	39,934	1
Ty	12,596	3.17
EA	6,688	5.97
Pin	37,578	1.06
Lut	114,566	0.35
Apig	151,131	0.26
Lig Agl	9,019	4.43

For ESI-MS detection, a and b values are coefficients of the linear calibration dependence $y = ax + b$ and RFs are calculated as RF (phenolic compounds) $= a_{Hyty}/a_{phenolic\,compound}$.

2.8. Statistics

As far as phenolic compounds are concerned, the results reported in this study are the averages of at least three repetitions ($n = 3$), unless otherwise stated. Tukey's honest significant difference multiple comparison (one-way ANOVA) and Pearson's linear correlations, both at $p < 0.05$, were evaluated using Statistica 6.0 (2001, StatSoft, Tulsa, OK). Data were also analyzed by Multivariate Exploratory Techniques in particular factor analysis and principal components and classification analysis (using Statistica 6.0) to evidence the correlation between the determined analytes and the differences in the samples. The analytical data were arranged in a matrix with the rows corresponding to the samples (objects) and the columns corresponding to the analytical indices (variables).

Results from breast cancer cell viability assays are expressed as the mean of three independent experiments. For each independent experiment, three replicate determinations were performed and a mean value was calculated. A two-way analysis of variance model, including the effects for cell line, dose and interaction between cell line and dose, was used to evaluate the relationship between cell viability and those effects.

3. Results and discussion

3.1. Development of a RRLC method

Typically, the separation of complex EVOO samples has required longer run time, in some cases more than 60 min, using conventional HPLC methods (column packed with 5 μm particles) [12,19,20]. The introduction of a new kind of chromatography RRLC allows working with columns of very small particle size (1.8 μm) and high flow withstanding high pressures. In this way, reducing the particle size the efficiency of the column increases and the resolution becomes independent of analysis time. Working with the flow and temperature we can get shorter analysis time and a maximum resolution between peaks. Shorter run times mean less peak capacity; therefore users should choose a balance between peak capacity and run time [34–36].

Based on the chromatographic conditions of a previous HPLC method used so far in our research group (Gemini C18 column: 3 mm × 250 mm, 5 μm particle size) [12], the optimization of a new RRLC method was carried out (Column Zorbax C18: 4.6 mm × 150 mm and 1.8 μm particle size). Length was reduced roughly by half in order to obtain faster analyses, maintaining a diameter of 4.6 mm, and a small column particle size was chosen to increase the efficiency of the separation with an excellent time of life and resistant to high pressures. The gradient, injection volume, flow rate, column temperature and dilution of the sample were optimized, following the general rules for the conversion of a HPLC method to RRLC method.

As a starting point for optimization, maintaining the composition of the mobile phase (phase A: H_2O + 0.5% acetic acid, phase B: ACN) and other variables from the original HPLC method (0.5 mL/min at 25 °C) different gradients were tested, changing the gradient slope until no significant reduction in resolution was observed. The optimum gradient was: from 5 to 30% B in 10 min, from 30 to 33% B in 2 min, from 33 to 38% B in 5 min, from 38 to

Table 5
Total polyphenol content (TPC) expressed as caffeic acid equivalents (Folin Ciocalteau method) and concentrations (mg/kg) of 19 phenolic compounds identified in eight EVOO samples using the combination of the total polyphenol content, the area percentage of each phenol in the total area of the chromatogram and the response factors of each particular phenol.

	P. Carbonell	P. Borges	A. Carbonell	A. Borges	H. Borges	H. Hojiblanca	H. Carbonell	P. Coosur
TPC (mg/kg caffeic acid)	189.39	260.98	102.89	109.61	190.2	230.6	219.5	169.8
Compounds								
Hyty	9.25 (a)	17.90 (b)	3.80 (c)	3.37 (c)	9.85 (a)	5.50 (d)	8.83 (a)	6.95 (e)
Ty	7.89 (a)	10.31 (b)	3.22 (c)	2.72 (d)	6.76 (e)	5.71 (f)	5.44 (f)	6.63 (e)
H-EA[a]	0.92 (a,b)	5.09 (c)	0.33 (b,d)	0.28 (d)	1.21 (a)	1.10 (a)	1.05 (a)	0.61 (b)
Hyty-Acet[b]	0.30 (a,b)	0.14 (b)	0.72 (c)	0.35 (a)	0.30 (a,b)	0.37 (a)	0.48 (a)	0.44 (a)
EA	47.81 (a)	67.48 (b)	13.17 (c)	12.26 (c)	38.85 (d)	46.92 (a,e)	52.18 (e)	35.79 (d)
H-DOA[c]	9.30 (a)	41.51 (b)	3.79 (c)	3.56 (c)	13.66 (d)	19.22 (e)	13.65 (d)	5.64 (f)
DOA[c]	192.33 (a)	301.44 (b)	111.01 (c)	127.46 (d)	254.25 (e)	352.34 (f)	295.45 (b)	169.14 (g)
Lut	3.45 (a)	2.11 (b)	4.82 (c)	4.84 (c)	3.73 (d)	5.03 (c)	6.29 (e)	2.12 (b)
Syri[d]	0.78 (a)	0.29 (b)	1.82 (d)	1.31 (e)	0.63 (a,c)	0.68 (a,c)	0.61 (a,c)	0.47 (c,b)
H-D-Lig Agl[c]	11.83 (a)	31.14 (b)	3.95 (c)	3.57 (c)	9.00 (d)	9.45 (d)	8.60 (d,e)	7.53 (e)
Pin	1.80 (a)	1.22 (b,c)	2.95 (d)	2.52 (d)	2.41 (d)	1.02 (c)	1.56 (a,b)	2.03 (a)
Ac Pin[d]	2.05 (a,b)	0.99 (c)	16.32 (d)	10.90 (e)	1.55 (a,c)	5.77 (f)	2.88 (b)	0.88 (c)
H-Ol Agl[c]	17.42 (a)	74.43 (b)	1.76 (c)	3.06 (c)	15.72 (a,d)	12.09 (e)	12.78 (d,e)	10.57 (e)
D-Lig Agl[c]	33.37 (a)	29.48 (b)	16.72 (c)	18.73 (c)	26.77 (b,d)	24.94 (d)	27.24 (b,d)	29.82 (b)
Methyl DOA[c]	5.69 (a)	6.91 (b)	1.40 (c)	2.32 (d)	4.54 (e)	5.19 (f)	5.62 (a)	4.45 (e)
Apig	1.28 (a)	0.74 (b)	1.83 (c)	1.57 (a)	1.26 (a)	3.12 (d)	1.95 (c)	0.64 (b)
Ol Agl[c]	310.00 (a)	366.43 (b)	64.45 (c)	107.93 (d)	261.36 (e)	315.05 (a)	306.45 (a)	305.71 (a)
Methyl Ol Agl[c]	24.93 (a)	22.22 (a)	2.78 (b)	2.91 (b)	17.22 (c)	11.65 (d)	14.87 (c,d)	34.96 (e)
Lig Agl	47.67 (a)	65.83 (b)	9.96 (c)	19.77 (d)	44.37 (a)	38.03 (e)	53.22 (f)	53.48 (f)

P: Picual; A: Arbequina; H: Hojiblanca. Means in the same row with different letters are significantly different ($p \leq 0.05$).

[a] Quantified by using response factor of EA.
[b] Quantified by using response factor of Hyty.
[c] Quantified by using response factor of Lig Agl.
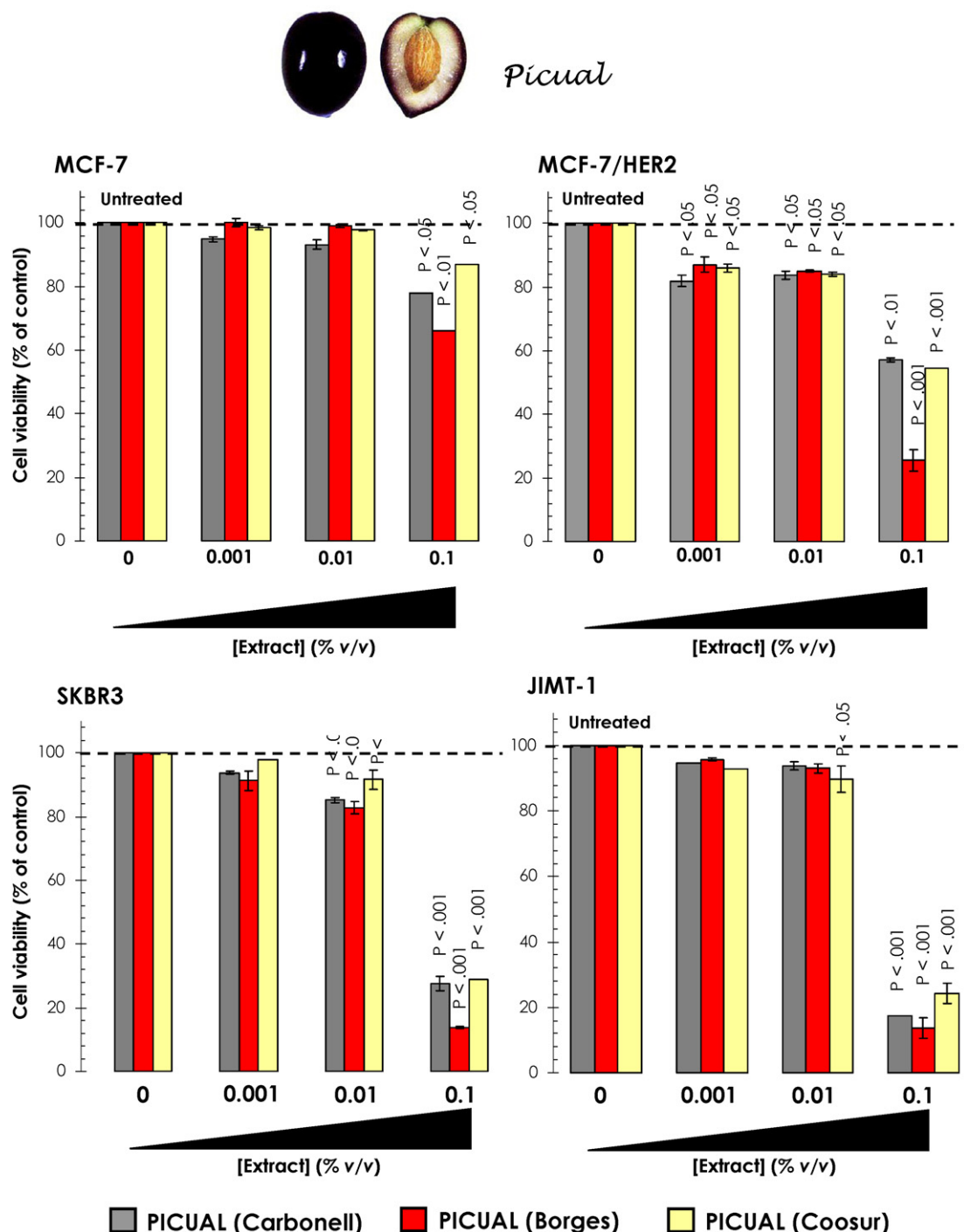[d] Quantified by using response factor of Pin.

Fig. 4. Antiproliferative effects of crude EVOO phenolic extracts (PICUAL variety) in human breast cancer cell lines. Cell viability (MTT assay) in MCF-7, MCF-7/HER2, SKBR3 and JIMT-1 cells after 5 days treatment with crude EVOO phenolic extracts obtained from the Picual variety. Cell viability is expressed as a ratio of the absorbance between treated cells and untreated control (=100% cell viability). Each point is a mean (*columns*) ± SE (*bars*) of three independent experiments performed in triplicate. Statistically significant differences are labeled.

50% B in 3 min, and from 50 to 95% B in 3 min; then the percentage of B was reduced again to 5% B in 2 min and we kept the initial conditions for 10 min. With the optimized gradient the runtime could already be reduced by 40%. In order to increase the resolution between peaks the injected volume was reduced from 20 to 10 μL.

In the next step, the flow rate was increased from 0.5 to 2.0 mL/min (passing through the following steps: 0.5, 0.8, 1.0, 1.5, 2.0 mL/min). With increasing flow rates the back-pressure of the system rises until to reach the maximum value (80–90% of the

pressure accepted by the chromatograph). However, if the temperature of the column also increases (25, 30, 35, 40 °C), the viscosity of the mobile phase decreases and the system back-pressure is reduced. Choosing a suitable temperature the flow could be further increased up to the maximum value. With acetonitrile as organic mobile phase the maximum flow rate until the maximum system back-pressure is reached is higher than for methanol because its viscosity is lower. In Fig. 1 we can observe that increasing the flow and the temperature shortened the run time without compromising too much resolution, but with temperatures above 40 °C there
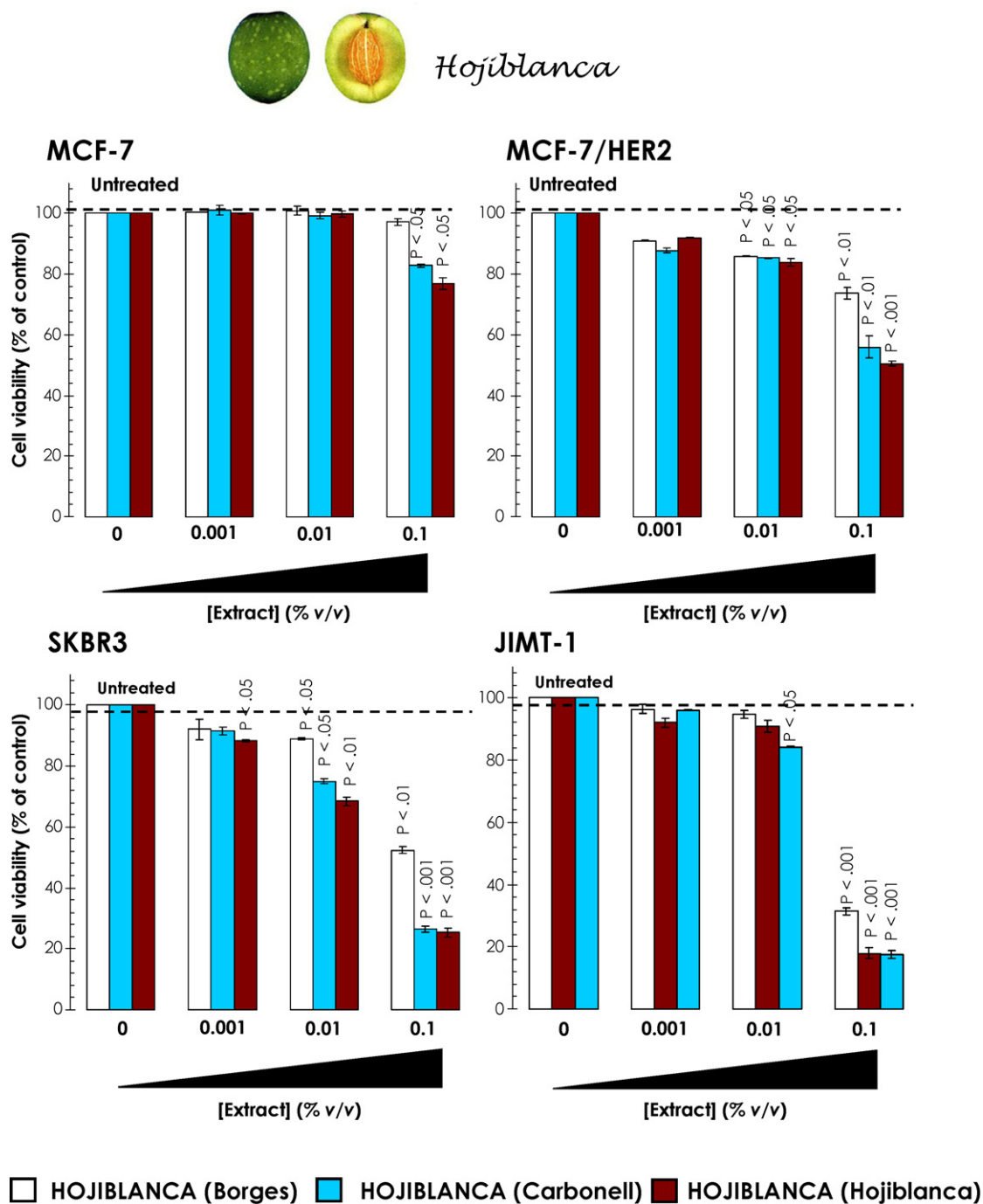
Fig. 5. Antiproliferative effects of crude EVOO phenolic extracts (HOJIBLANCA variety) in human breast cancer cell lines. Cell viability (MTT assay) in MCF-7, MCF-7/HER2, SKBR3 and JIMT-1 cells after 5 days treatment with crude EVOO phenolic extracts obtained from the Hojiblanca variety. Cell viability is expressed as a ratio of the absorbance between treated cells and untreated control (=100% cell viability). Each point is a mean (*columns*) ± SE (*bars*) of three independent experiments performed in triplicate. Statistically significant differences are labeled.

was overlapping of some peaks and loss of compounds. Finally, an optimum flow of 1.5 mL/min and temperature of 30 °C, maintained by a column thermostat, were selected since they ensure that the system pressure is not exceeded even later at the end of the run. The maximum pressure reached during this analysis was approximately 450 bar. As described in the experimental section this flow rate is too high for electrospray (ESI) so, it is necessary to use a splitter 1:6. The detection was carried out apart from mass spectrometry (TOF) with UV at two wavelength characteristics of the phenolic compounds of interest, 280 and 240 nm.

Fig. 2 shows the chromatograms of the same EVOO sample (Picual Borges) analyzed using the conventional HPLC and the RRLC method. With the optimum RRLC method the run time could be reduced from 60 min (Fig. 2a) to 20 min (Fig. 2b and c) and the analyst could achieve even better performance by using that method. This was achieved by using steeper gradients, increased temperature and higher flow rates. The peaks in Fig. 2b were narrower than those in Fig. 2a what means that the peak capacity increased. In general the results with the RRLC method were superior to those with the corresponding HPLC method providing better analysis time, separation and resolution.

3.2. Identification of EVOO phenolic compounds

Once analytical conditions for separation and detection were optimized, the RRLC-ESI-TOF method was used to identify and quantify the phenolic profile of eight commercial EVOOs. Peak identification was done by comparing both migration time and MS spectral data obtained from olive oil samples and standards (commercial standards or isolated compounds by HPLC); we also used the information previously reported [17,22,29] and the information

provides with time of flight analyzer (TOF) by the mass spectrometer. TOF MS instrumentation with excellent mass resolution and mass accuracy in combination with true isotopic pattern is the perfect choice for molecular formula determination of small molecules using the editor SmartFormula^{TM}. For routine screening practice, a SigmaFit^{TM} tolerance of 0.05 and a mass tolerance of 5 ppm were chosen. Table 1 summarizes the main compounds identified in the Picual Borges variety including the information generated by TOF analyzer: retention time; product ions obtained spontaneously in
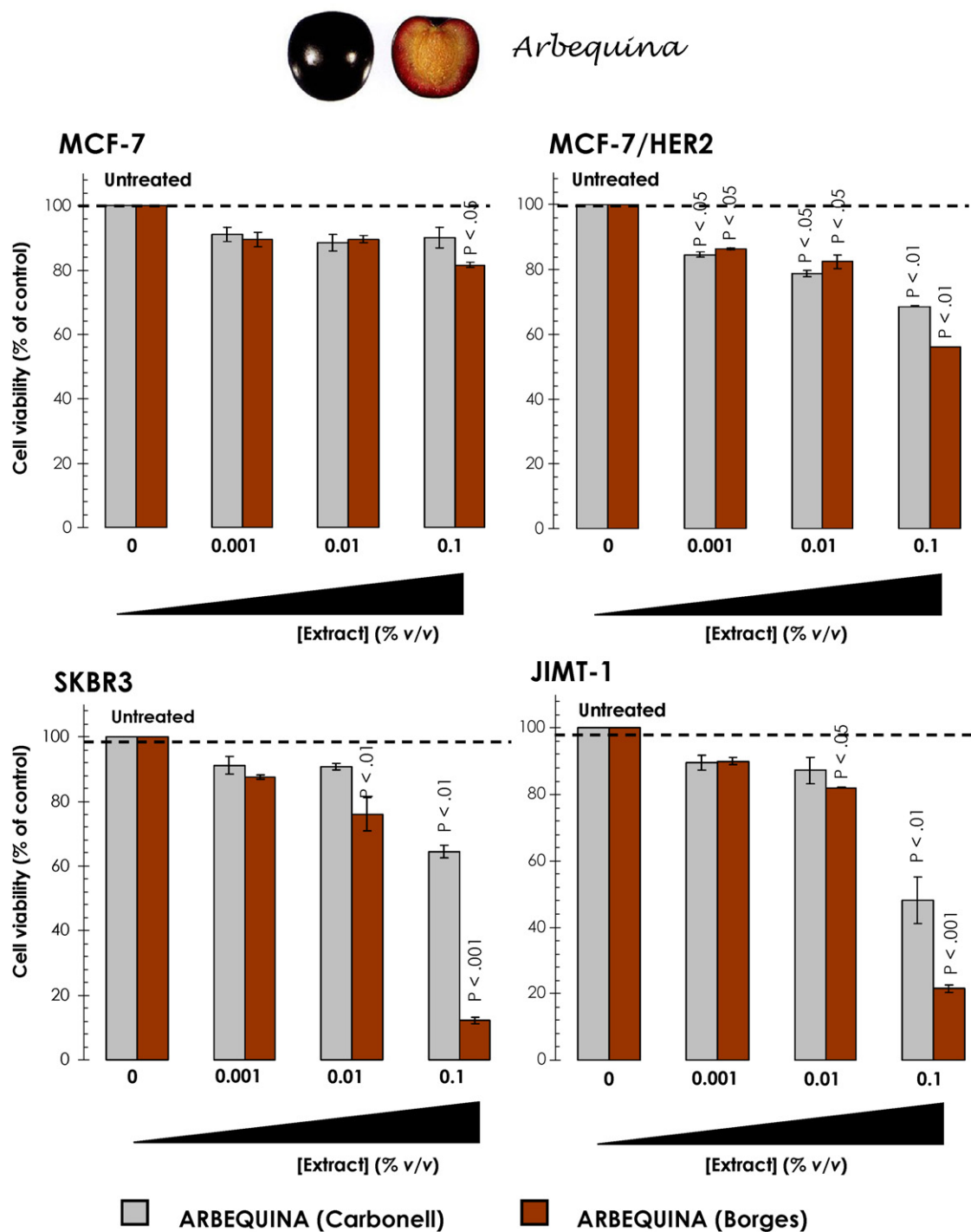
Fig. 6. Antiproliferative effects of crude EVOO phenolic extracts (ARBEQUINA variety) in human breast cancer cell lines. Cell viability (MTT assay) in MCF-7, MCF-7/HER2, SKBR3 and JIMT-1 cells after 5 days treatment with crude EVOO phenolic extracts obtained from the Arbequina variety. Cell viability is expressed as a ratio of the absorbance between treated cells and untreated control (=100% cell viability). Each point is a mean (*columns*) ± SE (*bars*) of three independent experiments performed in triplicate. Statistically significant differences are labeled.

the ionization source, m/z experimental and calculated, molecular formula, error and sigma value.

Finally, 19 phenolic compounds from different families (simple phenols, flavonoids, lignans and secoiridoids) were unequivocally identified. Fig. 3 represents the extracted ion chromatograms (EICs) of the main phenolic compounds identified in the Picual Borges variety and the base peak chromatograms (BPCs) obtained by RRLC-ESI-TOF for the eight EVOOs in the optimum conditions.

3.3. Quantification

3.3.1. Calibration curves

The quantification of seven phenols in the different EVOOs was carried out by both UV and ESI-TOF MS using commercially available standards and other pure standards previously isolated by semipreparative HPLC. Calibration curves were obtained using ten-point ($n = 3$) curves of each compound. Linear regression analysis using the least-square method was used to evaluate the MS and UV responses of each analyte as a function of its concentration. The responses fitted well to a straight line with r^2 values higher than 0.991 for both detectors. The limits of detection and quantification of the individual analytes in standard solutions were obtained by injecting diluted standard and were calculated according to the IUPAC method [37]. LODs were slightly better with MS detector in the range of 0.02–1.44 μg/ml. The linearity of the method was studied by injecting standard solutions in the range from 1 to 300 ppm obtaining less linearity responses in the mass spectrometer mainly because the degree of ionization in the ion source decreases when the amount of ions increases. Method precision based on within-day repeatability and expressed as relative standard deviation (RSD%), was estimated by measuring the peak areas of the different standards and, as it was expected, the repeatability was slightly better in UV. Table 2 shows the analytical parameters which enabled to evaluate the method performance: repeatability (% RSD), limits of detection (LOD) and quantification (LOQ), linearity, calibration curves and regression coefficient (r^2).

The described method was successfully applied to quantify these phenolic compounds in eight samples of different kinds of EVOOs. The phenolic compounds concentration was determined using the area of each individual compound (three replicates) and by interpolation in the corresponding calibration curve. Table 3 presents the content of the individual phenolic compounds found in commercial olive oils from RRLC-UV and RRLC-ESI-TOF MS. The results were statistically the same with both detectors for Hyty, Ty, Lut and Apig since no overlapping was detected in the zone of the chromatogram where these compounds were eluted. Opposite situation was found for Pin, EA and Lig Agl (in some cases), where some peaks could elute with the same retention time and create interferences in UV, making the quantification inaccurate by UV detector.

3.3.2. Folin Ciocalteu and response factor (RF)

In order to find a rapid and effective approach to quantify the largest number of olive oil phenolic compounds, a combination between the results obtained with the Folin Ciocalteau method and the percentage of each phenolic compound in the total area of the BPC in MS was developed by using the response factors (RFs) calculated for each compound.

Total polyphenol content (TPC) was analyzed according to the Folin Ciocalteau method and the results were given as caffeic acid equivalents. As a first approach, the content of each compound could be easily calculated without the necessity of calibration curves taking into account the percentage of each compound with respect to the total chromatogram area and the total polyphenol content calculated with the Folin Ciocalteau method. However, the main problem of this approach is that the mass spectrometry response for phenols changes significantly, mainly due to the dif-

ferent behaviour of compounds with variations in their chemical structure during the nebulization. Some parameters relating to the electrospray nebulizer (nebulizer pressure, gas flow rate, temperature, . . .) have a notable effect on RFs. This leads to systematic errors in the quantitation based on the relative peak areas. For this reason, a suitable approach for the quantitation of complex mixtures with a limited range of authentic standards is to use response factors (RFs).

We firstly had to look for an appropriate compound which could be our reference. To establish the mentioned reference compound, we compared the quantitation obtained with the calibration curves for the seven available standards and the one calculated directly with the total phenol content and the percentages of individual components. Finally, Hyty was chosen as reference because of its similar results with both ways of quantitation and its RF was set to 1. Other RFs were expressed in terms of this standard compound (Hyty) and linear concentration responses were calculated with the ratio of calibration slopes a(Hyty)/a(other phenolic compounds). RFs for the seven standards with respect to Hyty are shown in Table 4. As it can be observed some similarities can be found among the compounds which belong to the same family. So, EA and Lig Agl had very low responses, probably due to a poor electrospray nebulization. Better nebulization was shown for flavonoides with a very high response. The mass spectrometry response of pinoresinol was similar to that one of Hyty.

Finally, to calculate the real concentration of individual phenolic compounds in olive oil samples, the total polyphenol content is multiplied by the individual peak area percentage and then by the corresponding RF. The results obtained in this way for the seven standards are shown in Table 3 and, as it can be observed, are statistically the same as the previously found results achieved by using the calibration curves with RRLC-ESI-TOF MS. Due to the good results obtained with the new approach the quantitation of the other phenolic compounds identified in the eight EVOOs was carried out. In Table 5 we include the total polyphenol content as caffeic acid equivalents of the eight analyzed samples and the concentrations of the 19 phenols identified were calculated by using TPC, area percentages of each compound and response factors. The response factors of the other phenols which were not available as commercial standards were calculated assuming similar responses for compounds of the same family and with slight differences in their molecular structure.

Compared with other methods previously described in literature where external or internal standards are used, the proposed method using response factors together with total polyphenol content provides correct and faster quantitative results. The TPC and the calibration curve of the hyty are enough to quantify all the phenolic compounds using the response factors.

3.4. Inhibitory effects of crude EVOO phenolic extracts on proliferation in human breast cancer cells in vitro

Cell viability was evaluated by the MTT assay. First, we compared the anti-proliferative effects of crude EVOO phenolic extracts using two *in vitro* breast cancer cell models: MCF-7 breast cancer cells – which express physiological levels of HER2 (*i.e.* one single copy of HER2 gene) – and MCF-7/HER2 cells—an MCF-7 derived model engineered to overexpress HER2 gene (~70-fold increase in HER2 oncoprotein expression when compared to MCF-7 parental cells). Cells were treated with a series of ethanolic dilutions that were made by diluting full strength (100%) EVOO phenolic extracts. At concentrations ranging from 0.001 to 0.1%, HER2-negative MCF-7 cells were mostly unresponsive to all the crude EVOO phenolic extracts tested (Figs. 4–6). However, a completely different picture emerged when Picual-, Hojiblanca- and Arbequina-derived crude EVOO phenolic extracts were tested on

Table 6
Effects of crude EVOO phenolic extracts on breast cancer cell viability.

	Picual (Carbonell)	Picual (Borges)	Picual (Coosur)	Hojiblanca (Carbonell)	Hojiblanca (Borges)	Hojiblanca (Hojiblanca)	Arbequina (Carbonell)	Arbequina (Borges)
MCF-7	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A	N.A
MCF-7/HER2	0.11	0.062	0.105	0.106	N.A.	0.102	N.A.	0.106
SKBR3	0.063	0.052	0.070	0.055	0.102	0.044	0.110	0.045
JIMT-1	0.061	0.059	0.064	0.055	0.069	0.060	0.073	0.056

The metabolic status of MCF-7, MCF-7/HER2, SKBR3, and JIMT-1 breast cancer cells cultured in the absence or presence of crude EVOO phenolic extracts was evaluated using a MTT-based cell viability assay as described in Section 2. Upon construction of dose–response curves, IC_{50} values (*i.e.* the concentration of each extract needed to reduce cell viability by 50% relative to untreated control cells) were calculated by interpolation. Values are means (in %, v/v) from three independent experiments made in triplicate. N.A.: not available (*i.e.* >0.150%, v/v).

their growth inhibitory activities against MCF-7/HER2 cells. Thus, MCF-7 cells stably overexpressing high levels of the human HER2 oncogene became significantly more sensitive to crude EVOO phenolic extracts in terms of decreased cell proliferation. Importantly, the inhibition of viability in MCF-7/HER2 cells was significantly more pronounced in the presence of Picual-derived crude phenolic extracts than in the presence of Arbequina-derived crude phenolic extracts.

To further evaluate whether crude EVOO phenolic extracts from Picual variety preferentially exhibited tumoricidal effects against HER2-overexpressing breast cancer cells, we explored the anti-proliferative effects of crude EVOO phenolic extracts in SKBR3 and JIMT-1, two human breast cancer-derived cell lines naturally exhibiting HER2 overexpression. SKBR3 cells represent a widely used breast cancer *in vitro* model characterized by naturally bearing HER2 gene amplification and HER2 protein overexpression. HER2-dependency for cell proliferation and survival is reflected by the fact that SKBR3 cells are highly sensitive to anti-HER2 therapies, including the anti-HER2 monoclonal antibody trastuzumab and small molecule HER2 tyrosine kinase inhibitors (TKIs). HER2-overexpressing JIMT-1 cells, however, provide a valuable experimental model for the studies of resistance to HER2 targeting therapies, as they are insensitive to trastuzumab and other HER2-inhibiting drugs including HER2 TKIs. When tested against SKBR3

Fig. 7. (A) Projection of the variables on the factor-plane (PC1 × PC2) considering the 19 variables (phenolic compounds quantified in the current study). (B) Score plot for the two principal components showing the eight EVOO samples studied. The position of each sample depends on the variables shown in Section (A) of the current figure.

Fig. 8. Correlation between phenolic composition of crude EVOO extracts, cell viability effects, and HER2 oncogene status in human breast cancer cells.

cells, crude EVOO phenolic extracts exhibited the following anti-proliferative potencies: Picual > Hojiblanca > Arbequina (Figs. 4–6). Crude EVOO phenolic extracts failed to decrease JIMT-1 cell viability in a concentration-dependent manner. Intriguingly, exogenous supplementation with 0.1% (v/v) of Picual- and Hojiblanca-derived crude EVOO phenolic extracts drastically reduced JIMT-1 cell viability (>80% reduction; Figs. 4–6). This cytotoxic effect was less pronounced when JIMT-1 cells were cultured in the presence of crude EVOO phenolic extracts from the Arbequina variety.

3.5. Relationship between crude EVOO extracts' phenolic composition and anti-breast cancer activity

The above-mentioned findings strongly suggested that: (a) HER2-overexpressing cancer cells display exacerbated growth inhibitory responses when treated with crude EVOO phenolic extracts; (b) the anti-proliferative effects shown by crude EVOO extracts might relate to their different phenolic compositions (Table 6).

To evaluate the possibility of differentiating the samples taking into account the phenolic fraction, we applied a multivariate statistical analysis for the results of the RRLC-ESI-TOF MS analyses of the quantitative-phenolic profile. All the phenolic compounds quantified were considered to identify the two principal factors. Nineteen variables were selected for the PCA and the explained variance was higher than 80%. Fig. 7a represents the projection of the variables with regard to the single factor (PC1 or PC2) on the factor plane (PC1 × PC2). We can observe that each quadrant contains, at least, one of the variables. A map of samples (score plot) for the two principal components is shown in Fig. 7b. Extra-virgin olive oils made from Picual, Hojiblanca and Arbequina olives were shown to be quite different to the others, since the samples belonging to each family lied in different zones of the plot. The variables which were more decisive to discriminate among varieties were Ol Agl, Methyl-Ol Agl, DOA, Apig, Lut, Ac Pin and Syri; a finding which is in very good agreement with Carrasco-Pancorbo et al. [38]. The concentration of Ac Pin in the Arbequina extra-virgin olive oils under study was extremely high if we compare the oils from that variety with those from Picual and Hojiblanca. Some authors evidenced that fact previously; for example, Brenes et al. [39] observed the low Ac Pin content in Spanish EVOOs produced from Picual cultivar. Flavonoids, in general, were found at higher concentrations in Hojiblanca and Arbequina than in the Picual EVOOs analyzed. H. Hojiblanca was the richest variety concerning Apig and H. Carbonell, the richest in terms of Lut, a fact that could explain their position in the score plot. Picual EVOOs were found in a different quadrant of the plot, due to their high concentration in terms of secoiridoids (DOA, methyl-Ol Agl and Ol Agl).

However, we think it is important to stand out that our main aim was not only to discriminate among the analyzed samples, it was more to find out the phenolic compounds which could help to distinguish the samples. In that way, we could contribute to clarify what the phenols more responsible of the antiproliferative effect of the extracts from oil are.

Indeed, when we graphically represented EVOO varieties as a gradient of their major polyphenolic fractions, the anti-proliferative effects shown by crude EVOO phenolic extracts can be attributed to their enrichment in specific polyphenol classes (Fig. 8). Thus, crude EVOO phenolic extracts rich in lignans (i.e. Arbequina variety) appear to induce mostly cytostatic effects whereas secoiridoids-rich crude EVOO phenolic extracts (i.e. Picual variety) can be mostly related to strong cytotoxic effects. Moreover, our results further showed a close correlation between the ability of crude EVOO phenolic extracts to decrease cell viability and the expression status of HER2 in breast cancer cells (i.e. the effects on the cell viability were significantly more pronounced in the HER2-positive breast

cancer cell lines than in HER2-negative cells). These findings, altogether, strongly suggest that the anti-proliferative effects shown by the phenolic compounds contained in three different varieties of EVOO may be attributed to the ability of specific phenolics to block HER2-dependent breast cancer cell viability. These findings support previous reports from our group showing a more pronounced anti-HER2 activity of individual secoiridoids (e.g. oleuropein aglycon, ligstroside aglycon) when compared to that of individual lignans (e.g. pinoresinol). They also suggest that the apparent HER2-related anti-breast cancer properties of crude EVOO phenolic extracts may result from antagonistic/synergistic properties of its individual phenolic compounds against HER2 activity and/or expression.

4. Conclusions

To the best of our knowledge, we report for the first time an easy, fast and effective RRLC-ESI-TOF MS method for the characterization/quantification of EVOOs of which we have demonstrated their potential anticancer value. Using columns of very small particle diameter (1.8 μm) and higher flows the analysis time was reduced to less than 20 min, without compromising chromatographic quality, with good resolution and reproducibility. Eight olive oils were analyzed and the quantification of the main phenolic compounds was developed in three ways, finding a new approach using the combination of the total polyphenol content, the area percentage of each phenol in the total area of the chromatogram and the response factors of each particular phenol. When coupled to the occurrence of correlations between the phenolic composition of EVOO-derived crude phenolic extracts and their anti-proliferative abilities toward human breast cancer-derived cell lines, this novel methodological approach may enable a rapid and objective identification of EVOO with potential anti-cancer value. When compared with lignans-rich EVOO varieties, secoiridoids-rich EVOOs had a significantly strong ability to alter cell viability in four different types of human breast carcinoma cells.

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