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Uptake and metabolism of olive oil polyphenols in human breast cancer cells using nano-liquid chromatography coupled to electrospray ionization–time of flight-mass spectrometry

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ABSTRACT

Polyphenols from extra virgin olive oil (EVOO), a main component of the Mediterranean diet, have demonstrated repeatedly anti-tumor activity in several *in vitro* and *in vivo* studies. However, little is known about the efficiency of the absorption process and metabolic conversion of these compounds at cellular level. In this study, a nano liquid chromatography–electrospray ionization–time of flight mass spectrometry (nanoLC–ESI–TOF MS) method was developed to study the cellular uptake and metabolism of olive oil phenols in JIMT-1 human breast cancer cells. After incubation for different time periods with EVOO-derived phenolic extracts, culture media, cytosolic fraction and solid particles fraction were separated and analyzed. Most of the free phenols, mainly hydroxytyrosol, its secoiridoid derivatives, and the flavonoid luteolin, disappeared in the culture media in different ways and at different times. Besides, several metabolites were detected in the culture media, fact that may indicate absorption and intracellular metabolism followed by rapid cellular export. Low intracellular accumulation was observed with only traces of some compounds detected in the cytosolic and solid particles fractions. Methylated conjugates were the major metabolites detected, suggesting a catalytic action of catechol-O-methyl transferase (COMT) in cancer cells.

1. Introduction

Several studies have reported that the consumption of olive oil has a potential protective effect against several malignancies, especially in relation to colon and breast cancer [1–4]. The beneficial effects of olive oil are related not only to its high content in the monounsaturated fatty acid (MUFA) oleic acid [5–7] but also to the presence of a nonsaponificable fraction which contains biologically active phenolic compounds [8–10]. The main families of phenolic compounds in olive oil are: simple phenols, lignans, flavonoids and secoiridoids [11]. A large body of evidence indicates that polyphenols can exert chemopreventive effects against different cancers and our research group and some others have explored the anti-tumor activity of olive oil polyphenols. These *in vitro* studies mainly involved incubations of different types of human cancer cell lines (*e.g.*, colon, prostate, leukemia, and breast) with individual polyphenols or whole olive oil phenolic extracts [12–16]. These studies have suggested that olive oil polyphenols are capable of significantly affecting the overall process of carcinogenesis due to their abilities to inhibit cell cycle, cell proliferation or oxidative stress, improve the efficacy of detoxification enzymes, induce apoptosis, and stimulate the immune system. We have recently assessed the effects of olive oil polyphenols, supplemented individually or in combination, in different human breast cancer lines naturally exhibiting clinically relevant molecular markers. We have revealed that olive oil phenolics, especially those fractions enriched in lignans and secoiridoids, had a strong ability to decrease breast cancer cell viability by promoting an efficient blockade in the activity and expression of the tyrosine kinase receptor HER2 (*erb*B-2), one of the most commonly analyzed proto-oncogenes in human cancer studies [17–19].

Abbreviations: BPC, base peak chromatogram; COMT, catechol-O-methyl transferase; D-Lig Agl, deacetoxy ligstroside aglycon; DOA, deacetoxy oleuropein aglycon; EIC, extracted ion chromatogram; EVOO, extra virgin olive oil; Lig Agl, ligstroside aglycon; nanoLC–ESI–TOF MS, nano liquid chromatography–electrospray ionization–time of flight mass spectrometry; Ol Agl, oleuropein aglycon; RSD, relative standard deviation.

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Despite the strong experimental evidence suggesting that olive oil-derived polyphenols exhibit antitumor activity, the molecular mechanism(s) underlying both the uptake and the metabolism of these compounds still remains to be definitely elucidated. This prompted us to study the efficiency of absorption and metabolic conversion of different families of phenolic compounds in human breast cancer cells. Similar works have been recently developed with other cell types: Caco-2 cells (model system of the human intestinal epithelium) [20,21] and HepG2 (model system of the human liver) [22] to investigate the metabolism of these compounds at both the intestinal and hepatic level. Limited metabolism of olive oil phenolics was observed using Caco-2 cells and the methylated conjugates were the major metabolites detected. HepG2 cells showed extensive uptake and metabolism mainly as glucuronide and methylated derivatives.

A nano liquid chromatography coupled to electrospray ionization–time of flight mass spectrometry (nanoLC–ESI–TOF MS) method was developed to carry out the analyses. This technique has emerged as a powerful analytical tool, providing a wide number of important applications, especially in proteomics and also in fields such as pharmaceutical, environmental and enantiomeric analysis [23] and, in less extent, in food analysis [24,25]. We have recently studied its potential in the determination and quantification of polyphenols from olive oil samples [26]. This technique offered several advantages over classical analytical methods, highlighting its high sensitivity (when techniques to load large samples volumes are used) what becomes it in a very promising alternative for the analysis of samples with low concentration of analytes, as for instance biological samples. However, its potential in the metabolomic field has not been fully explored and to the best of our knowledge there are few works using nanoLC for the analysis of small bio-active molecules (*i.e.*, polyphenols) in biological samples (*i.e.*, human breast cancer cells).

The aim of this work was to study the uptake and metabolism of olive oil phenolic compounds in JIMT-1 human breast cancer cells using, for the first time in such an application, nanoLC–ESI–TOF MS.

2. Material and methods

2.1. Materials

All chemicals were of analytical reagent grade. For the extraction procedure methanol, hexane and ethanol were purchased from Panreac (Barcelona, Spain), hydrochloric acid from Scharlau (Barcelona, Spain) and ethyl acetate from Lab-Scan (Dublin Ireland). Acetonitrile from Lab-Scan (Dublin, Ireland) and acetic acid from Fluka (Buchs, Switzerland) were used for preparing mobile phase. Water was deionized by using a Milli-Q-system (Millipore, Bedford, MA, USA). Culture medium and fetal bovine sure were purchased from Sigma–Aldrich (St. Louis, MO, USA). EVOO sample of the Cornezuelo variety used for the incubation with cancer cells was obtained from CTAEX.

2.2. Extraction of phenolic compounds from virgin olive oil

The extraction procedure was based on a specific solid phase extraction (SPE) method with Diol-cartridges, previously described in literature [27]. Briefly, 60 g of EVOO were dissolved in 60 ml of hexane and passed through a column, previously conditioned with 10 ml of methanol and 10 ml of hexane. After removing the non-polar fraction with 15 ml of hexane, the phenolic compounds were eluted with methanol (40 ml). The final volume was dried in a rotary evaporator under reduced pressure at 35 °C and the residue was dissolved in 2 ml of methanol. For the supplementation to the cells, 1 ml of the methanolic extract was evaporated and reconstituted in 125 μl of ethanol.

2.3. Human breast cancer cell line and culture conditions

We took advantage of the *HER2* gene-amplified JIMT-1 cell line originally established from a ductal carcinoma pleural metastasis of a 62-year-old patient, who did not respond to HER2-targeted therapies [28,29]. JIMT-1 human breast cancer cell line was established at Tampere University and is available from the German Collection of Microorganisms and Cell Cultures (http://www.dsmz.de/). JIMT-1 cells were grown in F-12/DMEM (1:1) supplemented with 10% FBS and 2 mM L-glutamine. Cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Cells were screened periodically for *Mycoplasma* contamination.

When the cells reached 75–80% confluence were washed twice with serum-free medium and incubated overnight upon serum-free conditions. Cells were then stimulated to grow in low-serum (0.1% FBS)-containing medium in the presence of 0.01% of olive oil extracts dissolved in ethanol and were incubated for 0, 15 min, 30 min, 1 h, 2 h, 6 h and 24 h. Two control experiments were prepared strictly in parallel: culture medium with cells in the absence of olive oil extracts and culture medium containing only olive oil extract in the absence of cells.

At each of such time points, the supernatants were collected, centrifuged at 1000 × *g*, aliquoted, and immediately stored at −80 °C until use. Cells were rinsed with cold phosphate-buffered saline (PBS) and immediately solubilized in NP-40 lysis buffer [1% NP-40, 20 mmol/l Tris–HCl (pH 8.0), 137 mmol/l NaCl, 10% glycerol, 2 mmol/l EDTA, 1 mmol/l sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mmol/l phenylmethylsulfonylfluoride, and complete protease inhibitor cocktail (Sigma-Chemicals)] by rocking the lysates gently at 4 °C for 30 min. Following microcentrifugation at 14,000 × *g* for 5 min, supernatants (*i.e.*, cytosolic fraction) and membrane + nuclear fractions (*i.e.*, solid particles fraction) were obtained. Cytosolic fractions were transferred into a clean test tube and sample protein concentrations were determined using the Pierce Protein Assay Kit (Rockford, IL, USA) and stored immediately at −80 °C until utilization.

2.4. Sample preparation

A liquid–liquid extraction with ethyl acetate, including some slight differences depending on the type of sample, was used. For the culture medium the extraction procedure was as follows: 1 ml of the culture medium was mixed with 1 ml of ethyl acetate, vortexed for 10 min, centrifuged at 13,000 rpm for 10 min and the supernatant was evaporated to dryness. The dried sample was reconstituted in 200 μl of mobile phase (water + 0.5% acetic acid) with 20% MeOH.

For the cytosolic fraction, the extraction procedure consisted in stirring 100 μl of the sample with 100 μl of 0.5 M HCl/MeOH and 200 μl of ethyl acetate for 10 min using a vortex. The mixture was maintained in the freezer for 1 h at −20 °C. After samples reached the room temperature, they were centrifuged at 13,000 rpm for 10 min and the supernatant was evaporated to dryness. The dried sample was reconstituted in 150 μl of mobile phase (water + 0.5% acetic acid) with 20% MeOH (lower volumes may lead to erratic recoveries).

The solid particles fraction was firstly washed with 100 μl of hexane to remove lipids. The samples were vortexed for 10 min and then centrifugated for 10 min at 13.000 rpm. The supernatant was removed and the solid parts were extracted as described above for the cytosolic fraction.

2.5. Instrumentation

2.5.1. Nano LC

Commercially available instrumentation EASY-nLC™ (Bruker Daltonik GmbH, Bremen, Germany), composed by one module and equipped with three pumps, three pressure sensors, four valves, two flowsensors, an autosampler and a touchscreen was used for the study.

The chromatographic separation was performed in a capillary column BioSphere (75 μm ID, effective length 10 cm, 3 μm particle size) packed with C18 particles. An on-line short capillary trapping column (100 μm ID, effective length 20 mm, 5 μm particle size) was used before the nanoLC column in order to achieve both pre-concentration and clean up of samples. The mobile phases were composed of water + 0.5% acetic acid (phase A) and acetonitrile (phase B) with the following gradient: 0–20 min, 20–33% B; 20–45 min, 33–40% B; 45–48 min, 40–95% B. Finally, the B content was decreased to the initial conditions (20%) within 2 min and the column rinsed with these conditions for 5 min. Before starting the following analysis the pre-column and column were re-equilibrated with phase A at 6 μl/min for 2 min and 0.6 μl/min for 8 min, respectively. The flow rate used to elute the compounds in the analytical column was 300 nl/min at room temperature and 5 μl of the sample was injected into the loop (the use of trapping column before the analytical column allowed injecting higher sample volumes).

2.5.2. Mass spectrometry

The nanoLC column was coupled to a Bruker Daltonik microTOF mass spectrometer (Bruker Daltonik, Bremen, Germany) using a commercial sheatless nano-spray interface with a tapered fused silica sprayer tip. The key parameters of the nano-ESI were adjusted for the flow rate used (300 nl/min) to achieve stable spray across the entire gradient range: pressure 0.4 bar, dry gas flow 4 l/min and dry gas temperature 150 °C.

The mass transfer parameters (radio frequencies and voltages in the different skimmers, hexapoles and lenses) were optimized in recent works, where the same olive oil polyphenols were analyzed [30]. The mass spectrometer was run in the negative mode and was operated to acquire spectra in the range of 50–800 *m*/*z*. SmartFormula™ tool within DataAnalysis was used for the calculation of elemental composition of compounds: it lists possible molecular formulae consistent with the accurate mass measurement and the true isotopic pattern (TIP).

In order to obtain high mass accuracy, the mass spectrometer must be calibrated by analyzing a standard sample with known masses along the *m*/*z* scale and then applying a mass correction. The calibrant can either be measured within the sample itself (internal calibration) or, alternatively, can be introduced externally, for instance, with a pump at the beginning or at the end of the analysis (external calibration). With the instrumentation used in this work for the nanoLC analysis was not possible to do an external calibration because a system to introduce externally the calibrant has not been developed yet. Instead of this, an internal calibration was applied using culture medium samples spiked with olive oil extracts (quality control samples) that contained a mixture of phenols with well-known mass values that were used as calibrant, providing mass peaks throughout the desired range of 100–450 *m*/*z* [26]. The quality control samples were injected every three analyses and their calibration parameters were used for the next three samples injected in a row. This procedure resulted in mass accuracies less than 5 ppm.

Table 1
Recovery percentages (%) of the main phenolic compounds identified in olive oil from culture medium and cytoplasm spiked with ethanolic extracts of olive oil. Each determination was made in triplicate (in all cases RSD was lower than 5% of the mean value).

Compounds	*m*/*z*	Recovery %	
		Culture medium	Cytoplasm
Hydroxytyrosol	153.0557	82 ± 3.2	70 ± 1.4
Tyrosol	137.0608	73 ± 2.5	63 ± 0.8
Luteolin	285.0405	60 ± 1.2	57 ± 0.5
Apigenin	269.0455	64 ± 1.8	64 ± 0.6
Pinoresinol	357.1344	148 ± 3.7	119 ± 3.6
Acetoxypinoresinol	415.1398	157 ± 3.2	134 ± 3.7
Syringaresinol	417.1555	138 ± 2.5	123 ± 2.8
Ligstroside aglycon	361.1293	55 ± 1.6	68 ± 1.3
Oleuropein aglycon	377.1242	62 ± 1.3	48 ± 0.5
Deacetoxy ligstroside aglycon	303.1238	30 ± 0.9	4 ± 0.08
Deacetoxy oleuropein aglycon	319.1187	21 ± 0.2	3 ± 0.1

3. Results and discussion

3.1. NanoLC analysis of cancer cells treated with olive oil extracts

Different extraction procedures were assayed for the culture medium and cytosolic fraction in order to extract the major number of phenolic compounds and metabolites with good recoveries in a reproducible way. To simulate real samples culture medium and cytoplasm were spiked with olive oil extracts in ethanol.

These samples were also used to optimize chromatographic and mass spectrometry conditions of nanoLC–ESI–TOF MS based on a previous study with olive oil samples [26].

The most representative components of the olive oil phenolic fraction were observed during the optimization: simple phenols (hydroxytyrosol and tyrosol), flavonoids (luteolin, apigenin), lignans (pinoresinol, acetoxypinoresinol and syringaresinol) and secoiridoids (oleuropein aglycon (Ol Agl), ligstroside aglycon (Lig Agl), deacetoxy oleuropein aglycon (DOA) and deacetoxy ligstroside aglycon (D-Lig Agl)).

Table 1 shows the recoveries of the main olive oil phenolic compounds for both samples culture medium and cytoplasm. Extraction efficiency ranged from 63% to 82% for simple phenols and from 57% to 64% for the flavonoids. For the family of secoiridoids, Lig Agl and Ol Agl were obtained from 48% to 62% but recoveries were a bit lower for the deacetoxy derivatives (D-Ol Agl and D-Lig Agl) especially in the cytoplasm samples. Recovery percentages higher than 100% were obtained for the lignans, perhaps because of the presence in the medium of coeluting compounds that could interfere in the ionization of these analytes enhancing their signal.

The precision of the assay was evaluated by three consecutive injections ($n = 3$) of the quality control sample (culture medium spiked with the olive oil extract) in the same day (intra-day repeatability) and in three different days (inter-day repeatability) obtaining values of relative standard deviation (RSD%) on the peak area ratio below 3.8% and 18.6% respectively. These results showed good intra-day repeatability although poor inter-day repeatability, one of the disadvantages observed with the nanoLC technique.

The optimum nanoLC–ESI–TOF MS method was applied to analyze all the real samples (culture medium, cytoplasm and solid parts) before and at different times after the administration of the olive oil extract. Every three analyses, a quality control sample was injected for correcting the variability in terms of signal sensitivity.

Fig. 1 shows the base peak chromatogram (BPC) of the culture medium at time 0 h in the absence of olive oil (blank sample) (Fig. 1A) and just after the addition of the olive oil extract (Fig. 1B). In the same figure, the extracted ion chromatograms (EICs) of the most representative olive oil phenols recovered from the culture medium can be observed (Fig. 1C). The separation of the most

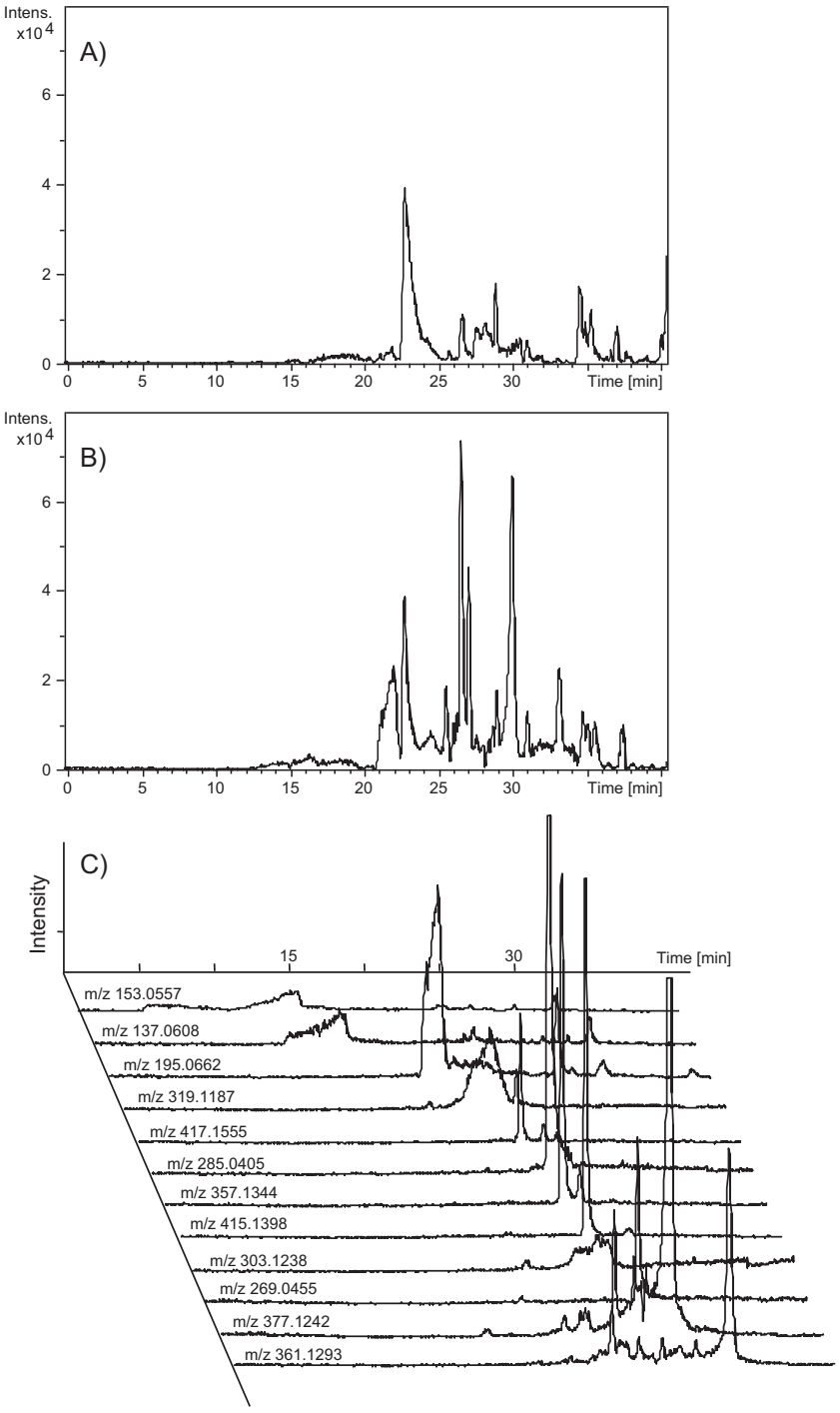

Fig. 1. Base peak chromatograms (BPC) obtained by nanoLC–ESI TOF MS at the optimum conditions for culture medium at time 0 h in the absence of olive oil (blank sample) (A) and just after the addition of the olive oil extract (B). Extracted ion chromatograms (EICs) of the most representative olive oil phenols obtained from Fig. 1B (C). *m*/*z* 153.0557: hydroxytyrosol; *m*/*z* 137.0608: tyrosol; *m*/*z* 195.0662: hydroxytyrosol-acetate; *m*/*z* 319.1187: deacetoxy oleuropein aglycon; *m*/*z* 417.1555: syringaresinol; *m*/*z* 285.0405: luteolin; *m*/*z* 357.1344: pinoresinol; *m*/*z* 415.1398: acetoxypinoresinol; *m*/*z* 303.1238: deacetoxy ligstroside aglycon; *m*/*z* 269.0455: apigenin; *m*/*z* 361.1293: ligstroside aglycon; *m*/*z* 377.1242: oleuropein aglycon.

hydrophilic compounds (hydroxytyrosol and tyrosol) showed low efficiency and resolution, probably due to the higher aqueous content used at the beginning of the gradient that could interfere in the spray stability [26]. The method proved specific, since no interfering endogenous compounds could be seen at the elution times of the polyphenols in the EICs when blank samples were analyzed.

The identification of the phenolic compounds in free form and the possible metabolites was easily performed by using the valuable information provided by TOF analyzer. TOF offers two dimensions of information to carry out a high-confidence identification of the compounds under study. Using the accurate mass and the isotopic pattern of the peak under investigation, TOF analyzer gives us a reduced number of possible molecular formulae than can be then matched against available databases. Although, as we have mentioned in Section 2, it was not possible to do an external calibration because of the lack of a suitable system to introduce the

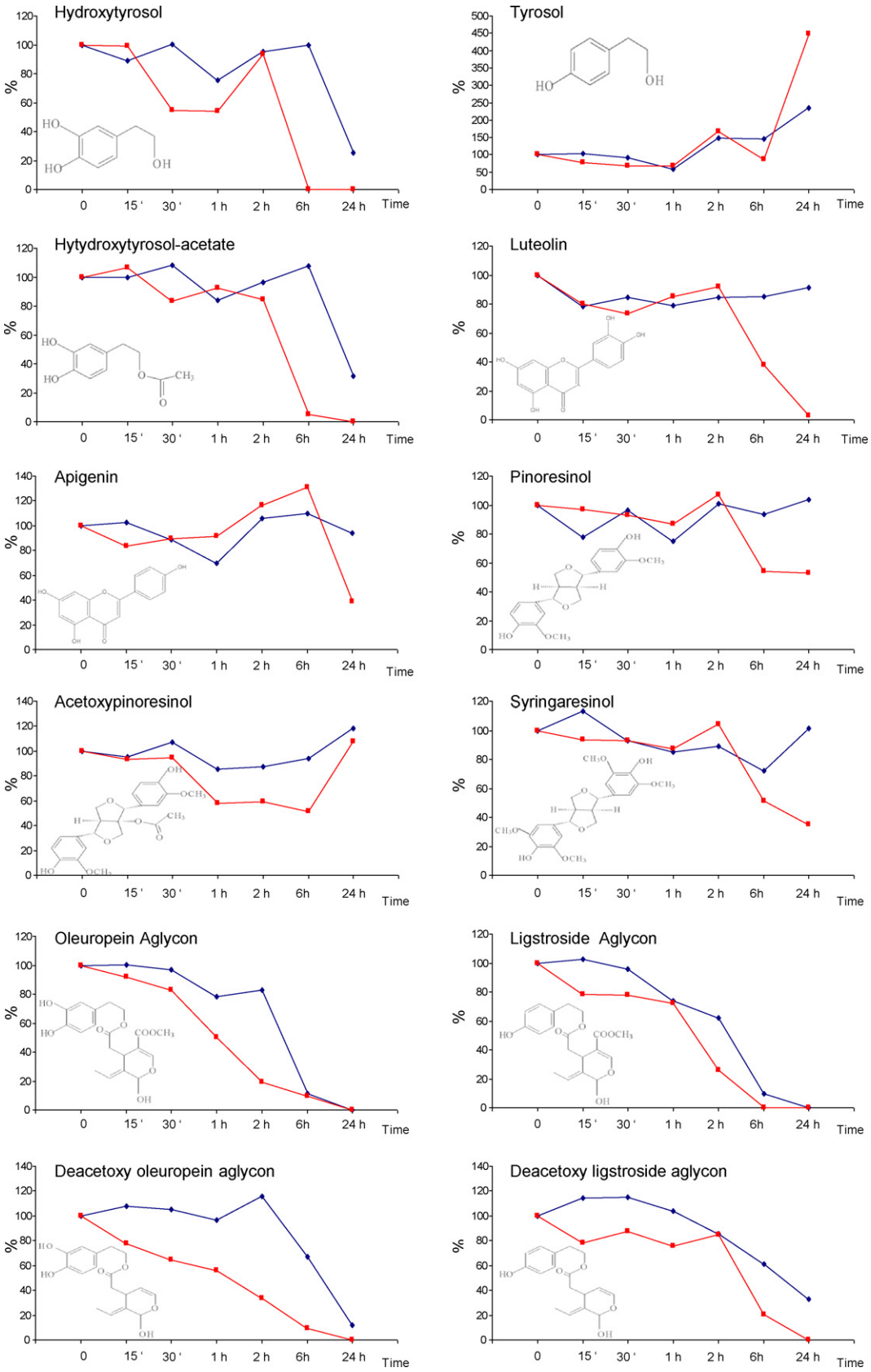

Fig. 2. Time course of cellular uptake of the parent olive oil phenolic compounds in the culture medium at different incubation times: 0, 15 min, 30 min, 1 h, 2 h, 6 h and 24 h. The values show the amount of compounds remaining in the culture medium expressed as % of the quantity initially incorporated. ■, culture medium in the absence of cells (red line) and ♦, culture medium in the presence of cells (blue line). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

calibrant, a satisfactory internal calibration was developed using a combination of well-known phenols present in the quality control samples.

Additionally, the comparison of both retention time and MS spectral data with those from available standards, the polarity of the compounds and the information previously reported in literature [11,26] contributed to corroborate the identity of the compounds.

Simple phenols, small molecules with high polarity (hydroxytyrosol, tyrosol and hydroxytyrosol acetate) appeared at the beginning of the chromatogram with errors below 2 ppm. The compounds with *m*/*z* 285.0405 and 269.0455 were identified as the flavonoids luteolin and apigenin, the luteolin eluting before apigenin because of the presence of one hydroxyl group more in the molecule. Lignans with masses 417.1555 (syringaresinol), 357.1344 (pinoresinol) and 415.1398 (acetoxypinoresinol) eluted close to each other and were identified with errors around 3 ppm. The less polar compounds Ol Agl (377.1242) and Lig Agl (361.1293) appeared at the end of the chromatogram and were identified with errors of 2.5 and 1.2 ppm respectively. Lig Agl, with one hydroxyl group less than Ol Agl, eluted the last. Their decarboxylated derivatives (DOA and D-Lig Agl) were detected in the same elution order that the original compounds but at shorter retention times. This group of secoiroids showed different isomeric forms that were very difficult to separate due to the similar polarity.

3.2. Cellular uptake

JIMT-1 human breast cancer cells were incubated in culture medium exogenously supplemented with olive oil extracts. The most representative compounds belonging to different families of phenolic compounds described in olive oil were monitored in the medium at different incubation times: 0, 15 min, 30 min, 1 h, 2 h, 6 h and 24 h. The time course of cellular uptake of the original compounds expressed as % of the quantity initially incorporated in the culture medium can be seen in Fig. 2. Considering that the disappearance of some compounds could be due to their rapid degradation in the medium, two experiments were carried out in parallel: one of them with the culture medium in the presence of cells to study how the compounds are absorbed and biologically metabolized and the other, in the absence of cells, to indicate the spontaneous or basal degradation of compounds.

A different uptake was observed for each compound in relation to their different chemical structures. In the case of flavonoids, luteolin decreased significantly in the culture medium from 2 to 24 h: 38% of the quantity added was present after 6 h of incubation and only traces of this compound were observed after 24 h. Apigenin seemed to be more slowly and poorly taken up with 39% of the nonmetabolized molecules detected in the culture medium after 24 h. This could be explained considering the limited diffusion into the cell of compounds with a lower degree of hydroxylation, as the case of apigenin with one hydroxyl group less than luteolin.

Regarding lignans, pinoresinol and syringaresinol remained practically intact in the culture medium for 2 h and were absorbed, although poorly, between 2 and 6 h. In the case of acetoxypinoresinol, the absorption started a few hours before (around 1 h) and the rapid increase between 6 and 24 h could be due to the appearance of the acetoxylated metabolite of pinoresinol.

The family of secoiridoids showed to be susceptible to spontaneous degradation in free cells culture media, particularly after 2 h of incubation. The cellular uptake seemed to differ slightly between the secoiridoids containing a molecule of hydroxytyrosol in their structure (Ol Agl and DOA) and those with a molecule of tyrosol (Lig Agl and D-lig Agl). Ol Agl and DOA were absorbed very quickly after the administration and a gradual uptake, starting from 15 min, was observed along the time. Both compounds were taken up at all exposure time points although the pattern of association differed in a time-dependent manner, probably due to the differences in their structures. During the first minutes, the absorption was a bit faster for DOA whereas the levels of Ol Agl decreased more slowly within 30 min and reached the maximum of absorption between 30 min and 2 h. Limited uptake of the secoiridoids derived from tyrosol (Lig Agl and D-Lig Agl) was observed at short incubation time: 15 min to 1 h, reaching the maximum of absorption a bit later, between 1 and 2 h for Lig Agl, and after 6 h of incubation for D-Lig Agl. This slower uptake could be due to the lower degree of hydroxylation, as mentioned before. The remaining amount of compounds disappeared between 2 and 24 h probably due to the spontaneous degradation, as can be observed in the experiments in the absence of cells.

The results obtained from simple phenols were more complex because part of them could come from the decomposition of their respective secoiridoids in the culture medium. Hydroxytyrosol, as in the case of its secoiridoid derivatives, was incorporated in the first minutes, reaching 50% after 1 h of incubation. The increase observed after 1 h could be due to the decomposition of Ol Agl and the rapid decrease after 2 h to the absorption and also to its spontaneous decomposition in the culture medium. Hydroxytyrosol-acetate showed high absorption from 2 h and only 4% of the original compound was detected after 6 h of incubation. In a previous work with HepG2 cells hydroxytyrosol and hydroxytyrosol acetate were present in the culture medium as free molecules at 2 h of incubation and a high absorption was observed after 18 h [22].

No significant changes were observed in the concentration of tyrosol throughout the incubation period what it is also in agreement with the results obtained in literature about the limited diffusion of this compound in HepG2 cells [22]. Besides, a high increase in the concentration of this compound was observed after 2 h, probably due to the decomposition of the secoiridoids containing this molecule in their structure.

Removal from the culture medium could suggest accumulation of these compounds in the cells but also in the cell membranes. However, we failed to distinguish these compounds when analyzing cytoplasm and solid parts of JIMT-1 cells, maybe due to the low intracellular accumulation as also occurs in HepG2 cells [22]. Therefore, the decrease of the amount of some compounds in the culture medium along the time may indicate the uptake of intact compounds by the cells.

3.3. Metabolism of olive oil phenolic compounds in breast cancer cells

Several compounds formed as a consequence of cell metabolism were observed in the extracellular culture medium at different incubation times. Table 2 shows all the metabolites tentatively identified in the culture medium grouped by families, including *m*/*z* experimental, retention time, error and sigma value and molecular formula. The time range needed for the appearance of these compounds and the time at which the highest intensity of each compound was reached are also indicated in the table. The EICs of all the metabolites tentatively identified when they showed the highest intensity can be seen in Fig. 3. Some EICs (*m*/*z* 335.1499, *m*/*z* 333.1347, *m*/*z* 391.1397) showed several peaks corresponding to different isomeric forms, typical of the family of secoiridoids. Control samples cell-free and control samples with no added phenolics were also analyzed because some compounds could also be formed spontaneously in the culture medium.

3.3.1. Simple phenols

One metabolite with very low intensity and *m*/*z* value of 167.707 was tentatively identified as the methyl conjugate of hydroxytyrosol. This was one of the most important metabolites

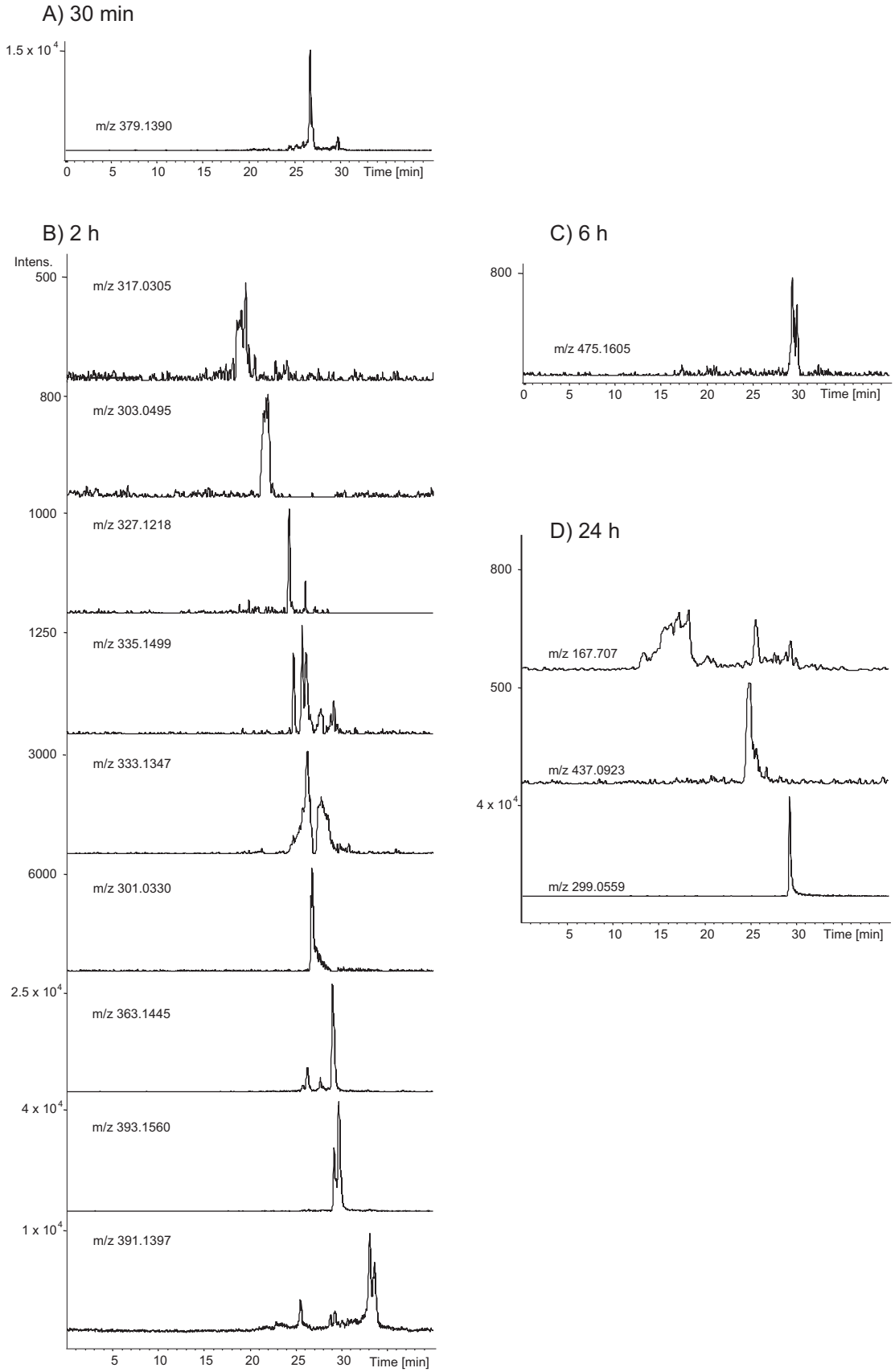

Fig. 3. Extracted ion chromatograms (EICs) of the main metabolites tentatively identified in culture medium of human breast cancer cells incubated with olive oil extracts. They are grouped according with the time at which they presented the highest intensity: (A) 30 min, (B) 2 h, (C) 6 h and (D) 24 h. The identification of the compounds is indicated in Table 2.

Table 2
Metabolites tentatively identified at different incubation times in the culture medium of cancer cells exogenously supplemented with olive oil extracts.

	m/z experimental	Retention time	Error (ppm)	Sigma	Molecular formula	Proposed metabolite	Maximum concentration (range)
Simple phenols	167.707[a]	18.097	4.2	56.5	$C_9H_{11}O_3$	Methyl hydroxytyrosol	24 h (2–24 h)
Secoiridoids	335.1499	25.605	0.3	17.8	$C_{18}H_{23}O_6$	Dihydrogenated methyl DOA	2 h (1–24 h)
	333.1347	26.190	−0.9	63.1	$C_{18}H_{21}O_6$	Methyl DOA	2 h (30 min to 6 h)
	379.1390	26.625	2.3	17.1	$C_{19}H_{23}O_8$	Dihydrogenated Ol Agl	30 min (15 min to 2 h)
	363.1445	28.933	1.3	4.9	$C_{19}H_{23}O_7$	Dihydrogenated Lig Agl	2 h (30 min to 24 h)
	393.1560	29.602	−1.3	5.3	$C_{20}H_{25}O_8$	Dihydrogenated methyl Ol Agl	2 h (15 min to 24 h)
	391.1397	33.599	0.4	28.2	$C_{20}H_{23}O_8$	Methyl Ol Agl	2 h (15 min to 2 h)
Flavonoids	317.0305[a]	19.501	−0.7	95.1	$C_{15}H_9O_8$	Dihydroxy luteolin	2 h
	303.0495[a]	21.692	5.2	94.9	$C_{15}H_{11}O_7$	Luteolin hydrated	2 h
	301.033	26.709	4.1	58.6	$C_{15}H_9O_7$	Hydroxy luteolin	2 h
	299.0559	29.117	0.8	3.6	$C_{16}H_{11}O_6$	Methyl luteolin	24 h
Lignans	327.1218[a]	24.250	6.0	135.6	$C_{19}H_{19}O_5$	Demethoxy pinoresinol	2 h (1–6 h)
	437.0923[a]	24.920	2.5	120.6	$C_{20}H_{21}O_9S$	Pinoresinol sulfate	24 h
	475.1605[a]	29.284	0.9	28.5	$C_{24}H_{27}O_{10}$	Acetoxy-syringaresinol	6 h (2–24 h)
Unknown	463.0865[a]	20.789	3.6	37.4	$C_{21}H_{19}O_{12}$		2 h (30 min to 2 h)
	349.0581	20.872	−4.6	32.5	$C_{16}H_{13}O_9$		2 h
	447.0939	22.026	−1.5	6.2	$C_{21}H_{19}O_{11}$		6 h
	433.1502	22.880	0.5	57.0	$C_{22}H_{25}O_9$		15 min
	431.1000	23.548	−3.8	4.8	$C_{21}H_{19}O_{10}$		6 h
	609.1249[a]	24.501	9.7	66.5	$C_{23}H_{29}O_{19}$		2 h
	273.1692	25.037	5.8	74.9	$C_{14}H_{25}O_5$		24 h
	281.1035	25.672	−1.7	31.4	$C_{14}H_{17}O_6$		2 h
	265.1072	27.010	3.5	110.7	$C_{14}H_{17}O_5$		2 h
	249.1120	29.267	5.1	2.6	$C_{14}H_{17}O_4$		2 h
	437.1130	29.736	−9.4	32.8	$C_{20}H_{21}O_{11}$		6 h
	403.1398[a]	36.609	0.2	306.2	$C_{21}H_{23}O_8$		1 h (15 min to 2 h)

[a] Compounds present in small traces.

found in experiments with Caco-2 and HepG2 cells incubated with hydroxytyrosol [20–22]. This compound started to appear in the culture medium after 2 h of incubation, when part of the original compound had already been absorbed, but reached the maximum concentration after 24 h. As it was mentioned before, tyrosol was poorly absorbed and subsequently none metabolites of this compound were found in the culture medium.

3.3.2. Secoiridoids

Compounds belonging to the secoiridoids were the most extensively metabolized, especially those derived from hydroxytyrosol (Ol Agl and DOA). Methylation was the preferential pathway for both compounds followed or preceded, in some cases, by a hydrogenation reaction. All the methylated forms increased their concentration with the time, in parallel with the decrease of the parent compounds in the culture medium, reaching the maximum value after 2 h of incubation. No methyl conjugates of secoiridoids derived from tyrosol (Lig Agl and D-Lig Agl) were observed, as could be expected considering the fact that methylation by catechol-O-methyl transferase (COMT) requires and ortho-diphenolic structure [20]. Two new peaks with *m/z* values of 379.1390 and 393.1560 were tentatively identified as dihydrogenated metabolites of Ol Agl and Lig Agl, respectively. This Ol Agl metabolite appeared in the medium with high intensity at earlier times (30 min), indicating a faster metabolism and excretion compared with the methylation. Regarding the Lig Agl, this was the only metabolite detected, confirming the poor metabolism of the secoiridoids which contain in their structure a molecule of tyrosol. Besides, this compound appeared with high intensity a bit later (2 h) than the same metabolite of the Ol Agl, probably because of the slower uptake of the parent compound, as indicated in the previous section. After 24 h of incubation these compounds were almost disappeared.

3.3.3. Flavonoids

As can be observed in the above section the cellular uptake of flavonoids was slow and only traces of three oxidation products of luteolin (hydroxylated, dihydroxylated and hydrated) were observed after 2 h, when the absorption of the compound is still very limited. At longer incubation time (24 h), an intensive peak with *m/z* 299.0559 and longer retention time was tentatively identified as methyl-luteolin. In previous works this metabolite has been described in the culture medium of Caco-2 cells after 24 h of incubation with luteolin [21]. The other flavonoid, apigenin, seemed to be poorly metabolized, and presumably this compound cannot take the same metabolic route as luteolin because of the lower degree of hydroxylation. Only a peak observed after 6 h of incubation with *m/z* 447.0939 could be identified as dehydrogenated apigenin glucuronide and the same metabolite of luteolin (dehydrogenated luteolin glucuronide) could be attributed to another small peak observed at a retention time of 20.789 min with *m/z* 463.0865. However, we have some doubts about this identification because both metabolites were detected in the culture medium at too early times (6 and 2 h) when the parent compounds were poorly absorbed. Besides, according to the literature, glucuronoconjugates have great difficulty to cross the membrane because of their polarity and it would be expected to appear at longer times or even not to appear. Further experiments would be necessary to confirm that human breast cancer cell contain UDp-glucuronosyl-transferase activity capable of causing glucuronoconjugated forms of flavonoids.

3.3.4. Lignans

The metabolites formed from the lignan precursors were practically absent, in part because of their low absorption into the cells. Two metabolites of pinoresinol appeared in small traces, one of them (*m/z* 327.1218) could correspond to the loss of a methoxy group and the other (*m/z* 437.0923) to its sulfoconjugated metabolite. The sulfate derivative was also observed at high

concentrations after 6–24 h of incubation with pinoresinol in Caco-2 cells [21]. At longer incubation time (24 h) a small peak with m/z 475.1605 was identified as acetoxy-syringaresinol, the same metabolite that was described for pinoresinol in the previous section (acetoxypinoresinol).

Other peaks with high intensity were detected in the culture medium at different incubation times but it was not possible to identify them with the instrumentation used in this study and we can only show their molecular formula. Some molecular formulae are very similar to each other and could come from the same family of compounds, as for instance: $C_{14}H_{17}O_4$, $C_{14}H_{17}O_5$ and $C_{14}H_{17}O_6$.

The identified metabolites were also studied in the cytosolic fraction but only small traces were observed after different hours of incubation. No metabolites were detected in the solid particles fraction either.

Therefore, the decrease of some compounds in the culture medium along the time and the appearance of metabolites could indicate that the compounds are absorbed, intracellularly metabolized to different forms and excreted. Alternatively, they could be produced directly in the medium by secreted cellular enzymes. Neither original compounds nor metabolites were detected in the cytosolic and solid particles fraction. This could be because the concentration of the added compounds is too low to detect them in these fractions (further studies with higher concentrations should be developed) or could suggest no intracellular accumulation of the original compounds and metabolites and a rapid excretion into the culture medium. New experiment will be necessary in the future to go into depth regarding this issue and confirm the hypothesis.

Since the methyl conjugates prevailed in the culture medium, these results might suggest that JIMT-1 human breast cancer cells possess the enzymatic activity of catechol-O-methyltransferasa (COMT), as it has been demonstrated in the case of cells obtained from the gastrointestinal tract (Caco-2 and HepG2) [20,22]. However further studies would be required to confirm that JIMT-1 human breast cancer cells possesses enzymatic activity for metabolizing flavonoids and lignans to glucuronide and sulfate conjugates.

4. Conclusions

The extent of uptake and the level of metabolism of olive oil polyphenols in cancer cells will be pivotal to understand the mechanisms by which these compounds act as chemopreventive agents affecting the overall process of carcinogenesis. In this work, the cellular uptake and metabolism of olive oil polyphenols in human breast cancer cells has been evaluated for the first time, taking advantage of the sensitivity of the nanoLC–ESI–TOF MS method developed. The potential of this analytical platform for the analysis of small molecules in biological samples has been demonstrated. The analysis of culture medium, cytosolic fraction and solid particles fraction at different incubation times, suggested that some compounds, mainly hydroxytyrosol, secoiridoids, and the flavonoid luteolin are absorbed, metabolized in the cytoplasmic compartment and excreted to the extra cellular medium. The secoiridoids, especially those derived from hydroxytyrosol (DOA and Ol Agl) appeared to be the most rapidly and extensively taken up and metabolized, mainly as methylconjugates. The methylated derivatives were also predominant for hydroxytyrosol and luteolin, this last one together with trace amounts of other oxidation metabolites. The presence of the methylconjugates suggests that this breast cancer cell line possesses catechol-O-methyltransferase activity. Limited metabolism was observed for the lignans, apigenin, tyrosol and their related secoiridoids (Lig Agl and D-Lig Agl), a phenomenon likely related to a less efficient transport through human breast cancer cell membranes.

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