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Chemical Screening of Olive Biophenol Extracts by Hyphenated Liquid Chromatography

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Chemical Screening of Olive Extracts

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Abstract

Chemical screening using reversed phase HPLC-photodiode array detection (RPLC-DAD) and RPLC-electrospray ionisation mass spectrometry (RPLC-ESI-MS) is widely applied as an approach to streamline natural products research. The full potential of this approach is demonstrated in this paper by application to the chemical screening of olive products including olive mill waste (OMW). Out of 100 biophenols previously reported in olive products, the on-line RPLC-DAD-ESI-MS was able to confirm the presence of 52 compounds in OMW. This included a number of simple phenols, flavonoids and secoiridoids. By careful examination of the combined DAD and ESI-MS data, extra information was elucidated including: the site of glycosidation on the phenol ring of hydroxytyrosol; the identity of the other luteolin-glucoside isomer as luteolin-4′-*O*-glucoside; identifying rutin rather than the previously reported hesperidin (and the reasons for possible mis-assignment); and the detection of diastereomers of 4-hydroxyphenylethyl alcohol-deacetoxy elenolic acid dialdehyde (4-HPEA-DEDA) and 3,4-dihydroxyphenylethyl alcohol-deacetoxy elenolic acid dialdehyde (3,4-DHPEA-DEDA).

Keywords: Liquid chromatography-electrospray ionisation-mass spectrometry, photodiode array detection, fluorimetry, olive mill waste, oil, fruit, secoiridoids, polyphenols

1. Introduction

Biophenols have attracted increased attention in recent years for diverse reasons including their wide range of bioactivities. They are relatively polar compounds and reversed phase HPLC (RPLC) has been widely used for their analysis [1]. Many biophenols have characteristic ultraviolet spectra (e.g. the flavonoids) [2] making them ideal candidates for photodiode array detection (DAD) and most of the reported work has used the combination of RPLC and DAD [1,3]. However, this has generally involved use of a single or at most 2-3 wavelengths and the full capabilities of DAD have not been exploited or even explored [4,5] despite the ability to differentiate between compounds within the same class when their UV-Vis spectra are sensitive to substitution patterns [2]. RPLC with DAD is well suited for chemical screening of biophenols in a range of matrices. In RPLC, the more polar molecules elute first and hence elution order can also provide valuable structural information [6]. The integration of chemical screening into bioactive compound discovery programs reduces the chance of missing novel and unidentified compounds, prevents replication during separation, and opens a new horizon for reassessment of traditional plants.

Nevertheless, other detection methods offer particular advantages as, for example, fluorimetric detection (FLD) which is both more sensitive and selective than DAD whereas mass spectrometry (MS) provides universal detection that allows simultaneous determination of molecular weight. Though complete structure elucidation is not usually possible with RPLC-MS, the fragmentation of the molecular ions can provide useful structural information. The number of compounds that have been detected in bio-extracts is increasing exponentially with the introduction of electrospray ionisation (ESI). RPLC-ESI-MS has recently been adopted as a routine screening technique for these extracts to identify previously reported biophenols, and allow identification of novel unidentified constituents [7-9]. However, ESI-MS is extremely sensitive to the elution conditions and suppression effects are not uncommon [10], while some compounds may fail to ionise [11].

Olive mill waste (OMW) resulting from processing of olive fruits represents a complex matrix that is rich in biophenols with a wide array of biological activities [5,12]. The complexity of the biophenolic fraction of OMW has been demonstrated [5,13]. In the present study, the potential of RPLC coupled with DAD, ESI and FLD was explored for its application for screening complex extracts. This is the most comprehensive study on the combination of MS and DAD to screen such a large number of biophenols from a diverse range of phenolic classes. Our main aim is to demonstrate a systematic approach to the integration of the full range of analytical data available – retention time, UV absorbance, fluorescence and mass spectrometric – to provide more information about a natural product extract than is usually derived from a conventional chemical screening program.

2. Experimental

2.1. Reagents and Standards

Reagents and standards were obtained and prepared as described previously [13].

2.2. Sampling and Sample Pre-treatment

OMW samples from a Pieralisi commercial two-phase olive oil mill (Sambuca, Italy) were obtained from "Riverina Olive Grove" (Wagga Wagga, NSW, Australia). The Frantoio OMW samples were collected in the 2003 season (26th May) and the Mission OMW samples were collected in the 2004 season (8th June). Fruit and virgin olive oil samples were collected for the Frantoio cultivar. The fruit and fresh waste were stored under liquid nitrogen without delay, freeze dried and stored at −20 °C [13]. Olive oil samples were stored in the dark at room temperature until extracted.

2.3. Extraction of Biophenols

Freeze-dried OMW: Sodium metabisulfite preserved extracts were prepared according to the method described previously [13]. This involved extraction of freeze-dried OMW (1 g) with methanol:water (60:40 v/v; 5 mL) containing sodium metabisulfite (2% w/w). The mixture was filtered, re-extracted and defatted with hexane. All extractions were performed at 20 ± 2 °C. Extracts without sodium metabisulfite were prepared using exactly the same procedures, but replacing the extraction solvent with acidified aqueous methanol (methanol + water, 80+20 v/v) adjusted to pH 2 with conc. HCl.

Olive Fruits: Freeze-dried Frantoio olive fruits (20.0 g) were blended using a household blender for one minute. The resulting paste (1.00 g) was extracted twice with 5 mL 60% aqueous methanol (2% sodium metabisulfite w/w) as indicated for the OMW samples.

Olive Oil: Extraction was carried out according to Kalua et al. [14], a process in which 15 mL olive oil was extracted with 3 mL methanol + water (50+50, v/v).

2.4. High Performance Liquid Chromatography

RPLC-DAD and RPLC-DAD-FLD: Routine analysis was performed with a Varian 9021 solvent delivery system equipped with a Varian 9065 Polychrom UV diode array detector (190-367 nm) [13]. Separation was performed by gradient elution on a Phenomenex Luna C-18(2) column, 5 µm particle size; (150 mm x 4.6 mm) (Sydney, Australia) attached to a Phenomenex SecurityGuard guard cartridge (Sydney, Australia). Analysis conditions were as described previously [13]. Thus, solvent A was a mixture of 100:1 water/acetic acid (v/v), and solvent B was a mixture of 90:10:1 methanol/acetonitrile/acetic acid (v/v/v). A six-step linear gradient analysis for a total run time of 60 min was used as follows: Starting from 90% solvent A and 10% solvent B, increase to 30% solvent B over 10 min and then isocratic for 5 min, increase to 40% solvent B over 10 min, to 50% over 15 min and to 100% solvent B over

10 min, and finally isocratic for 10 min. For fluorimetric detection, a Perkin Elmer LC-240 fluorescence detector was connected in series.

RPLC-MS: Liquid chromatography-mass spectrometry (LC-MS) of the olive extracts (used as a generic term referring to all of waste, fruit and oil) was performed on a Waters Micromass Quattro micro tandem quadrupole mass spectrometer (Manchester, UK). LC separation was provided by a Waters liquid chromatograph (Milford, MA, USA), consisting of a 2695 Separation Module and 2487 dual wavelength UV detector operated at 240 and 280 nm. An SGE Wakosil C18 column (150 mm × 2 mm; 5µm) (Melbourne, Australia) was used. Aqueous formic acid (1%) and methanol + acetonitrile + formic acid (89.5+9.5+1 v/v/v) served as solvents A and B, respectively. Analysis conditions were as described before [12] and involved a seven-step linear gradient analysis for a total run time of 75 min was used as follows: Starting from 90% solvent A and 10% solvent B increasing to 30% solvent B over 10 min, then isocratic for 5 min, increased to 40% solvent B over 10 min, to 50% over 15 min and to 100% solvent B over 10 min, back to 10% solvent B over 5 min and finally isocratic for 10 min.

RPLC-DAD-MS: Samples were analysed using a Beckman (Fullerton, CA, USA) liquid chromatograph, consisting of a 126 pump and a 168 diode array detector. A Micromass Quattro II (Manchester, UK) was used for the mass spectrometry analysis with C18 Phenomenex column (Sydney, Australia) at a flow rate 1 mL min^{-1} and applying the gradient previously described [15]. Solvent A was a mixture of water+formic acid 100+1 (v/v) and Solvent B was a mixture of methanol+acetonitrile+formic acid 90+10+1 (v/v/v). Data were acquired by MassLynx system for the mass spectrometer and Beckman system for diode array detection; two wavelengths, 280 nm and 520 nm, from the diode array were also recorded by the MassLynx system to allow data alignment. During LC-MS, scans were performed for both

positive and negative ions (*m/z* 120 to *m/z* 1000). Cone voltage fragmentation was also used in both ion modes. A splitter system on the solvent flow from the HPLC allowed approximately 150 µL of the flow to be directed to the electrospray source.

3. Results and Discussion

3.1. Spectroscopic study of reference biophenols

Before embarking on the analysis of the olive extracts, it was necessary to examine a series of standard biophenols, representative of different biophenolic classes commonly reported in olives and olive products, by RPLC-DAD-FLD and RPLC-ESI-MS (Tables 1 and 2) to ascertain structure/spectra relationships. From RPLC-DAD, various correlations between structure and UV spectrum (Table 1) were extracted. The most notable effects of substitution occurred for the *B*-band (the highest absorption maximum of benzene analogues) [16]. Thus, addition of one *o*-hydroxyl group to a monophenol to give the corresponding catechol resulted in a bathochromic shift of 5-15 nm as observed for tyrosol (273 nm) / hydroxytyrosol (278 nm) and *p*-coumaric acid (307 nm) / caffeic acid (321 nm). The addition of a further *o*-hydroxyl group to the catechol to give the corresponding pyrogallol derivative resulted in a decrease of 20 nm (e.g. protocatechuic acid, 291 nm versus gallic acid, 271 nm). Substitution at the *o*-hydroxyl group in a catechol caused minimal change (e.g. protocatechuic acid versus vanillic acid; caffeic acid versus ferulic acid). The addition of a *p*-carboxyl to a catechol resulted in an increase of ca 15 nm e.g. catechol and protocatechuic acid, while addition of a *p*-alkyl group slightly affected the λ_{max} as seen for catechol (275 nm) and hydroxytyrosol (278 nm). Esterification at the alcoholic hydroxyl group with elenolic acid did not affect the *B*-band e.g. hydroxytyrosol and oleuropein, yet the lower absorption maximum increased by ca 10 nm.

INSERT TABLES 1 and 2

For flavonoids, the λ_{max} (band I) increased in the series: flavanone (hesperidin) < flavone (luteolin) < flavonol (quercetin). The λ_{max} also provided an indication of the substitution within the same class as seen for apigenin and luteolin in which an increase in the number of hydroxyl groups increased λ_{max} [2]. Glycosidation at the chromophoric portion resulted in a substantial decrease in λ_{max} (e.g. quercetin and rutin), while away from the chromophore the absorption (band I) was not affected (e.g. apigenin and apigenin-7-glucoside).

Using an excitation and emission wavelength of 280 nm and 330 nm, respectively, only 11 of the reference standards were fluorescent (Table 1). These wavelengths represented the conditions that produced maximum fluorescence in olive extracts. Differences between these data and results reported by Ryan et al. [17] using the same solvents and instrumentation but an emission wavelength of 340 nm may be attributed to the de-oxygenation of mobile phases with helium in the earlier work; oxygen is well known as a fluorescence quencher. In the current study, hydroxylation abolished the fluorescence (protocatechuic acid) while methylation of the *o*-hydroxyl group restored the fluorescence (vanillic acid). Moreover, none of the cinnamic acids investigated in this study were fluorescent under the specified conditions.

The standard compounds were examined (Table 2) in both negative (NIM) and positive ionisation modes (PIM) under soft (−30 and +35 V) and "strong" ionisation conditions (−70 and +70 V). With the exception of the flavonoids, NIM was more sensitive by 20-50 fold than PIM. For the flavonoid aglycones, quercetin and luteolin, PIM was slightly more sensitive than NIM, while the flavonoid glycoside, rutin, showed slightly higher sensitivity in NIM. The monophenols, tyrosol and 4-hydroxyphenylacetic acid, did not give a detectable peak as

previously reported for tyrosol [11]. Under soft ionisation conditions in both NIM and PIM, limited fragmentation took place and the pseudomolecular ion was the most abundant peak in the mass spectra.

Using strong ionisation, small molecular weight biophenols and non-glycosylated flavonoids had their pseudomolecular ions $[M - H]^-$ as the base peak in the NIM mass spectra whereas verbascoside, rutin, and oleuropein had a small pseudomolecular ion peak in NIM. Adducts with formic acid $[M - H + 46]^-$ were detected for all the tested standards except for tyrosol and caffeic acid. Dimers were observed for large molecular weight biophenols in the NIM mass spectra. Though PIM was less sensitive than NIM for most biophenols, it provided more fragmentation and hence more structural information. Oleuropein was exceptional in that fragmentation in NIM was greater than for PIM. Excluding luteolin, the pseudomolecular ion did not form the base peak in any of the PIM mass spectra. Sodium adducts for the glycosylated standards, verbascoside, oleuropein, and rutin, were very prominent and sometimes more intense than the pseudomolecular ion itself.

The availability of suitable standards is a challenge for bioscreening studies and the power of the detection techniques used in this study is demonstrated by the identification of minor components in the commercial oleuropein standard. Thus, oleuropein (Extrasynthese standard) generated two main peaks in RPLC-DAD-FLD and three peaks in RPLC-ESI-MS. The peaks identified by RPLC-DAD-FLD exhibited identical UV-Vis spectra and both peaks fluoresced at 330 nm following excitation at 280 nm. The first eluting peak was the major component, oleuropein itself, while the later eluting peak represented about 10% of the peak area of oleuropein, had the same molecular weight (540 amu) and was previously identified as oleuroside (**Figure 1**) [18]. The additional peak seen with RPLC-ESI-MS eluted between oleuropein and oleuroside and had a molecular weight of 584 amu. Fragmentation behaviour

and its relative retention suggested that this compound was a carboxyl derivative of oleuroside. As carbon 3 is similar in oleuropein and oleuroside and only oleuroside formed the carboxyl derivative, it is more likely that this compound is oleuroside-10-carboxylic acid (**Figure 1**).

INSERT Figure 1.

3.2. Screening of Olive Biophenolic Extracts

Biophenolic extracts from fruit and oil (*cv*. Frantoio) plus OMW (*cv*. Frantoio and Correggiola) were screened for the range of biophenols previously reported in olive products such as fruit [4,7,8,19], oil [14,20-22] and OMW [5]. The full range of biophenols is presented in Table 3 but discussion is limited to those compounds that best illustrate the use of combining the data from DAD and MS to allow facile and efficient identification. Three screening strategies were used as follows. Firstly, where reference compounds were available as standards, the presence or absence of a particular biophenol was assessed by comparison of retention time, UV-Vis spectra, and fluorescence at 330 nm (excitation at 280 nm) with that of the reference. The presence was further confirmed by molecular mass data. Secondly, when reference compounds were not available, the TIC traces in both negative and positive mode at soft ionisation and strong ionisation conditions were scanned for appropriate pseudomolecular ions $[M + H]^{+}$ and $[M - H]^{-}$. When found, confirmation was performed by examining UV-Vis spectra and mass spectral fragmentation data at the expected elution time, depending on pre-identified compounds in the sample, the structure of the target compound, and the literature data. Thirdly, major peaks in TIC and UV-chromatograms that were not identified by either of the other screening processes were screened for novel compounds by generating UV-Vis spectra and mass spectra. In all, screening was conducted for approximately 100 biophenols from

different classes (Table 3). The main aim was to demonstrate the power of combined use of RPLC-DAD and ESI-MS in screening of olive extracts.

INSERT Table 3

The TIC traces of extracts showed a high level of background noise compared with spectrophotometric detection. The continuous flow of mobile phase components to the mass spectrometer usually results in increased background noise [36]. The most prominent peaks were *m/z* 129 in PIM and *m/z* 137 in NIM. The latter was identified as a cluster ion formed by mobile phase constituents corresponding to $[(HCOOH)_2.HCOO^-]$ [37]. The maximum intensity of these ions in the TIC was less than 10%, and almost all of these ion peaks did not appear as major peaks in the mass spectra of detected compounds. Thus, the effect of background noise was considered insignificant [36]. The TIC of OMW showed a sharp peak eluting at 51.23 min followed by a broad peak till the end of the run, this was only detected in PIM. The mass spectrum revealed a pattern characteristic for polyethylene detergents, the presence of which was attributed to remnants from washing glassware.

Simple phenols: By means of RPLC-DAD-ESI-MS, out of the sixteen previously reported non-acidic simple phenols (Table 3); it was possible to confirm the presence of six compounds and the absence of eight compounds while results for only two compounds were inconclusive. For simple phenols with a molecular weight less than approximately 200, if their concentration does not allow detection by DAD, the MS data alone did not permit confirmation of their presence due to background noise and the complexity of the matrices.

The presence of tyrosol and hydroxytyrosol was determined using screening strategy 1 by comparison of retention time and UV-spectrum of authentic compounds. In the case of tyrosol,

confirmation by MS data was not possible as ionisation did not occur under the analysis conditions. Glucosides of both compounds eluted prior to the parent biophenols and were identified from UV, mass spectral and fluorescence data, plus relative retention and partitioning behaviour [13] according to screening strategy 2 (Table 3). There are three possible isomers of hydroxytyrosol glucoside: 1-glucoside (HG1), 3'-glucoside (HG3) or 4'-glucoside (HG4) (Figure 2). Although hydroxytyrosol glucoside is frequently reported in the literature [17,34,38,39] the position of glycosidation is rarely specified. Indeed, the three isomers have been identified in olive oil extract by NMR [40]. HG1 was identified as the major hydroxytyrosol glucoside in olive leaves based on its fragmentation pattern in MS/MS [32] and in vegetation water by ^{1}H NMR [24]. The occurrence of HG1 in olive is not surprising as it is a hydrolysis product of verbascoside. However, HG4 has also been identified as a major component of OMW and olive fruit [38,39]. The hydroxytyrosol derivative detected in this study has its *B*-band shifted to lower λ_{max} (273 nm) which confirmed glycosidation at the ring hydroxyl groups i.e. HG3 or HG4. Further distinction between the two structures was not possible from UV or mass spectral data. .

INSERT Figure 2.

In contrast to most literature reports on Mediterranean olive oil, fruit and OMW, only two benzoic acids (vanillic acid and syringic acid) and three cinnamic acids (caffeic acid, *p*-coumaric acid and chlorogenic acid) were detected in the extracts (Table 3) and all were minor components as reported previously for Australian olive fruits [17,41] and olive oil [14]. Whether this is due to geographical differences between Australian and Mediterranean olives or results from processing methodology is not clear. In plants, phenolic acids are mostly found in conjugated forms [42]. However, the level of phenols in Australian olives did not significantly change in response to acid or base hydrolysis [17].

Flavonoids: The only peaks attributable to anthocyanins were detected in the chromatograms of Frantoio fruits at 520 nm. These were assigned as cyanidin-3-*O*-rutinoside and delphinidin-3-*O*-glucoside respectively, based on their UV-Vis spectra, molecular masses and fragmentation pattern. Cyanidin-3-*O*-rutinoside and luteolin-rutinoside(s) are isobars, however they were easily differentiated from each other by their UV-Vis spectra and expected retention times on the reverse phase system. Cyanidin-3-*O*-rutinoside absorbs in the visible region at 525 nm, while luteolin-rutinoside(s) have their maximum at 340-350 nm. The positively charged cyanidin-3-*O*-rutinoside appeared earlier than its aglycone, cyanidin, as was expected. The overall MS detection sensitivity of anthocyanins under the present experimental conditions was low, though PIM was more sensitive than NIM.

Using the second screening procedure, two luteolin mono-glucosides and two luteolin rutinosides were detected (Table 3). Cardoso et al. [8] also reported two isomers of luteolin rutinoside. They identified the first eluting compound as luteolin-7-*O*-rutinoside on RPLC [8]. In the current study, the identity of the first eluting rutinoside was confirmed as luteolin-7-*O*-rutinoside by comparing the UV-Vis spectrum with that of Cardoso et al. [8]. The later eluting rutinoside isomer had a UV-Vis spectrum with λ_{max} of 336 nm which suggested a 4′-substitution. Hence, the isomer is tentatively assigned as luteolin-4′-*O*-rutinoside. In the case of the two luteolin glucosides, the early eluting isomer was identified as the most commonly reported luteolin-7-*O*-glucoside, λ_{max} 350 nm, and the later eluting isomer was assigned as luteolin-4′-*O*-glucoside based on its λ_{max} of 337 nm, and relative retention time [7].

A peak eluting at 26.17 min was identified as rutin from retention and mass spectral data. Rutin and hesperidin are isobars, molecular mass 610 amu, and the situation is further complicated by having an identical glycone part (rutinose); and the aglycones (quercetin and hesperitin) are also isobars. This provided a good example of the power of the RPLC-ESI-MS

fragmentation pattern to discriminate between isobars. Generally, 3-substituted flavonoids (flavonol-3-*O*-glycosides) such as rutin but not hesperidin (flavanone) form sodium adducts more easily [43]. Thus, the peak at *m/z* 633 is attributable to $[M + Na]^+$ of rutin. Furthermore, the peak at *m/z* 300 in NIM was due to quercetin radical aglycone, as flavonols such as rutin but not flavanones (hesperidin) can generate radical aglycone $[Y_0 - H]^{-\circ}$ at high energy ionisation [10]. The fragmentation system described by Cuyckens and Claeys [6] is used in this study. Methoxy flavonoids including hesperidin lose a methyl radical (15 amu) from the $[M + H]^+$ so readily that the $[M + H - CH_3]^+$ ion dominates the spectrum of the aglycone [10]. No peaks corresponding to the loss of methyl radical were found in the PIM mass spectrum, which again excluded the possibility of hesperidin. The retro-Diels-Alder fragment ion $^{0,2}B^+$ appeared at *m/z* 137 for quercetin and at *m/z* 151 for hesperitin due to the different substitution pattern on the C-ring. While *m/z* 137 was detected in the present study which confirms that the peak was due to rutin, it appears that previous studies misinterpreted the spectrum [14].

Isochromans and Lignans: Hydroxyisochroman derivatives are minor components that have recently been discovered in Italian commercial extra virgin olive oil [22]. There are no reports of their presence in olive fruits; this may be because they are formed during processing or storage of olive oil or their part per trillion concentrations (ng kg^{-1}). None of the two reported derivatives in olive oil were found in our samples (Table 3). Similarly, lignans have recently been identified in extra virgin olive oil [44] as major components. The major peak in the 280 nm chromatogram and TIC of Frantoio oil sample was identified as a mixture of pinoresinol and acetoxypinoresinol, λ_{max} 226 & 277 nm. Smaller amounts of hydroxypinoresinol and another late eluting derivative with molecular mass 432 amu were detected. The late eluting isomer was tentatively assigned as acetylhydroxypinoresinol. However, lignans were not detected in OMW or fruit samples.

Secoiridoids: These are an important group of compounds in olive. For example, oleosides are oleaceae-specific secoiridoids that are commonly esterified to a phenolic moiety as in oleuropein and ligstroside. As the non-phenolic secoiridoids are co-extracted with the phenolic fraction, they are included in this study. Although DAD was not particularly useful in screening for secoiridoids, RPLC-ESI-MS provided valuable insight and, of 22 previously reported secoiridoids, it was possible to confirm the presence of 17 compounds and absence of 2 compounds while results were inconclusive for 3 compounds (Table 3).

A number of isomers and/or isobars of the same secoiridoid molecular mass were detected. For instance, scanning TIC traces for the parent glycoside at *m/z* +541 and –539 revealed only two peaks eluting at 29.59 and 34.10 min corresponding to oleuropein and oleuroside, respectively (Table 3). Scanning for the aglycone at *m/z* +379 and –377 showed nine peaks (Figure 3). This is not unexpected as oleuropein is stabilised by the presence of the glucose residue. The removal of the glucose exposes the labile hemiacetal carbon C-1 (Figure 1) that undergoes ring opening, and a series of subsequent transformations can occur. Figure 4 summarises these transformations which can occur physiologically through biotransformations during fruit maturation or as artefacts during olive oil extraction or subsequent sample handling. The situation is further complicated by the possibility of five isobars of oleuropein aglycone, molecular mass 378. These can be identified as the cyclic aglycone (II), the acyclic enol form of oleuropeindial (I), the acyclic dialdehyde form of oleuropeindial (XII), the cyclic mono-aldehyde (XXV), and the lactone product of Cannizzaro reaction (XIV) (Figure 4). Keto-enol tautomerism results in racemisation around C-4 giving two diastereomers of oleuropeindial (XII) and also the cyclic mono-aldehyde (XXV) is diastereomeric. Similarly, for the oleuropein isomer, oleuroside (Table 3) (Figure 1), a range

of products is expected upon deglycosylation of the glycoside. Thus, the single biophenol, oleuropein can theoretically give rise to many compounds in an olive extract.

INSERT Figures 3 and 4

It was not possible to assign structures to the nine compounds of Figure 3. However, 11 of the compounds in Figure 3 are aldehydic and are expected to undergo nucleophilic addition reactions under acidic conditions i.e. hydration with water, acetylation with methanol, and sulfite adduct formation with bisulfite ions (Figure 5). In the present study, hydrates were not detected and methanol acetals were observed when sodium metabisulfite was not used in the extraction solvent. The formation of these adducts with characteristic mass spectral fragment ions, $[M - H + 82]^{-}$ and $[M + H + 82]^{+}$ due to the sulfite addition product, and $[M - H + 32]^{-}$ and $[M + H + 32]^{+}$, due to the methanol hemiacetal, provided a useful tool to confirm the aldehydic nature of eight of the nine compounds; namely, those eluting between 25 and 41 min (Table 3). Further structural assignment of these peaks to the individual aldehydic structures in Figure 4 was not feasible. The ninth peak, eluting at 15.68 min, was reported previously in Hardy's Mammoth olive fruits [7] and was identified as oleuropein aglycone. However, this peak can not be an oleuropein aglycone based on its UV-Vis spectrum (λ_{max} 225 and 333 nm), short retention time and mass spectral data (Table 3). Unlike the other 378 amu isobars, peak 9 (Figure 3) did not form addition products with methanol or with sodium metabisulfite indicating the absence of aldehydic groups.

INSERT Figure 5

An analogous situation exists for ligstroside and its corresponding aglycone with a molecular mass of 362. However, with ligstroside an oleuroside analog was not detected and only three ligstroside aglycone derivatives were observed of which one was aldehydic (Table 3).

Scanning for *m/z* −319 and +321 corresponding to oleuropein decarboxymethyl aglycone (3,4-dihydroxyphenylethyl alcohol-deacetoxy elenolic acid dialdehyde; 3,4-DHPEA-DEDA) (VIII, Figure 4), also known as 4-noroleuropein aglycone [45] and oleacin [9] revealed two peaks eluting at 27.28 and 27.71 min with similar fragmentation pattern suggesting diastereomers. The aldehydic nature of both peaks was confirmed by peak shifting due to formation of bisulfite addition products (eluting approximately 3 min earlier) and methanol acetals. The tyrosol analog, deacetoxyligstroside aglycone (4-hydroxyphenylethyl alcohol-deacetoxy elenolic acid dialdehyde; 4-HPEA-DEDA or oleocanthal [50]) also eluted as two peaks (Table 3). Both 3,4-DHPEA-DEDA and 4-HPEA-DEDA have been identified previously as their dialdehyde form in Australian olive fruit and olive oil [7,14]. The diastereomers of 4-HPEA-DEDA were not investigated any further due to their ultra-trace concentration. Both NIM and PIM had good sensitivity; nevertheless PIM was more sensitive.

While oleoside (XVII, Figure 4) (Figure 6) was detected as a single peak in TIC for Frantoio OMW and Correggiola OMW, in Frantoio fruit another peak with the same molecular mass of 390 amu eluted one minute after oleoside. Two isomers (isobars) of oleoside are known i.e. secologanoside and secologanol (Figure 6). Both isomers are expected to elute after oleoside on RPLC. Close examination of the fragmentation pattern of the detected oleoside isomer revealed a neutral loss of 44 (Table 3) which indicates a free carboxyl group. Hence, the second peak was tentatively assigned as secologanoside which was supported by the detection of secologanoside derivatives e.g. oleuroside.

INSERT Figure 6.

A peak with molecular mass 552 amu, eluting at 31.74 min was detected in fruit and waste extracts but not in oil extract (Table 3). A compound with the same mass spectrum was previously identified as a caffeoyl ester of secologanoside [30] and also tentatively assigned as the disaccharide, 6′-β-glucopyranosyl oleoside [8]. However, in the Cardoso study [8], oleoside eluted at 16.2 min and the compound assigned as 6′-β-glucopyranosyl oleoside eluted at 44.6 min which violates the basic principles of RPLC where glycosidation decreases the retention time. This compound had λ_{max} of 327 and 245 nm with a shoulder at 305 nm which suggested the presence of a caffeoyl moiety. This was supported by mass spectral fragmentation data although not unambiguously. The caffeic acid can be attached to the carboxyl group of the secologanoside (as in oleuroside and oleuropein) or to the glucose residue (as in verbascoside). The bathochromic shift of the caffeic acid in the compound under investigation suggested that the esterification happened with the carboxyl of the caffeic acid to a hydroxyl group on the sugar residue. This was also confirmed by the detection of *m/z* 325 in PIM which is characteristic for caffeoylhexose [6] and the compound was tentatively assigned as caffeoyl-6′-secologanoside (cafselogoside).

3.3. Biophenol profiles in fruit, oil and waste

Application of the screening strategy to fruit, oil and waste allowed some insights into transfer and transformation of biophenols from fruit to end products – oil and waste. Although olive fruit, oil and OMW were dominated by the same biophenolic classes, namely, secoiridoids, simple phenols, and flavonoids, the biophenol profile of the fruit and OMW were similar but differed significantly from that of oil. The distribution of these classes between oil and OMW in general followed a simple partitioning model, where the glycosides

found in the fruit were transferred into the OMW without much hydrolysis or degradation. None of the fruit glycosides were detected in the olive oil. Previous reports of glycosylated derivatives in olive oil samples [40,51] can be ascribed to the use of three-phase processing and formation of microemulsions in these earlier studies. The only flavonoid aglycones identified in the olive oil were luteolin and chrysoeriol, whereas apigenin, which is more lipophilic than luteolin, surprisingly did not appear in the oil. Anthocyanin content was not high in the midseason olive fruits; only a small amount of cyanidin rutinoside and delphinidin glucoside were detected. Neither of these anthocyanins was detected in OMW most probably due to their degradation or complexation to form a red pigment [13]. Apart from anthocyanins and some minor flavonoid glycosides, the flavonoidal profile of the fruit is mostly transferred to OMW. Similar to flavonoids, only the aglycones of the secoiridoids, oleuropein aglycones, ligstroside aglycones and elenolic acid were detected in olive oil. All the fruit secoiridoids were present in OMW except for demethyloleuropein and secologanoside.

Lignans were totally absent in the fruit and OMW, yet they constituted a major biophenolic class in the oil. Pinoresinol and its derivatives were artefacts that were formed during the processing and/or the short storage of the olive oil.

Conclusion

The chemical diversity of the various biophenolic classes comprises an analytical challenge for any comprehensive chemical screening program. In this work, FLD was inappropriate for general screening, but has potential for targeted screening of specific compound(s) or a class of compounds e.g. hydroxytyrosol and tyrosol derivatives. Neither DAD nor ESI-MS alone accommodated the diverse nature of the compounds encountered in olive samples. It was their combined use in the on-line mode with RPLC that enabled fast, efficient and facile screening of such a plethora of biophenols. Out of nearly 100 previously reported biophenols screened

in this study, it was possible to confirm the presence of 52 compounds. By careful examination of the combined DAD and ESI-MS data, extra information was elucidated including: the site of glycosidation on the phenol ring of hydroxytyrosol; the identity of the other luteolin-glucoside isomer as luteolin-4′-*O*-glucoside; identifying rutin rather than the previously reported hesperidin (and the reasons for possible mis-assignment); and the detection of diastereomers of 4-HPEA-DEDA and 3,4-DHPEA-DEDA. The demonstration that multiple classes of biophenols can be screened in a single run is an advance on traditional approaches whereby typically a single class is evaluated based on a predetermined extraction solvent (e.g. phenolic acids extracted into ethyl acetate).

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Table 1. Retention times (T_R), UV-Vis spectra and fluorescence data ($\lambda_{excitation}$ 280 nm, $\lambda_{emission}$ 330 nm) of reference biophenols

Standard Biophenol	T_R	Maximum Absorbance (λ_{max}) nm	Fluorescence
Gallic acid	4.63	226, 271	No
Homogentisic acid	5.80	219, 288	Yes
Hydroxytyrosol	7.05	219, 277	Yes
Protocatechuic acid	7.61	217, 257, 291	No
Catechol	9.00	218, 275	Yes
Tyrosol	9.77	217, 273	Yes
4-Hydroxybenzoic acid	10.88	209, 252, 286	Yes
Chlorogenic acid	10..92	238, 303, 324	No
4-hydroxyphenyl acetic acid	11.60	226, 275	Yes
Vanillic acid	11.92	257, 289	Yes
Caffeic acid	12.39	214, 238, 299sh, 321	No
Homovanillic acid	12.46	223, 276	Yes
Syringic acid	12.84	220, 272	Yes
Cyanidin chloride	14.20	272, 520	No
Loganin	16.50	233	No
***p*-Coumaric acid**	19.16	223, 307	No
Ferulic acid	20.86	233, 292sh, 320	No
Sinapic acid	21.03	233, 322	No
Verbascoside	23.14	219, 242, 305sh ,329	No
Rutin	26.15	219, 253, 353	No
***o*-Coumaric acid**	26.75	273, 323	No
Ellagic acid	27.50	210, 250, 305sh, 351sh,& 366	No
Hesperidin	27.70	221, 281, 327	No

Oleuropein	29.59	228, 278	No
Apigenin-7-*O*-glucoside	31.50	220, 263, 335	Yes
Cinnamic acid	36.82	215, 275	No
Quercetin	39.00	220, 252, 370	No
Luteolin	40.62	251, 348	No
Apigenin	44.64	221, 263, 335	No

Table 2. ESI-MS data of reference biophenols at a cone voltage 70 V.

Standard Biophenol	Major peaks in negative ion ESI-MS	Major peaks in positive ion ESI-MS
Gallic acid	**215** (7), **169** (100), **125** (15), **97** (5)	**359** (26), **199** (8), **171** (13), **153** (100), **125** (27), **99** (15), **97** (21), **81** (30)
Hydroxytyrosol	**199** (17), **153** (100), **123** (35), **97** (2)	**155** (18), **137** (100), **119** (24), **102** (12), **91** (23), **88** (11)
Tyrosol	**137** [a]	[b] **—**
4-hydroxyphenyl acetic acid	**197** (17), **151** (100), **107** (51)	[b] **—**
Caffeic acid	**179** (100), **135** (36)	**181** (2), **163** (18), **135** (15), **117** (14), **89** (100)
Verbascoside	**1247** (2), **669** (1), **623** (30), **461** (9), **207** (15), **179** (7), **161** (100), **153** (2), **135** (11)	**647** (70), **625** (2), **479** (4), **471** (4), **325** (27), **163** (100), **135** (36), **117** (26), **89** (45)
Rutin	**655** (7), **611** (6), **610** (29), **609** (88), **461** (2), **302** (16), **301** (97), **300** (100), **179** (7), **161** (12), **151** (4)	**633** (37), **611** (2), **519** (10), **485** (4), **465** (1), **331** (9), **304** (28), **303** (100), **219** (3), **169** (3), **137** (5), **85** (33)
Oleuropein	**1079** (3), **585** (8), **539** (60), **377** (18), **307** (30), **275** (44), **223** (13), **153** (6), **151** (1), **149** (49), **139** (59), **101** (79), **95** (50), **89** (100)	**563** (40), **541** (1), **401** (14), **361** (9), **165** (7), **137** (41), **119** (12), **91** (100)
Quercetin	**603** (7), **369** (4), **301** (100), **265** (1), **247** (1), **147** (2), **97** (8)	**325** (14), **303** (98), **285** (10), **257** (29), **229** (65), **201** (25), **165** (25), **153** (100), **137** (47), **121** (19), **111** (27)

Luteolin	**571** (1), **285** (100), **241** (3), **175** (20), **151** (33), **133** (87), **107** (34)	**309** (3), **287** (100), **241** (6), **213** (4), **185** (12), **161** (7), **153** (70), **135** (25), **117** (2)

Peaks are in bold, the pseudomolecular ions are underlined, and intensities relative to the base peak are given in parentheses, [a] very low intensity; [b] No peaks detected

Table 3. Screening of reported biophenols in olive samples with retention times and mass spectral data.

Class/ Biophenol	T_R	MW [a]	Major ESI$^-$ Peaks	Major ESI$^+$ Peaks	FO	FF	FW	CW	Comment [b]	Ref.
Simple phenols										
Catechol	9.00	**110**			-	-	-	-	I	[23]
Cornoside		**316 A**			-	-	-	-	III	[24]
3,4-dihydroxyphenylglycol		**170 B**	169	193, 171,	*	*	*	*	III	[23,24]
4-methylcatechol		**124**			-	-	-	-	II	[23]
Halleridone		**154 C**			-	*	*	-	IV	[5]

D(+)-erythro-1-(4-hydroxy-3-methoxy)-phenyl-1,2,3-propantriol	7.80 or 8.95	**214**	427[e], 259[c], 213, 195, 151	429[f], 237[d], 215, 197, 155	+	++	+	+	III	[23]
Hydroxytyrosol	7.05	**154 C**	307[e], 153	155, 137	+++	+++	+++	+++	I	[13]
Hydroxytyrosol glucoside	6.19	**316 A**	315, 179, 153	317, 155	-	+++	+++	+++	II	[13]
Hydroxytyrosol acetate		**196 D**			-	-	-	-	IV	[5]
Tyrosol	9.77	**138 F**			++	++	++	++	I	[13]
Tyrosol glucoside (Salidroside)	8.55	**300 E**	599[e], 299, 227	601[f] , 323[d], 301, 229	-	-	-	++	III	[25]
Tyrosol acetate		**180 G**			-	-	-	-	IV	[5]
3-methoxy-4-hydroxyphenyl ethanol		**168 H**			-	-	-	-	IV	[5]
1-*O*-[2-(3,4-dihydroxy) phenylethyl]-(3,4-dihydroxy)phenyl-1,2-ethandiol		**306**			-	-	-	-	IV	[23]
1-*O*-[2-(4-hydroxy) phenylethyl]-(3,4-dihydroxy)phenyl-1,2-ethandiol		**290**			-	-	-	-	IV	[23]
4-[β-D-xylopyranosyl-(1→6)]-β-D-glucopyranosyl-1,4-dihydroxy-2-		**434**	479[c], 433, 281, 199, 149	457[d], 435, 303, 284, 229, 141	-	++	++	-	II	[23]

methoxybenzene	4.93									
Benzoic acids										
Gallic acid	4.63	**170 B**			-	-	-	-	I	[5]
Protocatechuic acid	7.61	**154 C**			-	-	-	-	I	[5]
2,4-Dihydroxybenzoic acid		**154 C**			*	*	*	*	IV	[5]
2,6-Dihydroxybenzoic acid		**154 C**			*	*	*	*	IV	[5]
4-Hydroxybenzoic acid	10.88	**138 F**			-	-	-	-	I	[23]
4-Hydroxybenzaldehyde [i]		**122**			*	*	*	*	IV	[26]
3,4,5-Trimethoxybenzoic acid		**212**			*	*	*	-	IV	[26]
Syringic acid	12.84	**198**	197, 152	221[d], 199, 154	+	-	-	-	I	[5]
3,4-dimethoxybenzoic (Veratric) acid		**182 I**			-	-	-	-	I	[26]
2,6-Dimethoxybenzoic acid		**182 I**			-	-	-	-	II	[26]
Syringaldehyde [i]		**182 I**			-	-	-	-	II	[26]
Homovanillic acid	12.46	**182 I**			-	-	-	-	II	[5]

Vanillic acid	11.92	**168 H**			++	++	++	-	I	[4]
3,4-Dihydroxyphenylacetic acid		**168 H**			-	-	-	-	I	[23]
2,5-Dihydroxyphenylacetic (homogentisic) acid	5.80	**168 H**			-	-	-	-	I	
Vanillin [i]		**152 J**			-	-	-	-	II	[26]
4-hydroxyphenylacetic acid	11.60	**152 J**			-	-	-	-	I	[5]
3,4-dimethoxyphenyl acetic (Homoveratric) acid		**196 D**			-	-	-	-	II	[27]
Cinnamic acids										
Caffeic acid	12.39 co	**180 G**	179	361[f], 181	+	++	++	++	I	[4]
Cafeoylglucose		**342**			-	-	-	-	II	[26]
Caftaric acid		**312**			-	-	-	-	II	
5-caffeoylquinic acid (Chlorogenic acid)	10.92 co	**354**	353, 191, 179	355	-	+	+	+	I	[4]
Cinnamic acid	36.82	**148**			-	-	-	-	I	[26]
o-Coumaric acid	26.75	**164 K**			-	-	-	-	I	[4]

p-Coumaric acid	19.16	**164 K**	163	165	+	-	-	-	I	[4]
Ferulic acid	20.86	**194**			-	-	-	-	I	[25]
Sinapic acid	21.03	**224**			-	-	-	-	I	[25]
Verbascoside	23.14	**624 L**	669[c], 623, 605, 461, 299, 161	647[d], 625, 479, 471, 325, 163, 147	-	+++	+++	+++	I	[12]
Isoacteoside	26.58	**624 L**	669[c], 623, 459, 297, 161, 135	647[d], 625, 479, 471, 325, 181, 163	-	++	++	++	II	[28]
β-Hydroxy verbascoside		**640**			-	-	-	-	IV	[29]
β-Ethanol-acteoside		**668**			-	-	-	-	IV	[30]
Verbascoside (ferulic) derivative		**638**			-	-	-	-	IV	[30]
Flavonoids										
Cyanidin	14.20	**287 M**			**-**	-	-	-	I	[26]
Cyanidin-3-*O*-glucoside		**449 N**			**-**	-	-	-	II	[4]
Cyanidin-3-*O*-rutinoside	7.74	**595 P**		595, 449, 287	**-**	++	-	-	II	[4]
Delphinidin		**303**			-	-	-	-	II	[5]
Delphinidin-3-*O*-glucoside	6.74	**465**		465, 303	-	+	-	-	II	[5]

Apigenin	44.64 co	**270**	269, 227, 161, 153, 139	271, 229, 149, 121	-	++	++	++	I	[4]
Apigenin-7-*O*-glucoside	31.50	**432 Q**	431, 269, 199, 179	455[d], 433, 271	-	+	-	-	I	[4]
Apigenin-7-*O*-rutinoside	29.75	**578**	577, 431, 269	579, 433, 293, 271	-	++	++	-	II	[4]
Luteolin	40.62	**286 M**	285, 151, 133	287, 153, 135	+	+++	+++	+++	I	[5]
Luteolin-4′-*O*-glucoside	30.39	**448 N**	447, 377, 285	449, 287	-	++	++	++	II	[5]
Luteolin-6-*C*-glucoside (Homoorientin)		**448 N**			-	-	-	-	II	[4]
Luteolin-7-*O*-glucoside	24.80	**448 N**	447, 285	449, 287	-	++	++	+++	I	[4]
Luteolin-8-*C*-glucoside (Orientin)		**448 N**			-	-	-	-	II	[4]
Luteolin-3′,7-*O*-diglucoside		**610 R**			-	-	-	-	II	[4]
Luteolin-7-*O*-rutinoside	24.40	**594 P**	593, 447, 285	595, 449, 287	-	++	++	++	II	[4]
Luteolin-4′-*O*-rutinoside	25.90	**594 P**			-	++	++	++	II	[4]
Quercetin	39.00	**302 S**	301	303	-	-	-	+	I	[12]
Quercetin-3-rhamnoside (Quercetrin)	32.35	**448 N**	447, 301, 300	449, 303	-	-	+	-	II	[4]

Rutin (Quercetin-3-rutinoside)	26.17 co	**610 R**	609, 301, 300	633[d], 611, 465, 303, 137	-	+++	+++	+++	I	[13]
Hesperitin		**302 S**			-	-	-	-	II	[4]
Hesperidin (hesperitin-3-rutinoside)	27.70	**610 R**			-	-	-	-	I	[4]
Chrysoeriol	44.92	**300 E**	299, 284, 149	323[d], 301, 286, 151, 135	+	+	+	-	III	[31]
Isochromans										
1-(3'-methoxy-4'-hydroxy)phenyl-6,7-dihydroxyisochroman		**288**			-	-	-	-	IV	[5]
1-phenyl-6,7-dihydroxyisochroman		**242 T**			-	-	-	-	IV	[5]
Lignans										
Pinoresinol	25.13 co	**358**	357, 339	359, 341	+++	-	-	-	II	[19]
Hydroxypinoresinol	24.77	**374**	373, 357, 339, 151	375, 359, 341	++	-	-	-	III	[19]
Acetoxypinoresinol	24.99 co	**416 U**	831[e], 461[c], 415, 373, 371,151	833[f], 439[d], 417, 359, 357, 319	+++	-	-	-	II	[19]

Acetylhydroxypinoresinol	27.14	**432 Q**	431	433[d], 417,	++	-	-	-	III	
Secoiridoids										
Demethyloleuropein	21.12	**526**	525, 447, 323, 241	549[d], 527, 365, 347, 137	-	++	-	-	III	[5]
Demethylligstroside		**510**			-	-	-	-	IV	[5]
Oleuropein	29.59	**540 V**	539, 377, 307, 275, 223	563, 541, 379, 361, 243, 225, 207, 165, 137	-	++	++	++	I	[4]
Oleuroside	34.10	**540 V**	539, 377, 307, 275, 223	563[d], 541, 379, 361, 243, 225, 207, 165, 137	-	++	++	++	II	[5]
Ligstroside	31.16	**524**	523, 385, 303, 223	547[d], 525, 387, 305, 225	-	+	+	+	III	[24]
Oleuropein diglucoside	25.43	**702**	701, 539, 377	703, 541, 379, 361, 225, 137	-	-	-	+	IV	[32]
Nüzhenide	25.86 [co]	**686**	685, 523, 229, 223	709[d], 687, 525, 507, 369, 225	-	++	++	++	III	[5]
Oleuropein aglycone derivative 1 (aldehyde)	25.57	**378 W**	459[h], 377, 307, 275, 241, 153, 149, 139	483, 461[h], 379, 347, 189, 137	++	++	++	++	III	

Oleuropein aglycone derivative 2 (aldehyde)	27.20	**378 W**	459[h]	461[h]	++	++	++	+	III
Oleuropein aglycone derivative 3 (aldehyde)	28.30	**378 W**	459[h], 377, 153	461[h], 379, 361, 137	++	++	++	++	III
Oleuropein aglycone derivative 4 (aldehyde)	35.61	**378 W**	459[h], 377, 241, 153	461[h], 379	-	++	++	-	III
Oleuropein aglycone derivative 5 (aldehyde)	36.90	**378 W**	459[h], 377	461[h], 379	-	+	+	-	III
Oleuropein aglycone derivative 6 (aldehyde)	38.12	**378 W**	459[h], 377	461[h], 379	-	+	+	-	III
Oleuropein aglycone derivative 7 (aldehyde)	40.19	**378 W**	459[h], 377	461[h], 379	-	+	+	-	III
Oleuropein aglycone derivative 8 (aldehyde)	40.91	**378 W**	459[h], 377	461[h], 379	-	+	+	-	III
Ligstroside aglycone derivative 1 (aldehyde)	32.17	**362 X**	443[h], 361, 291, 241, 137	445[h], 363, 245	-	+	+	+	III
Ligstroside aglycone derivative 2	35.75	**362 X**	361	363	-	+	+	+	III
Ligstroside aglycone derivative 3	27.00	**362 X**	361, 333	363	+	-	-	+	III

3,4-DHPEA-DEDA (Oleuropein aglycone decarboxymethyl dialdehyde form)	27.28, 27.71	**320**	401[h], 319, 301, 195	403[h], 321, 303	++	++	++	+++	III	[33]
4-HPEA-DEDA (Ligstroside aglycone decarboxymethyl dialdehyde form)	34.02, 34.59	**304**	385[h], 303, 285, 179	387[h], 327[d], 305, 287	++	++	++	+++	III	[4]
Elenolic acid 1	18.55	**242 T**	323[h], 241, 139	325[h], 265[d], 243	+++	++	++	++	III	[5]
Elenolic acid 2	19.27	**242 T**	323[h], 241	243	+++	++	++	++	III	
Elenolic acid 3	14.00	**242 T**	241	243	++	-	-	-	III	
Elenolic acid glucoside (Oleoside-11-methyl ester)	13.96	**404**	807[e], 449[c], 403, 241,	809[f], 427[d], 405, 243, 225, 165	-	++	+	+	III	[7]
Elenolic acid diglucoside		**566**			-	-	-	-	IV	[34]
Oleoside	11.38	**390 Y**	435[c], 389, 244, 183	413[d], 391, 229, 211, 193	-	++	+	++	III	[8]
Secologanoside	12.10	**390 Y**	389, 345, 225, 183	391, 362, 211	-	++	-	-	III	
2*H*-pyran-4-acetic acid, 3-hydroxymethyl-2,3-dihydro-5-(methoxycarbonyl)-2-methyl-methyl ester		**258**	257	259	*	*	*	-	IV	[25]

3-[1-(hydroxymethyl)-1-propenyl]-δ-glutarolactone		**184**	183	185	*	-	-	-	IV	[35]
3-[1-(hydroxymethyl)-1-propenyl]-δ-glutarolactone hydrate		**202**	201, 183	225[d], 203, 185	-	*	*	*	IV	[35]
Caffeoyl-6′-secologanoside	31.74	**552**	551, 507, 389, 385, 341, 303, 281, 251, 179, 161	575[d], 553, 325, 305, 181, 163	-	++	++	++	III	[30]
comselogoside	36.32	**536**	535, 491, 389, 345, 265, 163, 145	559[d], 537, 489, 309, 293, 165, 147	-	+++	+++	+++	III	[15]
hydroxytyrosyl acyclodihydroelenolate (HT-ACDE)	27.05	**382**	763[e], 427[c], 381, 363, 349, 245, 227, 153, 151	765[f], 405[d], 383, 365, 229, 137	-	+++	+++	-	III	[15]
Unknown compounds										
Molecular Mass 408	7.51	**408**	815[e], 443[c], 407, 245, 227, 151	839[g], 817[f], 431[d], 409, 391, 247, 229, 211, 197, 169, 155	-	++	++	++	III	
Molecular Mass 402	12.53	**402**	447[c], 401, 351, 269,	425[d], 403, 353, 271,	-	++	++	++	III	

			153	229, 177					
Molecular Mass 378	15.68	**378 W**	377, 197, 153	401[d], 379, 217, 199	-	++	++	++	III
Molecular mass 416	17.12	**416 U**	461[c], 415, 241	439[d], 417, 243	-	-	++	-	III
Molecular Mass 366	33.03	**366**	411[c], 365, 347, 333, 227	389[d], 367, 349, 229, 121	++	++	++	++	III

MW= Molecular weight; ESI$^-$ = Negative Ion Mode; ESI$^+$ = Positive Ion Mode; FO= Frantoio oil; FF= Frantoio Fruit; FW= Frantoio OMW; CW= Correggiola OMW; T_R= Retention Time (min); in the case of aldehydic species this is quoted for the sulfite addition compound; [a] isobars are indicated using same uppercase letter; [b] I- Identification confirmed by the use of reference compound; II- Identification confirmed by the use of DAD and comparison with literature values; III- Tentative Assignment of structure based on fragmentation pattern; IV- Detection by scanning of TIC traces (generating reconstructed ion chromatograms); [c] $[M - H + HCOOH]^-$; [d] $[M + Na]^+$; [e] $[2M - H]^-$; [f] $[2M + H]^+$; [g] $[2M + Na]^+$; [h] pseudomolecular ion of bisulfite addition product [M + 82]; [i] Aldehydes are grouped under the corresponding phenolic acid class; [co] coeluting

compounds are described as: (-) absent, (+) traces, (++) present (detected equally well in DAD and MS), (+++) a major peak in DAD chromatograms, and (*) the evidence for its presence is inconclusive.

Figure 1. Numbering system used for elenolic acid chemical structures of biophenols present in oleuropein standard

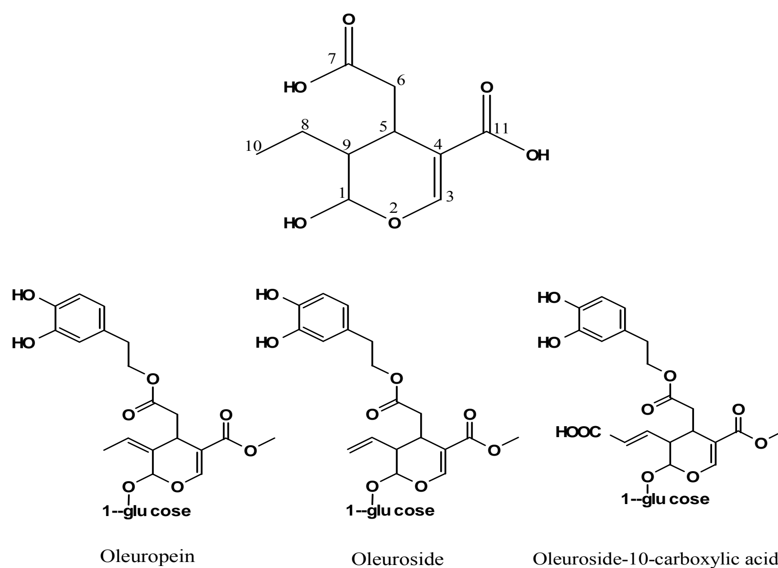


Figure 2. Chemical structures of different hydroxytyrosol glucosides

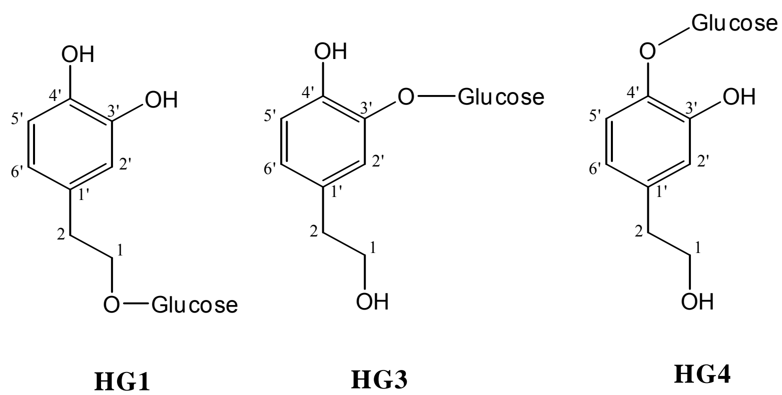


Figure 3. Reconstructed mass chromatograms for oleuropein aglycones (molecular mass of 378 amu) and the corresponding bisulfite adduct (molecular mass 460 amu) in Frantoio OMW extract. Only peak (9) was non aldehydic (did not form bisulfite addition product with sodium metabisulfite).

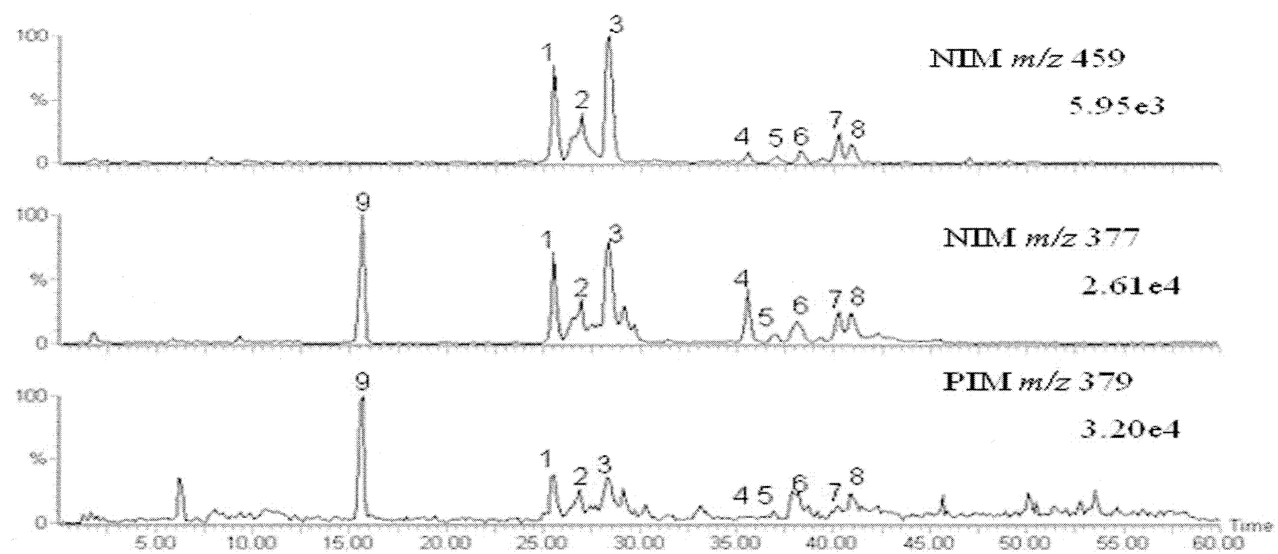
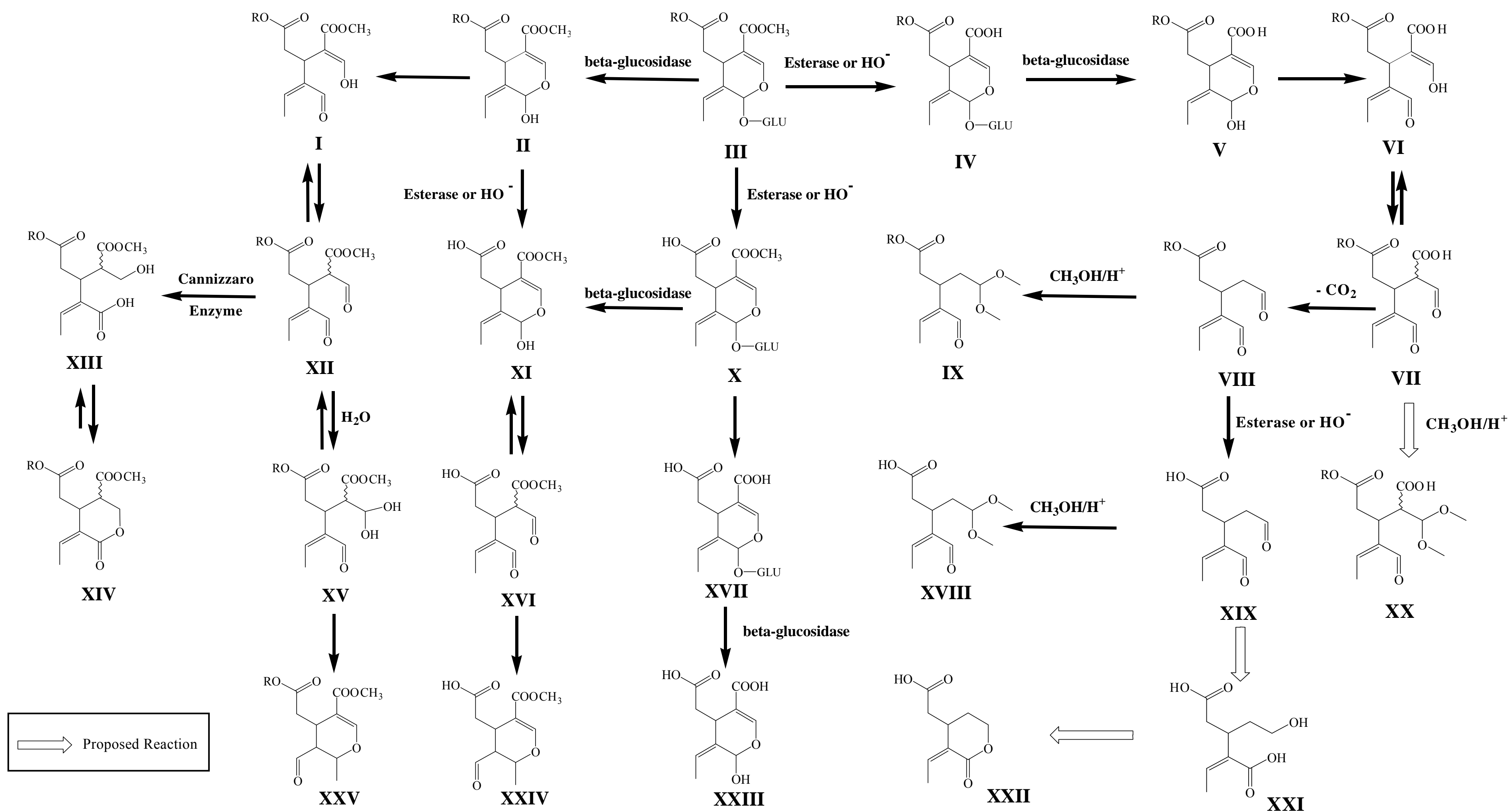


Figure 4. Transformations of oleuropein (R = hydroxytyrosol) during maturation (biotransformation), processing, extraction, and sample handling. Compounds (with molecular mass) are identified as follows: (I) oleuropeindial, enol form (378), (II) oleuropein aglycone (378), (III) oleuropein (540), (IV) demethyloleuropein (526), (V) demethyloleuropein aglycone (364), (VI) enol form of demethyloleuropein aglycone (364), (VII) demethyloleuropein aglycone dialdehyde (364), (VIII) 4-noroleuropein aglycone (3,4-dihydroxyphenyl ethyl alcohol decarboxymethyl elenolic acid dialdehyde or 3,4-DHPEA-DEDA) (320) [33,45] (IX) 3,4-DHPEA-DEDA acetal (366) [33], (X) oleoside methyl ester (404), (XI) elenolic acid (242), (XII) oleuropeindial (keto form) (378), (XIII) Cannizzaro-like product of oleuropeindial (396) [46], (XIV) lactone of XIII (378) [46]; (XV) oleuropeindial (monohydrate), (XVI) elenolic acid dialdehyde (242), (XVII) oleoside (390), (XVIII) acetal of XIX, (XIX) decarboxymethyl elenolic acid dialdehyde DEDA (184), (XX) demethyloleuropein aglycone acetal (410), (XXI) Cannizzaro-like product of XIX, (XXII) lactone form of XXI [25], (XXIII) demethyl elenolic acid (228), (XXIV) elenolic acid mono-aldehyde (rearrangement product) [47], (XXV) hydroxytyrosol elenolate (oleuropein aglycone aldehyde form or 3,4-DHPEA-EA) (378) [48,49]. A corresponding range of compounds is observed for ligstroside in which R = tyrosol.


beta-glucosidase
Esterase or HO$^-$
beta-glucosidase
Esterase or HO$^-$
Esterase or HO$^-$
Cannizzaro
Enzyme
beta-glucosidase
CH_3OH/H^+
- CO_2
H_2O
Esterase or HO$^-$
CH_3OH/H^+
CH_3OH/H^+
beta-glucosidase
Proposed Reaction
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
XIII
XIV
XV
XVI
XVII
XVIII
XIX
XX
XXI
XXII
XXIII
XXIV
XXV

⇨ Proposed reaction

Figure 5. Structure of secoiridoid dialdehydes and their extraction solvent artefacts: (I) monohydrate derivative forms in water; (II) methanol hemiacetal derivative forms in methanolic solvents; (III) bisulfite addition product forms when sodium metabisulfite is used to preserve the hydroalcoholic extract.

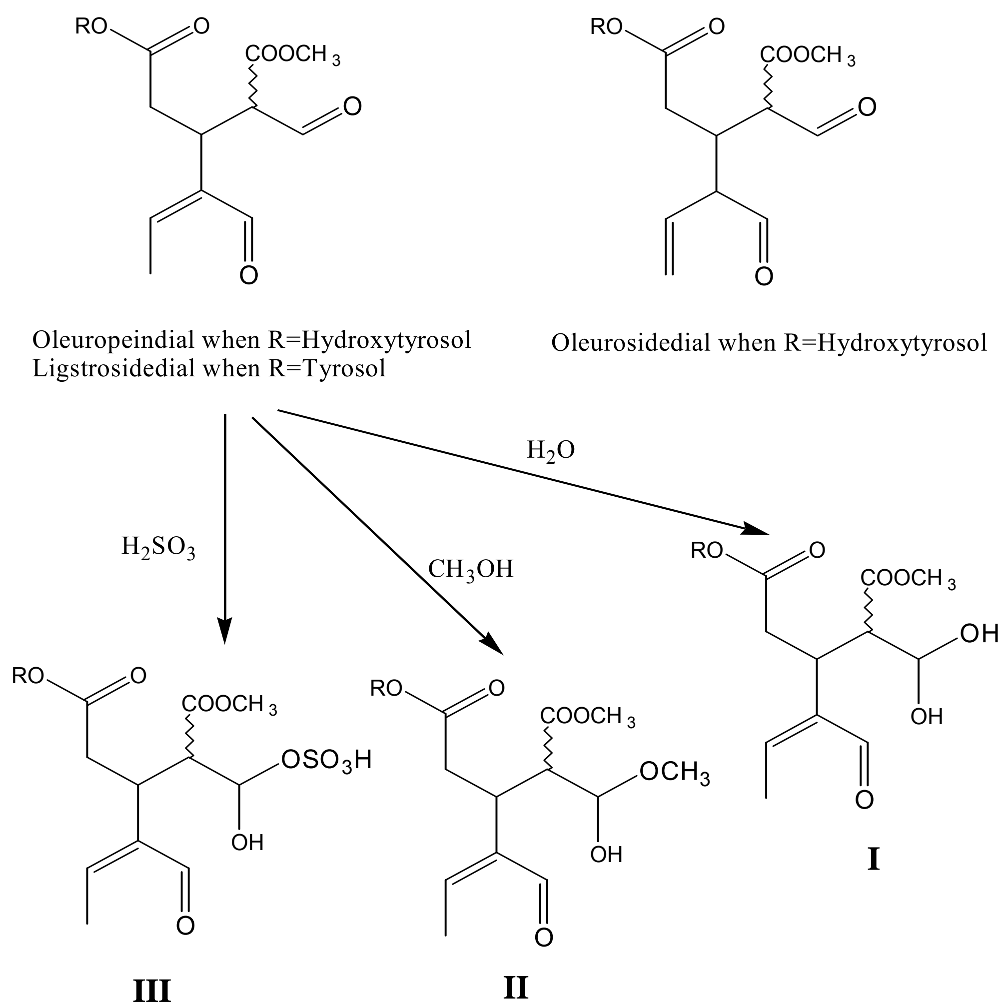


Figure 6. Isobars of oleoside (see XVII in Figure 4).