UNIVERSITY OF NOVA GORICA
GRADUATE SCHOOL

OLIVE FRUIT PHENOLS IN OLIVE OIL PROCESSING: THE FATE AND ANTIOXIDANT POTENTIAL

DISSERTATION

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Nova Gorica, 2014

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ABBREVIATIONS AND SYMBOLS

AOP - antioxidant potential

DAD - diode array

DPPH - 2,2-diphenyl-1-picrylhydrazil radical

DW - dry weight

EIC - extracted ion chromatograms

ESI - electrospray ionisation

FW - fresh weight

GRAS - generally recognized as safe

HR-MS - high resolution mass spectrometry

MW - molecular weight

NIM - negative ionisation mode

PIM - positive ionisation mode

RP-LC - reversed phase liquid chromatography

R_t - retention time

TIC - total ion chromatograms

TOF - time of flight

TP - total phenols

UPLC- ultra high pressure liquid chromatography

US - ultrasound

USLE - ultrasound-assisted solid liquid extraction

US-LLE - ultrasound-assisted liquid liquid extraction

UV-Vis - ultraviolet-visible

PREFACE

Phenolic compounds have a long history of scientific investigations, and yet they are still attracting considerable research efforts. The study investigating the fate of olive fruit phenols during olive oil processing was conducted due to increasing evidence of their double nature role, being recognized as food antioxidants and environmental pollutants.

The new demands for sustainability and functionally in olive oil industry are continuously stimulating the search for new technological improvements. To cope with emerging trends, the fruit phenols transfer, transformation and partition trail was evaluated in relation to some of the prominent technological variables as only such knowledge can facilitate manipulation of their levels and occurrence in the food and waste matrices. Understanding of the relationship between the initial and final products is essential for exploiting different operative conditions, and the variability of input and output matrices assessment important in the recognition of their quality and value to mankind.

The following doctoral thesis is organised into seven main and several sub-chapters each covering a single topic. The introductory chapter briefly presents the current knowledge of research topic and its related problems, while the research objectives are presented below. The theoretical background reports a state-of-the art review of phenolic compounds and their occurrence in olive matrices with emphasis on the technological variables influencing their behaviour during olive oil processing. The fourth chapter describes the experimental design of doctoral research, while the fifth presents its results along with interpretative discussion. The concluding remarks from all are given in conclusions and some of the forward research perspectives are provided at the end.

The ultraviolet-visible (UV-Vis) and mass spectra (MS) of individual phenolic compounds are placed in the annexes along with an already published papers, covering phenols extraction optimisations from olive fruit, olive mill wastewater and olive oil matrices (**Annex B1–B3**) and their fate assessment during commercial olive oil processing preliminary evaluated at industrial-scale level (**Annex B4**). These results are placed in the appendix with publishers' permission and are not separately presented among the main body of doctoral results, but rather in a comparative way discussed with yet unpublished results obtained in a fully controlled lab-scale olive oil processing trial using the same starting fruit material and improved ultra high pressure liquid chromatography system coupled with diode array and electrospray ionisation time-of-flight high resolution mass spectrometry (UPLC-DAD-ESI-QTOF-HRMS) detections.

Likewise, the study using kinetic 2,2-diphenyl-1-picrylhydrazil (DPPH) radical scavenging method for the antioxidant potential (AOP) assessment of olive fruits is not separately presented (**Annex B5**). One of the initial research aims involved its application for AOP behaviour evaluation during olive oil production, but has owing to long reaction times not been further used for the samples entailed in the processing. Their AOP behaviour was only assessed using a shorter, *i.e.* static DPPH method at industrial-scale, comparing three commercially available extraction systems, and described in the paper placed in the appendix (**Annex B4**). However, as the latter was positively correlated with the total phenol (TP) content of individual olive matrices, we reasonably not assessed it again in a lab-scale experiment conduced on Abencor system.

All the work, including conceptual and experimental design, analyses, interpretation of results, up to the writing of thesis and of already published papers were performed by the author Tina Jerman Klen, while the other co-authors of papers contributed to their results in terms of the technical, scientific and/or moral support.

1 INTRODUCTION

Olive phenols have drawn increased attention over the past few decades owing to diverse range of bioactivities such as antioxidant, antimicrobial, anticancer and others (Obied et al., 2007a; Obied et al., 2005) assigning them as one of the most valuable and perspective dietary compounds ever. The majority of research interests till now have mainly focused on their quali- and quantitative analyses, new discoveries, structure elucidations and/or to their health-related studies. The benefits from latter are now widely documented (Servili et al., 2009; Covas, 2007; Fitó et al., 2007; Covas, 2006) adding on to a continuous growth of their products consumption, in particular of olive oil. However, as olive phenols may beneficially influence the human health, they on contrary contribute to serious environmental problems *via* toxicity against some of terrestrial and aquatic organisms (Obied et al., 2007b). Olive oil processing has been recognised as one of the most problematic in terms of environmental pollution, as it, next to olive oil (20%), produces huge amounts of two waste matrices known as pomace (30%) and wastewater (50%) causing trouble for its sustainable development. Interestingly, both of them are in turn valorised by several pharmaceutical and food industries mainly due to their high phenolic content. Olive phenols double nature thus constitutes a challenge from many aspects and their regulation during olive oil processing one of the key research/technological opportunities nowadays.

However, only in recent years the research attention has focused on the processing and its role in the phenol profile shaping of priced olive matrices. The former consists of three essential steps, including olives crushing, paste malaxation and olive oil separation, with each highly affecting the quali- and quantitative composition of the final products, albeit not necessarily favourably. In fact, olive oil processing is associated with a huge loss of valuable phenolic compounds owing to biological (*e.g.* fruits enzymatic level), technological (*e.g.* malaxation conditions) and other limitative factors (*e.g.* phenols liposolubility problems *etc.*) facing industry with several challenges yet to approach. The route of olive phenols from fruits to paste and its final products – oil and wastes (pomace and wastewater) is poorly known and not yet well established, as the chemistry behind is very complex and diverse, arising from all – the transfer, transformation and partition phenomena. Consequently, the

studies investigating such entities are rarely found in the existing literature, though significant from all – the health, economic and ecological perspectives. In 2002, Rodis et al. reported that only 1–2% of the available fruit phenols are transferred to olive oil, while the rest (98%) are lost with the wastes produced. Such out-breaking results highlight the importance of a detailed quantitative study as well as raises some serious technological (in)efficiency considerations behind the current extraction approaches employed. Even so, no new work with novel partition rate calculations appeared since that report, though several attempts have been made toward value-adding, *i.e.* phenols-enriched olive oil production, including the control of malaxation time and temperature (Stefanoudaki et al., 2011; Inarejos-García et al., 2009; Gómez-Rico et al., 2009; Parenti et al., 2008; Ranalli et al., 2003; Ranalli et al., 2001), limitation of water inclusion (Di Giovacchino et al., 2002a), addition of NaCl, talc and/or cell-wall-degrading enzymes (Ben-David et al., 2010; Pérez et al., 2008; Vierhuis et al., 2001), up to the application of fruits de-stoning process and nitrogen flush (Yorulmaz et al., 2011). However, none of them have yet quantified their impact on phenols entire partition trail as the results for the corresponding matrices, except for oil, were mostly omitted. Moreover, their proponents have clearly failed to state the processes mass balance data, overlooking some of the key environmental concerns. Many questions regarding their potent application thus remained unanswered, and yet, they are all imperative for forward technological improvements.

As olive oil processing continues to grow and the world becomes more diet-conscious, the investigations of olive fruit phenols entailed in olive oil production will likely to expand. In response to such upcoming trends, the present doctoral research was undertaken as one of the early attempts to evaluate their fate during olive oil processing in relation to some of the prominent technological variables. As such, the study allowed some basic insights into olive fruit phenols transfer, transformation and partition trail, holding potential for forward research investigations and conceivably some of the scale-up applications. In addition, the study provided an important information of the quality and quantity of available phenolic compounds in Slovenian olive fruits and their commercial waste matrices, significant for the national olive sector development and its stakeholders.

2 RESEARCH OBJECTIVES

The main objective of present doctoral thesis was to investigate the fate of olive fruit phenols during olive oil processing in relation to some of the prominent technological variables. The study aimed to contribute to a better understanding of their behaviour throughout the processing as well as to improve their partition to olive oil and reduce their lost with wastes.

The research conducted hereby was divided into three independent, but highly connected tasks, each with defined objectives as follows:

1) Olive oil processing trial

a) To study the transfer, transformation and partition trail of olive fruit phenols during lab-scale olive oil processing trial through all operative steps – from fruits (peel/pulp and stone) to paste and its final products, *i.e.* pomace, wastewater and oil, considering thirteen technological variables united within three experimental studies; the impact of malaxation time and temperature (30 and 60 min/25, 35 and 45 °C), addition of lukewarm water (200 and 300 mL/30 and 60 min) and addition of co-adjuvants (NaCl, talc and NaCl + talc).

a) To study the mass balance of thirteen trails described above.

2) Istrska belica *cv*. fruits seasonal and geographical phenol profile variation

a) To evaluate the quality and quantity of phenolic compounds in the main Slovenian olive fruit cultivar, *i.e.* Istrska belica *cv*. in relation to the harvest season and orchard location with aim to foreseen their availability for transference to olive oil, and their variability under different environmental conditions.

3) Slovenian commercial olive mill wastes phenolic composition

a) To study the phenol profile variation of Slovenian commercially generated olive mill wastes as a function of extraction system (2- *vs.* 3-phase centrifuges) and to assess their potential as natural source of valuable phenolic compounds.

3 THEORETICAL BACKGROUNDS

3.1 Phenolic compounds

3.1.1 Definition

Phenolic compounds are the most abundant secondary metabolites of plants. Chemically they are defined as substances possessing an aromatic ring with one or more hydroxyl substituents, although a more precise definition is based on their metabolic origin and defines them as substances derived from the shiikimate pathway and phenylpropanoid metabolism, in detail presented elsewhere (Ryan et al., 2002a; Ryan et al., 1998).

3.1.2 Terminology

Many terms are used in existing literature to refer to these compounds such as phenols, phenolics, polyphenols, biophenols and others, depending on the matrix investigated. However, two of them were adopted as the most preferred ones when dealing with *Olea europaeae* L. matrices, *i.e.* olive phenols and/or olive phenolic compounds and were hence thoroughly used in the thesis as well. These two terms refer to all the phenols found in *O. europaeae* L. matrices and are sometimes erroneously named as polyphenols due to a nomenclature borrowed from a wine production (Uccella, 2000).

3.1.3 Classification

Classification of phenols is a very complex task, but a convenient one for the plant phenolic compounds is the one that distinguishes the number of constitutive carbon atoms in conjunction with the structure of the basic phenolic skeleton. The range of phenols is thus vast and currently comprises more than 8000 representatives of known structures, ranging from simple phenols (*e.g.* phenolic acids) up to a highly polymerized substances like tannins (Dai et al., 2010; Antolovich et al., 2000).

3.1.4 Functions

Plant phenols embrace a considerable range of functions that could easily fall into two broad categories, reflecting the focus on activity in plants or bioactivity in

humans *via* food consumption. Many reviews and monographs have been published describing both, however here, only two have been provided revising their functions in plants (Lattanzio et al., 2006) and – in man (Fraga et al., 2010). Some of the most vital are briefly presented below to elucidate the scientific importance of plant phenols research.

Functions in plants. Plant phenols have been associated with several roles. They can act as sun-screeners by absorbing the damaging UV light prior the photo-oxidation of cell constituents occurs (*e.g.* flavonoids), as signalling molecules enabling communication of plant with its environment or as visual signals for the pollinators and seed-dispersing animals (*e.g.* anthocyanins). They also have an important role in the plant's defence mechanism and in their physiological processes; for example, they are involved in the synthesis of structural polymers (*e.g.* ferulic acid in lignification), they can affect seed germination and dormancy (*e.g.* ferulic acid) or can directly regulate the growth of plant *via* control of auxins biosynthesis (*e.g.* some flavonoids) (Lattanzio et al., 2006).

Functions in humans. The putative health benefits of plant phenols have been mostly linked with their antioxidant activities and their protective effects toward some of the major diseases such as cancer, cardiovascular, neurodegenerative and others.

Hydrogen donators. Antiradical antioxidants can donate hydrogen atoms to the free radicals and stop the oxidation chain reactions associated with different types of cell oxidative damages and various pathological conditions (Fraga et al., 2010).

Enzyme inhibitors. Some plant phenols have the ability to suppress the free radical formation by inhibiting enzymes involved in their generation such as cytochrome P450 isoforms, lipoxygenases, cyclooxygenases and xanthine oxidases (Fraga et al., 2010).

Metal chelators. The antioxidant activity of plant phenols may also be utilized *via* capability to chelate metal ions involved in the production of free radicals, though an opposite behavior was also observed (Fraga et al., 2010).

Anticancer, anticardiovascular, antineurodegenerative and other actions. In addition to antioxidant activities, some plant phenols are thought to exert also others *via* interference with molecular processes. For example, they appeared to be involved in the neuroprotection (Rammassamy, 2006), cardiovascular protection (Manach et al., 2005), menopause and osteoporosis prevention (Cornwell et al., 2006). Another emerging role of plant phenols is their protective role in some types of cancers, which may be exert *via* removal of carcinogenic agents, modulation of cancer cell signalling and antioxidant enzymatic activities as well as through the induction of apoptosis and cell cycle arrest (Hu, 2011).

3.2 *Olea europaea* L.

Olea europaea L. belongs to the Tribe Oleae and family Oleaceae, comprising around 600 specious and some 25 genera, including *Olea* – which contains an economically important European olive tree known as *Olea europaea* L. The latter is one of the oldest known cultivated trees in the world with archaeological evidences back in 6000 BC in the region corresponding to ancient Persia and Mesopotamia. The Phoenicians were the first who introduced it to the western regions, first Greek islands and later to the Mediterranean Basin (Spain, Italy *etc.*) through the colonies of Greeks and Romans. In the 15th century AD, *O. europaea* L. also reached a newly discovered America and today it is farmed all around the world except in the Antarctic (Kapellakis et al., 2008; Wallander et al., 2000).

Olive tree is a polymorphous, medium-sized (up to 10 m) with a furrowed trunk. It has greyish-green leaves (5–6 cm long, 1–1.5 cm wide) with smooth edges and a short peduncle. The tree is well adapted to extreme environmental conditions, but requires high-intensity light and aerated soil. It is also known for alternating its fruit production – providing high yields in one, and low in the next year, causing major problems in olive industry. The fertilized fruit development begins with the appearance of floral buds, followed by pollination, fertilization, fruit bearing and ripening. The length and nature of each phase depends on environmental conditions, but normally it begins in April and ends-up in November, when fruits attain their maximal weights and their colour change from green to brownish red and black (Ramírez-Tortosa et al., 2006).

3.2.1. Olive fruit

3.2.1.1 Significance

Olive fruits are of economic, historical, religious, cultural and of aesthetic importance, providing a wide range commercialised products used for dietary and non-dietary purposes. Olives are rarely consumed as a natural fruit due to their extreme bitterness, but rather in one of the two edible forms – olive oil or table olives. Although the latters are the prime reason of their cultivation, other *i.e.* non-dietary products, are likewise gaining significance in the olive market *via* rise of cosmetic and pharmaceutical industries, producing several products used in the anti-aging and body care (Mataix et al., 2006; Ryan et al., 1998).

Oleiculture plays an important role in the economy of olive fruits growing countries. According to the FAOSTAT statistical data 2011, the world's olive fruit production accounts of 20 million tonnes (20.421.286) with EU being the main olive grower (13.459.112 tonnes). The significance of olive sector in EU is apparent as it involves about 2.5 million producers – roughly one third of all EU farmers. However, Spain continues to maintain its prominent role as the world's top grower with more than 7 million tonnes produced, followed by Italy (3.182.200 tonnes) and Greece (2.000.000 tonnes). Other countries exceeding 1 million tonnes are Morocco (1.364.690), Turkey (1.750.000) and Syrian Arab Republic (1.095.040), while others produces less, but yet of significant quantities (in tonnes); Tunisia (863.000), Egypt (459.650), Portugal (443.800), Algeria (420.000), Argentina (170.000) and Peru (160.914). For comparison, the cultivation in Slovenia in 2011 accounted 1704 tonnes (FAOSTAT, 2011; Niaounakis et al., 2006).

3.2.1.2 Composition

According to Bianchi (2003), the fruit of *O. europaea* L. can be structurally divided into three distinct anatomical parts as follows.

Epicarp. Skin has a protective role against the mechanical damage, fungal and pests attack. It accounts 1–3% of drupe's weight and is covered by a thin layer of wax. During maturation its colour changes from the bright to pale-green, straw yellow,

purple pink and finally to the black as a result of various pigments combinations, *i.e.* chlorophylls, anthocyanins and carotenoids.

Mesocarp (pulp or flesh). Pulp presents 70–80% of the whole fruit's weight and serves as a reserve for its constituents such as water, oil, sugars, proteins, minerals and phenols.

Endocarp (stone). The woody endocarp is characteristic for each olive variety and presents 18–22% of the fruit's total weight. It encloses embryo (the kernel) comprising 2–4% of the stone's weight and contains 22–27% of the oil.

The average composition of olive fruit is difficult to define due to its remarkable diversity, producing high compositional variability. However, the water and fat are indubitably the main constituents, beside other water-soluble compounds (sugars, organic acids, nitrogenous compounds, phenols) and insoluble fraction of colloids such as hemicelluloses, celluloses, pectins, enzymatic and structural proteins (Servili et al., 2012). The average composition of fruit's main constituents is provided in **Table 1**.

Table 1 Olive fruit composition (Adapted by Ryan et al., 1998)

Constituent	Flesh (%)	Stone (%)	Seed (%)
Water	50–60	9.3	30.0
Oil	15–30	0.7	27.3
N matter	2–5	3.4	10.2
Sugars	3–75	41.0	26.6
Cellulose	3–6	38.0	1.9
Ash	1–2	4.1	1.5
Phenols	2–2.5	0.1	0.5–1.0
Others	–	3.4	24

3.2.1.3 Cultivars

The typical Mediterranean *O. europaea* L. includes the two main varieties, *sylvestris* (wild olive) and *macrocarpa* (domesticated olive), of which only the latter is being cultivated for the fruit production. There are approximately 2500 inventoried varieties, of which 250 are classified as commercial cultivars included in the World

Catalogue of Olive Varieties published by International Olive Oil Council (IOOC). These olive cultivars are used for either olive oil or table fruits production, depending on the content of oil and the size of the fruit. The fruits used for olive oil production are medium in size, averaging less than 3.5 g in weight with a low pulp/stone ratio and a high oil yield (16–18%). By contrast, the table olives are medium to large, weighting 5–6 g (up to 17 g) with a high pulp/stone ratio and a little oil content. In general, different cultivars are used for the oil and table fruits production, though a double-use cultivars are also known (Mataix et al., 2006; Ryan et al., 1998).

The differences between the thousands of varieties can be very subtle and the cultivars identification complicated due to all environmental factors influencing phenotype of the plant. The methods for a primary olive characterization follow a list adopted by the Conseil Oléicole Internationale (COI), which refers to the analysis of 32 different morphological characteristics including the tree, leaves, flowering, fruits and endocarp (Pinheiro et al., 2005). Further identification at molecular level is based on genetic fingerprinting using different DNA markers like RAPDs, RFLPs, SSRs and others, allowing genetic differentiation among the cultivars (Alba et al., 2009; Wünsch et al., 2002; Besnard et al., 2001).

3.2.1.3.1 Cultivars grown in Slovenia

In Slovenia, there are more than 30 olive fruit varieties grown in the western part of the country. Olive orchards are spreading from Slovenian Istria in the south, through the Karst and Vipava Valley, up to the northern Primorska, *i.e.* Goriška Brda region. As documented, Črnica *cv*. was the predominant olive variety in the past (60–70%), followed by Istrska belica *cv*., Drobnica *cv.*, Buga *cv*. and others. After the big frost in 1956, the varietal structure of olive orchards has drastically changed – the old cultivars were grafted and the new cultivar seedlings imported, mainly from Italy (Mazi, 2006). Today, according to the official statistical data and out of 652 ha orchards inventoried, the orchards' cultivar structure consists of Istrska belica *cv*. (62%), Leccino *cv*. (24%), Pendolino *cv*. (3%), Maurino *cv*. (2%), while others (*e.g.* Črnica *cv.*, Frantoio *cv.*, Buga *cv.*, Ascolana tenera *cv.*, Štorta *cv.*, Oblica *cv.*) are presented in less than 1% (Orchards olives varietal …, 2010).

Istrska belica *cv*.

Synonyms: Belica, noble Belica, Bijelica, Istarska Bjelica, Bianchera, Bianca Istriana, Biancara

Istrska belica *cv*. is the most widely spread olive variety in Slovenia, accounting more than 60% of olive orchards. According to the oral tradition, this cultivar was brought from the area of Trieste (Italy) and introduced to the Slovenian region after the end of 19th century. This variety is not an indigenous, but rather domesticated like others of foreign origin, being cultivated in Slovenia for more than 50 years and well adapted to its climatic conditions. The fruits are medium in size (3.0 ± 0.4 g) with colours varying from light green to the dark violet that ripen late, from mid-November to mid-December. Due to its high oil content, the fruits are mainly pressed to oil, characterised as fresh, bitter and savoury (Bandelj Mavsar et al., 2005).

3.2.2 Olive oil

3.2.2.1 Definitions and classification

Olive oil is defined as oil obtained solely from the fruit of olive tree, while virgin olive oil is the one obtained from the fruit solely by mechanical or other physical means under conditions that do not lead to alterations, and which have not undergone any treatment other than washing, decantation, centrifugation and filtration (IOOC, 2014).

Virgin olive oil. It can be classified as described below.

Extra virgin olive oil. Virgin olive oil with max free acidity, expressed as oleic acid, of 0.8 g per 100 g and the other characteristics of which correspond to those fixed for this category.

Virgin olive oil. Virgin olive oil which has a free acidity, expressed as oleic acid, of not more than 2 g per 100 g and the other characteristics of which correspond to those fixed for this category.

Ordinary virgin olive oil. Virgin olive oil with max free acidity, expressed as oleic acid, of more than 3.3 g per 100 g and the other characteristics of which correspond to those fixed for this category.

Lampante virgin olive oil. Virgin olive oil with max free acidity, expressed as oleic acid, of more than 3.3 g per 100 g and the other characteristics of which correspond to those fixed for this category. It is intended for refining or for the technical use.

Refined olive oil. Is the oil obtained from virgin olive oils by refining methods, which do not lead to alterations in the initial glyceridic structure. It has a free acidity, expressed as oleic acid, of not more than 0.3 g per 100 g and its other characteristics correspond to those fixed for this category.

Olive oil. Is a blend of refined olive oil and virgin olive oils fit for consumption. It has a free acidity, expressed as oleic acid, of not more than 1 g per 100 g and its other characteristics correspond to those fixed for this category.

Olive-pomace oil. Is the oil obtained by treating olive pomace with solvents or other physical treatments to the exclusion of oils obtained by re-esterification processes and of any mixture with oils of other kinds. It can be marketed as a crude olive pomace oil, refined olive pomace oil or olive pomace oil.

3.2.2.2 Significance

Olive oil has for centuries been used for the consumption, medical, cosmetic, textile, illumination and other purposes *etc.* (Mataix et al., 2006). Nowadays, it is undoubtedly one of the most valuable sources of fat and the principle one in Mediterranean diet associated with several health benefits (Riachy et al., 2011a).

The significance of olive oil market in olive industry is apparent as it consumes approximately 90% of the annual olive fruits production (Ryan et al., 1998). Currently, the world's olive oil production exceeds 3 million tonnes (3.075.000) with EU being the largest producer – 2.209.000 tonnes (73%). The breakdown of this percentage between its chief producing countries shows the shares of 46% for Spain, 15% for Italy, 10% for Greece and 2% for Portugal. For comparison, Slovenia produces only 0.700 tonnes. The annual world consumption of olive oil in 2010/11

was 3.061.000 tonnes, of which 80% were consumed within IOC member countries, while the share of EU countries was 64%. The provisional data for further harvest seasons forecasts a continuous growth of its production and consumption, projected to rise for at least 1.5% (IOOC, 2014).

3.2.2.3 Composition

Olive oil is composed of two main fractions – the major and minor ones.

Major fraction. It is known as saponifiable or glyceride fraction, constituting 98–99% of oil's weight and is mainly composed of triacylglycerols, though some free fatty acids, monoglycerols and diglycerols can also be found. The typical fatty-acid profile of virgin olive oil embraces oleic acid (68–81.5%), which is the predominant and classifies it among MUFA (monounsaturated fatty acid) oils, as well as others such as linoleic, palmitic, stearic acid *etc.* (Ramírez-Tortosa et al., 2006). The ratio between *n*-6 and *n*-3 fatty acids is not of highest, *i.e.* 16 (published ranges for vegetable oils: 0–738) and hence its main putative health benefits are typically linked with its minor fraction (Dubois et al., 2007).

Minor fraction. A minor fraction of olive oil presents 1–2% of its total weight and comprises more than 230 different constituents. These compounds can be divided into several groups such as non-glyceride esters (*e.g.* waxes), aliphatic and triterpenic alcohols, sterols (*e.g.* campesterol), hydrocarbons (*e.g.* squalene), polar pigments (e.g. chlorophylls), tocopherols, phenolic compounds (*e.g.* hydroxytyrosol) and volatiles (*e.g.* benzaldehyde) (Ramírez-Tortosa et al., 2006). However, only few were recognized as bioactive and are along with their benefits reviewed by Covas et al. (2006).

3.2.2.4 Olive oil processing

Olive oil processing encompasses four technological steps, each affecting the final quality and quantity of olive oils obtained (Di Giovacchino et al., 2002a).

Leaf removal and washing. They are important for the mechanical safety of equipment and for the organoleptic quality of olive oils by removing extraneous

matter such as leaves, twigs, soil, dust, mineral and stone impurities (Di Giovacchino et al., 2002a).

Crushing. Is the first step of olive paste preparation in which fruits are crushed down to break the tissues and liberate the oil droplets contained in the cells. The press systems are generally equipped with the granite mill stones (2–6) and the resulting paste is additionally squeezed by hydraulic press, whereas the centrifugation systems are typically equipped with metallic crushers such as mobile or fixed hammers, toothed discs, cones or rollers. The type of crushing method is known to affect the extraction yield, organoleptic and nutritional quality of olive oils, in particular the phenols and volatiles (Di Giovacchino et al., 2002a).

Malaxation. Is the second stage of olive paste preparation in which paste is slowly mixed and heated (25–35 °C) to aggregate the small oil droplets into large drops, favouring the formation and release of "free oil". It is carried out in semi-cylindrical vats fitted with a horizontal shaft, with rotating arms and stainless steel blades of different shapes and sizes. These vats are equipped with a heating jacket with circulating hot water that mildly heats the paste. Malaxation also affects the extraction yields and qualitative characteristics of olive oil, which depend on paste's rheological characteristics and operative parameters such as the time and temperature, addition of water, co-adjuvants and others (Di Giovacchino et al., 2002a).

Oil separation. This operation enables the separation of olive oil from the solid (pomace) and liquid (wastewater) phases, and is performed by using either pressure, percolation or centrifugation systems in detail described below (Di Giovacchino et al., 2002a).

3.2.2.4.1 Extraction systems

From early 1970s, the olive oil technology has undergone evolutionary changes with the introduction of new continuous systems, *i.e.* 3- and 2-phase centrifuges, replacing traditional presses being used for centuries. First, a 3-phase centrifuge was introduced to the market with aim to improve the production capacity, oil extraction

yields and to reduce the labour costs. Later on, in 1990s, a new system, *i.e.* a 2-phase centrifuge was developed with a view to minimise the volume of water consumption and wastes production of a 3-phase decanter (Niaounakis et al., 2006; Tsagaraki et al., 2007; Azbar et al., 2004). The technological development of both is in detail presented elsewhere (Ranalli et al., 1995; Amirante et al., 1993).

However, the various extraction systems mainly differ in the two key aspects, namely, in the physical forces used to recover the oil, and in the amount of water added to olive paste during processing (Ryan et al., 1998). Currently, the commercial olive oil production is carried out at both continuous (centrifugation) and batch (traditional press) approaches, though the former have been far more widely used (Servili et al., 2012). The schematic presentation of three main extraction systems is shown in **Figure 1** and in depth described below, while the mass balance, water and energy data for each are provided in **Table 2**.

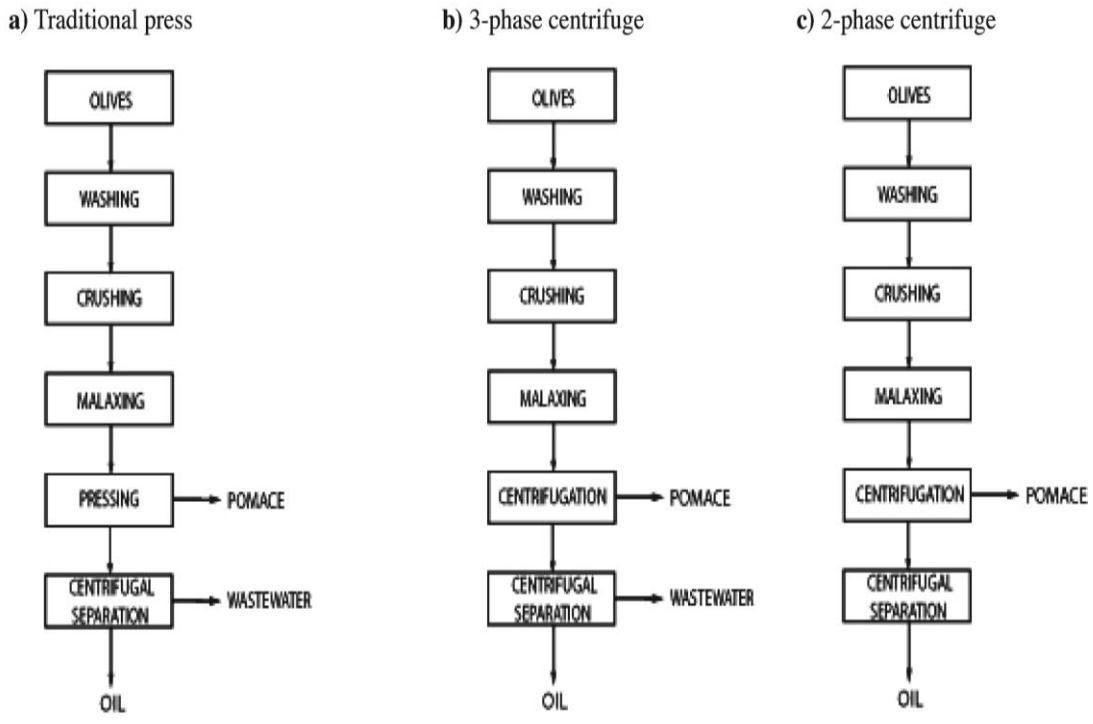


Figure 1 Extraction systems; traditional press, continuous 3- and 2-phase centrifuge

Traditional press. It is the oldest, but still quite widely spread olive oil extraction system used. In this system, olives are washed, crushed (milled) and malaxed with the addition of water (3–5 L/100 kg fruits weight). Then, the resulting paste is

pressed by hydraulic press to drain the oil, obtaining two products – the pomace and the liquid phase known as oily must (oil + wastewater). The latter is further separated by vertical centrifugation or decantation, while the pressed pomace can be de-oiled elsewhere by using special facilities. The process itself produces the three final products, *i.e.* the oil, pomace and wastewater (Tsagaraki et al., 2007; Azbar et al., 2004; Amirante et al., 1993).

The main advantage of traditional press over centrifuge systems is a lower capital cost and a relatively small quantity of wastes produced, while the main disadvantages are a lower capacity, process control and automation, more difficult cleaning, longer processing/storage time and a bigger space requirement and manpower costs (Azbar et al., 2004). Nevertheless, the oils produced by traditional presses are often associated with defects due to frequent contaminations (Ranalli et al., 2000).

3-phase centrifuge. This system is based on a 3-phase decantation, where olives are washed, crushed and malaxed, and then the lukewarm water is added to a horizontal centrifuge (40–60 L/100 kg fruits weight) separating pomace from the oily must (oil + wastewater). Such dilution increases the difference between the specific weights of liquid and solid phase needed to obtain their separation (solids > liquid). Then, the oil must is passed on to a vertical centrifuge, where oil is separated from the wastewater. A 3-phase centrifuge at the end generates the three final products – the oil, pomace and wastewater, on the basis of which it was also named, *i.e.* 3-phase (Di Giovacchino et al., 2002a).

Its main advantage is a continuous performance allowing a high productive capacity – up to 3-times higher *vs.* traditional press (30–32 tons *vs.* 8–10 tons per day). It is also characterised by an elevated automation, needs a smaller space and minimises the labour costs. On the other hand, this technology has much higher investment costs, energy and water consumption, and delivers larger quantities of wastes, causing troubles in terms of sustainability and treatment costs. Nevertheless, it produces olive oils with lower phenol yields, but of less mat flavour and acidity (Niaounakis et al., 2006; Azbar et al., 2004; Di Giovacchino et al., 2002a; Amirante et al., 1993).

In 1993, Amirante et al. proposed an alternative to this system with aim of reducing some of its drawbacks and developed a 3-phase centrifuge with a wastewater recycle. The concept of working is similar to a conventional 3-phase centrifuge, but instead of fresh lukewarm water addition to horizontal centrifuge, the wastewater is recycled. Its testing results from experimental trials showed a major reduction of water consumption (up to 50%) and wastewater production (20–40%) as well as a major increase of total phenols in olive oils (up to 64%).

2-phase centrifuge. This extraction system is also known as "ecologic" or "water-saving" as it requires no water addition and reduces wastewater generation up to 80%. The concept of working is similar to that of a 3-phase centrifuge, except that horizontal centrifuge has no or lower need of the water addition due to superior g values. This system hence delivers only two final streams – the oil and a single waste (pomace + wastewater), *i.e.* a very wet olive pomace also known as alperujo (Niaounakis et al., 2006; Tsagaraki et al., 2007; Di Giovacchino et al., 2002a).

The main advantages of 2-phase centrifuge *vs.* 3-phase is a lower water and energy consumption, resulting in a lower total capital costs. Likewise, the generation of wastes is reduced, though they are much more difficult and expensive to treat. Moreover, oils produced by this system have higher phenol yields, while other qualitative characteristics are rather similar (Niaounakis et al., 2006; Tsagaraki et al., 2007; Di Giovacchino et al., 2002a).

Besides the conventional 3- and 2-phase centrifuges widely spread, there are also some other extraction systems used such as:

2-phase and half centrifuge (mixed). It is a compromise between 3- and 2-phase centrifuges with the same working concept, but requires lower lukewarm water adds (0–30 L/100 kg fruits weight) and delivers the three final streams, *i.e.* the oil, pomace (50–60% moisture content) and wastewater (5–30 L). It is quite commonly employed in Italy (Di Giovacchino et al., 2002a).

Percolation (selective filtration). This is an old system still used in some of the countries and is mainly known as Sinolea. The steel plates are dipped into the paste, where they get coated with oil due to different surface tensions. The latter is then removed by using either pressure (in the past) or centrifugation (nowadays) (Di Giovacchino et al., 2002a).

Figure 2 illustrates the proportions of different types of olive oil extraction systems employed in EU countries. As evident, only Spain and Croatia have a high proportion of 2-phase centrifuges (98% and 55%), while in other countries the latter presents less than 5% of olive mill plants. The traditional press clearly dominates in Portugal (84%), followed by Italy, Croatia (42%) and Malta (40%), whereas a 3-phase centrifuge dominates in Greece and Cyprus (82%), Malta (60%) and Italy (46%).

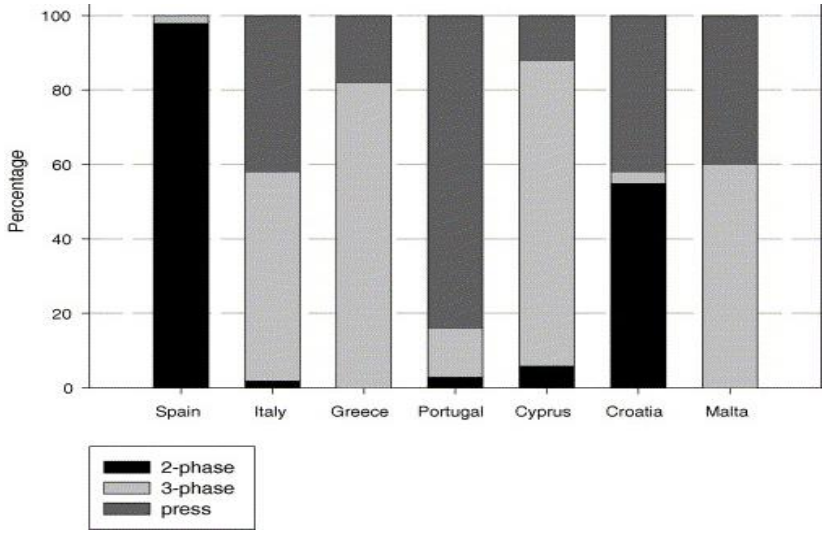


Figure 2 Extraction systems used by European olive mills (Adopted by Roig et al., 2006)

In Spain, the use of 2-phase centrifuges has been supported by the national programs and replacements of existing facilities benefited from the public foundings, while in other EU countries not. The reason for such reluctance mainly arrives from the expensive and difficult alperujo treatment, a non-sufficient awareness for the water conservation and from the premise equipped with an already existing systems (Tsagaraki et al., 2007; Azbar et al., 2004). On the other side, the 3-phase decanters can easily be changed into 2-phase ones by simply adjusting the crusher (Ranalli et al., 1995). In Slovenia, the share of 2-phase centrifuges constitutes one third of all

olive mills and is therefore equally employed as others; traditional press (1/3), 3-phase, 2-phase and half (1/3) (MORE, 2006).

3.2.2.4.2 Environmental concern

Olive processing is one of the fastest growing agro-food sectors in EU with more than 4% of annual growth rate. This trend is likely to continue due to intensive olive tree cultivation projected for the future with new plantations of higher density and of more intensified production using pesticides and fertilizers (Niaounakis et al., 2006). This affects the environment in several, unfortunately, negative ways. While olive farming is related to soil erosion, degradation of habitats and landscapes, run-offs to water bodies and exportation of scares water resources (Beaufoy, 2000), the olive oil industry is mainly facing with enormous waste disposal problems (Roig et al., 2006).

The huge amounts of by-products production poses an economic and environmental burden on olive oil industry and is of growing concern in terms of sustainability (Obied et al., 2007b). In general, next to olive oil (20%), it produces two environmentally problematic waste matrices known as olive mill wastewater (50%) and olive pomace (30%), though the quantity and type of by-products largely depend on the type of extraction system employed (Niaounakis et al., 2006). **Table 2** compares the three main extraction systems in terms of mass balance, water and energy consumption.

Table 2 Approximate input-output data of three main olive oil extraction systems (Adapted by Azbar et al., 2004)

Extraction system	Input	Amount of input	Output	Amount of output (kg)
Traditional press	Olives	1 ton	Oil	~ 200
	Wash water	0.10–0.12 m^3	Pomace (25% water + 6% oil)	~ 400
	Energy	40–63 kWh	Wastewater (88% water + solids + oil)	~ 600
3-phase centrifuge	Olives	1 ton	Oil	200
	Wash water	0.10–0.12 m^3	Pomace (25% water + 6% oil)	500–600
	Decantation water	0.50–1.00 m^3		
	Polish water	~ 10L	Wastewater (88% water + solids + oil)	1000–1200
	Energy	90–117 kWh		
2-phase centrifuge	Olives	1 ton	Oil	200
	Wash water	0.10–0.12 m^3	Wet pomace (60% water + 3% oil)	800–950
	Energy	< 90–117 kWh		

Olive mill wastes negatively affect the terrestrial and aquatic ecosystems *via* various pathways. For example, their high phosphorous content accelerates eutrophication of water bodies and their high sugar content allows faster microbial growth on account of others, while some aquatic organisms are severely intoxicated when exposed to olive mill wastewaters. Other side effects include a strong odour due to acidity and microorganisms, a reduced availability of some microelements, the soil's fertility change and others (Tsagaraki et al., 2007). Different ecotoxicological effects of olive mill wastewater have been just recently reviewed by Justino et al. (2012) concluding that this by-product is highly toxic not only to microorganisms, but also to some invertebrates and primary producers. The main phytotoxic and antimicrobial properties were linked to its high phenolic content and to some of organic acids accumulated during microbial growth. These phenols, however, inhibited the seed germination and were active against several bacteria and fungi (Tsagaraki et al., 2007; Obied et al., 2005; Fiorentino et al., 2003).

Some of the available environmental/toxicological data for the selected olive phenols are presented in **Table 3**.

Table 3 Environmental/toxicological data for the selected olive phenols

Phenolic compounds	Environmental/toxicological data				
	EC_{50} [A] *D. magna*	LC_{50} [A] *T. platyurus*	LC_{50} [A] *B. calyciflorus*	IC_{50} [A] *P. subcapitata*	$LogK_{ow}$ [B]
Simple phenols					
Catechol	10	8	17	34	0.87
Hydroxytyrosol	11	4	9	120	–
Tyrosol	861	296	47	210	0.69
Benzoic acids					
Vanillic acid	386	431	1	255	1.42
Vanillin	–	–	–	–	1.19
Protocatechuic acid	413	589	385	344	0.76
Cinnamic acids					
p-Coumaric acid	290	591	108	225	–
Caffeic acid	326	626	359	120	1.29

[A]Median effective concentration for particular organisms (μM), where EC_{50} refers to the immobilisation, LC_{50} to mortality and IC_{50} to inhibition (Fiorentino et al., 2003). [B]Partition coefficient in pure water at 298.15K (Noubigh et al., 2009).

3.2.2.4.3 Olive mill waste matrices, management & potential

Olive mill wastewater (alpechin). It is a mixture of fruits vegetative water, soft tissues and technological water used during processing, and is composed of pulp, mucilage, pectin, oil and others suspended in a relatively stable emulsion. It is the most environmentally problematic waste due to its characteristics listed below, high volumes and treatment costs. Though its chemical composition is variable and dependable on fruits variety, growing techniques, harvesting period and technology, some of the main characteristics are general, proving its strong nature as industrial waste (Tsagaraki et al., 2007):

- strong offensive/acidic smell
- high organic content (COD up to 220 g/L, $COD/BOD_{5:}$ 2–5, hardly degradable)
- pH: 3–5.9
- high phenolic content (up to 80 g/L)
- high content of solids (up to 20 g/L)

The significance of environmental concern is apparent as in terms of pollution the effect of 1 m^3 of olive mill wastewater is equivalent to 100–200 m^3 of domestic sewage. Hence, its direct discharge into environment is strictly forbidden unless managed properly (Tsagaraki et al., 2007).

Olive pomace. It is composed of a mixture of olive pulp, stones and some of the vegetative and washing water. A 2-phase pomace, also known as alperujo, olive wet husk, wet pomace or wet cake, is the most problematic with a high moisture content (55–77%), a slightly acidic pH (4.9–6.8) and a high organic content (mainly fibres). It also contains a lot of fats, proteins, water-soluble carbohydrates and active fraction of phenols (Morillo et al., 2009; Roig et al., 2006).

The intense investigation of olive wastes treatment options, which have been manifested for the past few years, accounts for many reviews, published in the form of papers (Morillo et al., 2009; Paraskeva et al., 2006; Roig et al., 2006; Azbar et al., 2004) or books (Buessing, 2012; Muscolo, 2010; Niaounakis et al., 2006) and much

of these work is already covered by patents (Takac et al., 2009). Considering that this research area is relatively new, it has faced a remarkable rate of progress.

However, olive wastes were initially recognized solely as pollutants and their treatment methods were oriented only toward detoxification using several physical, thermal, physico-chemical and biological processes and/or their combinations. Only later in 1990s, this trend has turned toward their valorisation with emphasis on recycling and recovery of valuable compounds, including phenols. In the following years, a number of projects have been established and several studies published promoting different methods for their isolation, focusing mainly on hydroxytyrosol, tyrosol and oleuropein. Collectively, they have been reviewed in the above-mentioned literature.

In spite of several treatment and valorisation options proposed, their practical application mainly depends on a country's individual legislation, its social, agricultural and industrial environment. Yet, the most common practise is still composting, in addition to other uses such as the use for for animal feed, substrate for high added-value products (*e.g.* biopolimers, enzymes *etc*.), source for residual oil recovery and energy production (Roig et al., 2006).

Practice in Slovenia. Between 2007–2010, the Slovenian olive mill waste management practise has been evaluated within the international IEE project MORE (Market of Olive Residues for Energy) and some of the main issues identified. The results showed that Slovenian olive residues are not exploited due to legislation restrictions, which treats them solely as waste and does not determine their management neither promotes their utilization as secondary products. In Slovenian Istria, which is the main olive fruit cultivation region, more than 90% of olive solid residues are composted, typically for 3 to 6 months, and then returned to the orchards as fertilizers. Less than 5% are used for the energy generation as only two households are using them for private heating. There are no pit separators nor drying facilities and refineries available for their proper treatments. Nevertheless, some of the wastewaters' parameters exceeded the limits allowed to be discharged into public sewage system (MORE, 2006).

3.3 Olive phenols

3.3.1 Classification

Phenolic composition of olive matrices is often extremely complex displaying a large diversity in structures. According to Obied et al. (2007a) olive phenols can be classified into seven groups, which have been along with their representatives listed in **Table 4**.

Simple phenols. Hydroxytrosol and tyrosol are the most important phenolic alcohols characteristic for this class of phenols (Bendini et al., 2007). They are phenyl ethanol derivatives and can be found in several forms.

Hydroxytrosol. It belongs to the group of *o*-diphenols and has been associated with strong antioxidant properties (Riachy et al., 2012a). It can be found in a free form and esterified form; i) with elenolic acid – forming oleuropein, its aglycone (3,4-DHPEA-EA) or its decarboxymethylated aglycon (3,4-DHPE-EDA), ii) with caffeic acid – forming verbascoside, and iii) in glucosidic form as hydroxytyrosol glucoside.

Tyrosol. Analogously as hydroxytyrosol, it is present in a free or in esterified form; i) with elenolic acid – forming ligstroside, its aglycone (*p*-HPEA-EA) or its decarboxymethylated aglycon (*p*-HPEA-EDA), and ii) in glucosidic form as tyrosol glucoside also known as salidroside.

Benzoic acids. This class comprises a wide range of phenolic acids such as gallic, vanillic, homovanillic, syringic, protocatechuic acids, vanillin and others (Obied et al., 2007a).

Cinnamic acids. They are phenolic acids characterised by cinnamic, *p*-coumaric, *o*-coumaric, ferulic, sinapic and other acids or by their glucosides and derivatives (Obied et al., 2007a).

Verbascoside. It is a heterosidic ester of caffeic acid and hydroxytyrosol and can be found in several analogous structures. It is also known as the most powerful radical scavengers with a 3-fold higher activity that hydroxytyrosol (Savarese et al., 2007; Aldini et al., 2006) and is hence of considerable pharmacological interests.

Flavonoids. Are the widest group of plant phenols frequently present as glycosides. This group is represented by several subclasses such as flavones (*e.g*. luteolin, apigenin and their glucosides), flavonols (*e.g*. rutin, quercitrin), anthocyanins (*e.g*. cyanidin-3-*O*-glucosides) and flavanones (Harborne et al., 1999).

Isochromans. They are 3,4-dihydro-1H-benzo[c]pyran derivatives generally present in the nature as part of complex fused ring systems. Two of them have been found in olive oil; 1-phenyl-6,7-dihydroxyisochroman and 1-(3'-methoxy-4'-ydroxy)phenyl-6,7-dihydroxyisochroman (Bianco et al., 2001).

Lignans. They are chemically related to polymeric lignins of plants cell walls. There are three structural classes of oxygenated lignans, namely simple lignans, lignanolides (*e.g*. pinoresinol) and cyclolignans (Harborne et al., 1999). Brenes et al. (2000) was the first that assigned the presence of pinoresinol and acetoxypinoresinol in olive oil, which are the most common lignans reported in *O. europaea* L. matrices.

Secoiridoids. Are the most characteristic of *Oleaceae* family, derived from iridoids and characterised by the presence of elenolic acid in its glucosidic or aglyconic forms in their molecule (Bendini et al., 2007). Two groups of secoiridoids are known, those containing exocyclic 8,9-olefinic functionally (oleosides) and those containing 8,10-exocyclic functionally (Ryan et al., 2002a).

Oleosides. Are unique to oleaceous plants and are frequently found in olive fruits. They are not phenolic compounds by themselves, but may involve a phenolic moiety as a result of esterification, which classifies them as phenolic oleosides. The most significant representatives are oleuropein, demethyloleuropein, ligstroside and oleoside often referred as secoiridoid glucosides (Ryan et al., 2002a).

Oleuropein. It is typically the predominant phenolic compound found in olives, where it can reach up to 90 mg/g of fruits dry weight (DW). It is an ester of elenolic acid glucoside with hydroxytyrosol and is responsible for the intense bitterness of olive fruit (Ryan et al., 2002a).

Demethyloleuropein. It is a derivative of oleuropein with no methyl group present in the carboxylic group on pyranosic ring and may originates from oleuropein by the activity of esterase during fruit ripening (Savarese et al., 2007).

Other representatives of secoiridoids are typically the derivatives of secoiridoid glucosides formed during oil extraction such as oleuropein aglycone, 3,4-DHPEA-EDA, ligstroside aglycone, *p*-HPEA-EDA and others (Servili et al., 2004).

The chemical structures of phenols covered by the doctoral study are illustrated in **Table 4**.

3.3.2 Olive fruit phenols

3.3.2.1 Main representatives

Olive drupes contain high concentration of phenols ranging between 1–3% of the fresh pulp's weight (Servili et al., 2004). Hydroxytrosol and tyrosol are the main fruit representatives of simple phenols, while verbascoside is typically the most abundant cinnamic acid derivative. Among secoiridoids, oleuropein, demethyloleuropein, ligstroside and oleoside are generally reported as predominant, whereas flavonoidal profile is mainly composed of flavone glycosides (luteolin-7-*O*-glucoside, apigenin-7-*O*-glucoside) and flavonols (mainly quercitrin). In the dark-coloured fruits two anthocyanins are typically found, *i.e.* cyanidin-3-*O*-rutinoside and cyanidin-3-*O*-glucoside, while the most abundant benzoic acids are gallic and vanillic acid (Goulas, 2012; Ryan et al., 2002a; Ryan et al., 1998). By contrast, lignans have only scarcely been found in the fruits (López et al., 2008). Other phenols associated with olive fruits are listed in **Table 5**.

Table 4 Chemical structures of selected olive phenols

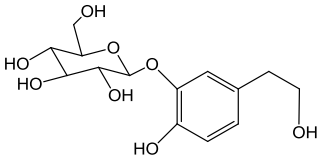
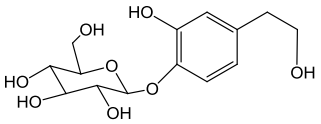
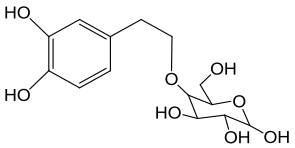
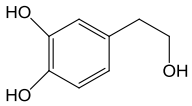
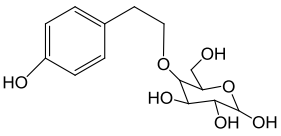
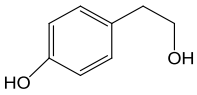
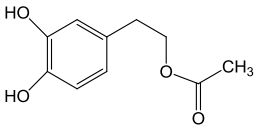
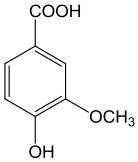
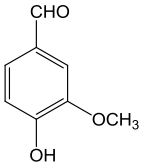
Class/Phenolic compound	Molecular formula	MW[A]	Chemical structure	Ref
Simple phenols				
Hydroxytyrosol glucoside	$C_{14}H_{20}O_8$	316	*Option 1*  *Option 2* 	Obied et al., 2007a
Hydroxytyrosol-1-β-glucoside	$C_{14}H_{20}O_8$	316		Obied et al., 2007a
Hydroxytyrosol / 3,4-DHPEA / DOPET	$C_8H_{10}O_3$	154		Servilli et al., 2004
Tyrosol glucoside / Salidroside	$C_{14}H_{20}O_7$	300		Guo et al., 2012
Tyrosol	$C_8H_{10}O_2$	138		Servilli et al., 2004
Hydroxytyrosol acetate / 3,4-DHPEA-AC	$C_{10}H_{12}O_4$	196		Gordon et al., 2001
Benzoic acids				
Vanillic acid	$C_8H_8O_4$	168		Obied et al., 2005
Vanillin	$C_8H_8O_3$	151		Artajo et al., 2006a

Table 4 *(Cont)*

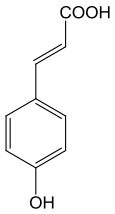
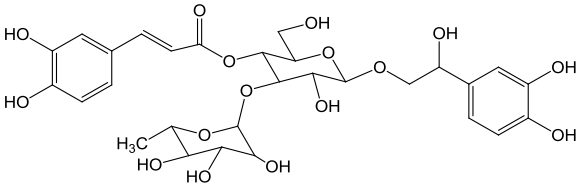
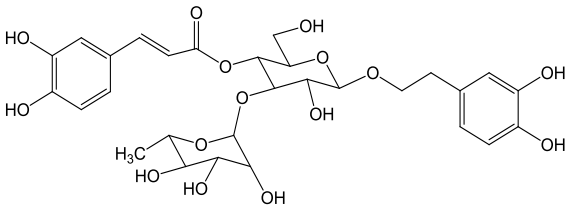
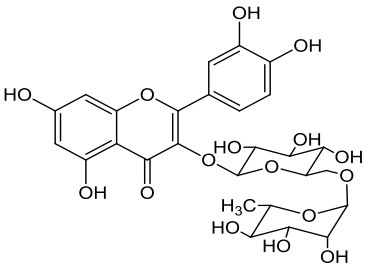
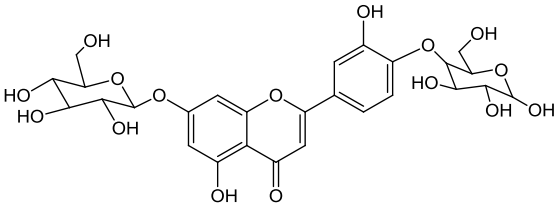
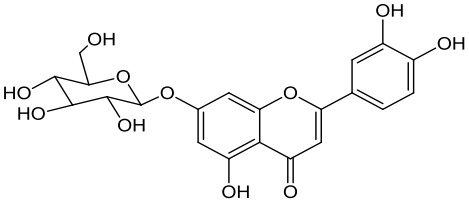
Class/Phenolic compound	Molecular formula	MW	Chemical structure	Ref
Cinnamic acids				
p-Coumaric acid	$C_9H_8O_3$	164		Obied et al., 2005
β-OH verbascoside	$C_{29}H_{36}O_{16}$	640		Innocenti et al., 2006
Verbascoside / Acteoside	$C_{29}H_{36}O_{15}$	624		Innocenti et al., 2006
Flavonoids				
Rutin	$C_{27}H_{30}O_{16}$	610		Obied et al., 2005
Luteolin-4',7-*O*-diglucoside	$C_{27}H_{30}O_{16}$	610		I[B]
Luteolin-7-*O*-glucoside	$C_{21}H_{20}O_{11}$	448		Obied et al., 2005

Table 4 *(Cont)*

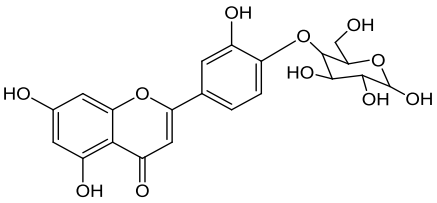
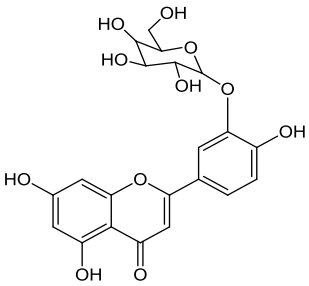
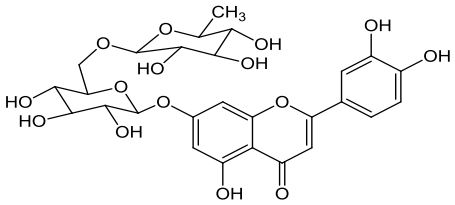
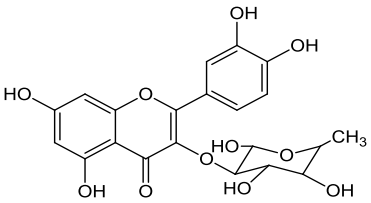
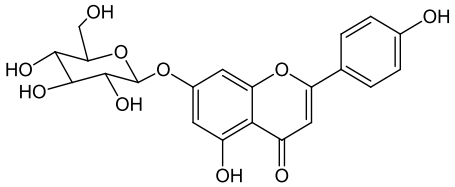
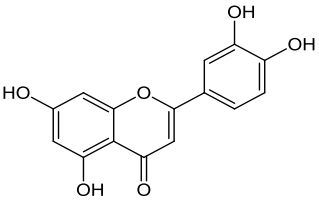
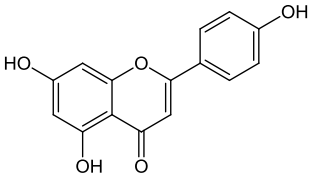
Class/Phenolic compound	Molecular formula	MW	Chemical structure	Ref
Luteolin-4'-*O*-glucoside	$C_{21}H_{20}O_{11}$	448		Obied et al., 2005
Luteolin-3'-*O*-glucoside	$C_{21}H_{20}O_{11}$	448		I[B]
Luteolin-7-*O*-rutinoside	$C_{27}H_{30}O_{15}$	594		Obied et al., 2005
Quercitrin	$C_{21}H_{20}O_{11}$	448		Obied et al., 2005
Apigenin-7-*O*-glucoside	$C_{21}H_{20}O_{10}$	432		Ryan et al., 2002a
Luteolin	$C_{15}H_{10}O_6$	286		Artajo et al., 2006a
Apigenin	$C_{15}H_{10}O_5$	270		Ryan et al., 2002a

Table 4 *(Cont)*

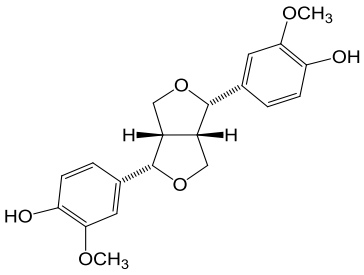
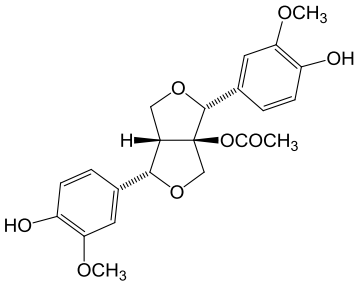
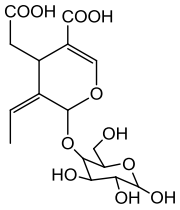
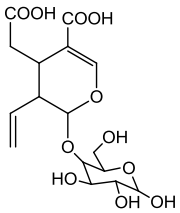
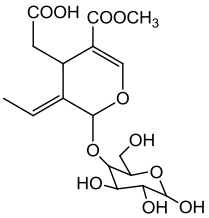
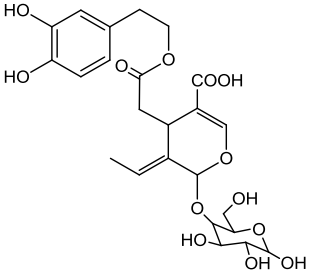
Class/Phenolic compound	Molecular formula	MW	Chemical structure	Ref
Lignans				
Pinoresinol	$C_{20}H_{22}O_6$	358		Bendini et al., 2007
Acetoxy-pinoresinol	$C_{22}H_{24}O_8$	416		Bendini et al., 2007
Secoiridoids				
Oleoside	$C_{16}H_{22}O_{11}$	390		Obied et al., 2007a
Secologanoside	$C_{16}H_{22}O_{11}$	390		Obied et al., 2007a
Elenolic acid glucoside / 11-Methyl oleoside / Oleoside 11-methyl ester	$C_{17}H_{24}O_{11}$	404		Ryan et al., 2002a
Demethyl-oleuropein	$C_{24}H_{30}O_{13}$	526		Ryan et al., 1998

Table 4 *(Cont)*

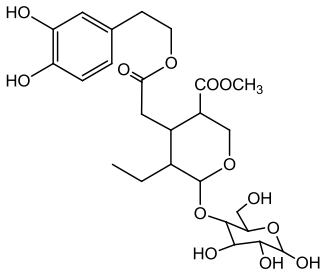
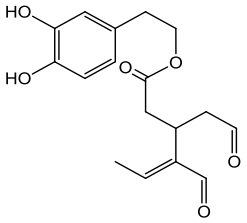
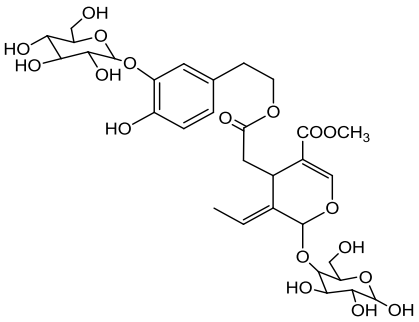
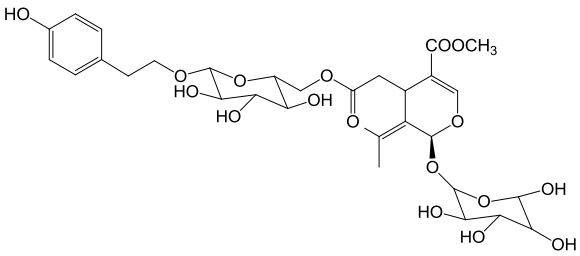
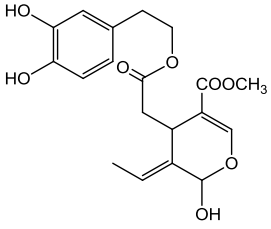
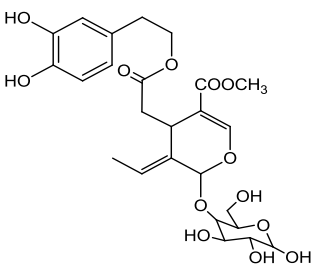
Class/Phenolic compound	Molecular formula	MW	Chemical structure	Ref
Dihydro-oleuropein	$C_{25}H_{36}O_{13}$	544		Obied et al., 2008a
3,4-DHPEA-EDA / Dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol / DOA	$C_{17}H_{20}O_6$	320		Servilli et al., 2004
Oleuropein diglucoside	$C_{31}H_{42}O_{18}$	702		Fu et al., 2010
Nüzhenide	$C_{31}H_{42}O_{17}$	686		Silva et al., 2010
Oleuropein aglycone / 3,4-DHPEA-EA	$C_{19}H_{22}O_8$	378		Servilli et al., 2004
Oleuropein	$C_{25}H_{32}O_{13}$	540		Ryan et al., 1998

Table 4 *(Cont)*

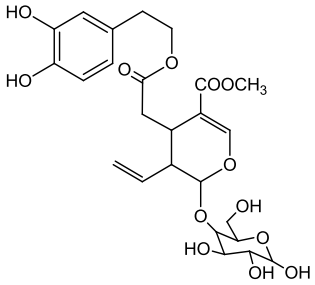
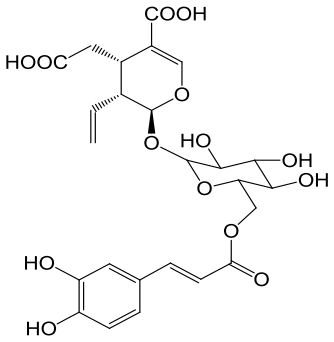
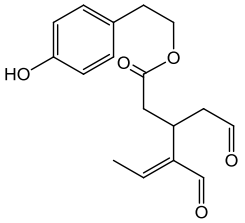
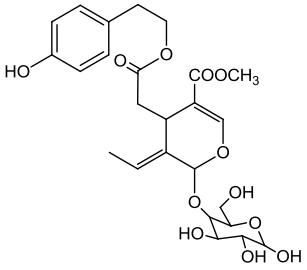
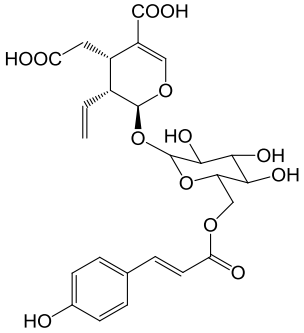
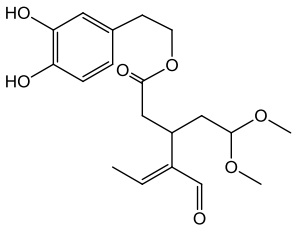
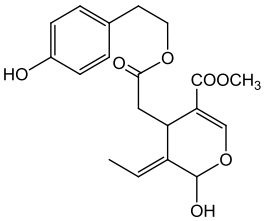
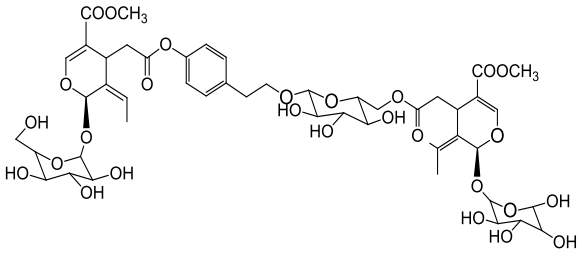
Class/Phenolic compound	Molecular formula	MW	Chemical structure	Ref
Oleuroside	$C_{25}H_{32}O_{13}$	540		Obied et al., 2007a
Caffeoyl-6'-secologanoside / Cafselogoside	$C_{25}H_{28}O_{14}$	552		Obied et al., 2008a
p-HPEA-EDA / Dialdehydic form of decarboxymethyl elenolic acid linked to tyrosol / DLA /	$C_{17}H_{20}O_5$	304		Servilli et al., 2004
Ligstroside	$C_{25}H_{32}O_{12}$	524		Ryan et al., 1998
Comselogoside	$C_{25}H_{28}O_{13}$	536		Obied et al., 2008a

Table 4 *(Cont)*

Class/Phenolic compound	Molecular formula	MW	Chemical structure	Ref
Acetal of 3,4-DHPEA-EDA	$C_{19}H_{26}O_7$	366		Obied et al., 2007a
Ligstroside aglycone / *p*-HPEA-EA	$C_{19}H_{22}O_7$	362		Servilli et al., 2004
Nüzhenide 11-methyl oleoside	$C_{48}H_{64}O_{27}$	1072		Silva et al., 2010

[A]Molecular weight (Da). [B]Chemical structures were drawn based on literature data for flavonoidal structures (Cuyckens et al., 2004).

3.3.2.2 Tissue distribution

The studies examining olive phenols tissue distribution are scarce, encompassing only few reports that separately monitored all the constituental parts of fruit, *i.e.* peel, pulp and stone (Servili et al., 1999a; Servili et al., 1999b). Other studies have focused only on a single tissue, mainly pulp and/or pulp and peel together, while stone has been rarely investigated (Silva et al., 2010; Servili et al., 2007; Silva et al., 2006; Ryan et al., 2003; Ryan et al., 2002b; Fernández-Bolaños et al., 1998; Maestro-Durán et al., 1994). However, the peel and pulp together contain more than 90% of total fruit phenols, while the rest are confined in the seed (Servili et al., 2012).

Peel. It is mainly represented by two secoiridods, *i.e.* demethyloleuropein and oleuropein, one cinnamic acid (verbascoside) and two flavonoids, *i.e.* luteolin-7-*O*-glucoside and rutin. Other phenolic species, with an exception of hydroxytyrosol, are present in much lower quantities (Servili et al., 1999a).

Pulp. It is the main reservoir of olive fruit phenols composed of several representatives collectively gathered in **Table 5** under the nomen – olive fruit.

Stone. Olive seed and husk, *i.e* stone, contains much lower phenolic species, but of all major classes (**Table 5**). Nüzhenide is generally reported to be the predominant phenol and restricted to the seed compartment (Ryan et al., 2002a).

3.3.2.3. Functions

Fruit preservation. Luteolin, apigenin and quercetin were recognized as strong UV sun-screeners protecting fruits against photooxidation (Ryan et al., 1998), while some phenolic acids were proved to be involved in the growth and alternate fruit regulation (Lavee et al., 1985).

Disease resistance. Many fruit phenolic representatives such as oleuropein, caffeic acid and others were proved to possess antimicrobial activities against various pathogenic bacteria and fungi, molluscicidal properties and preventive role in several parasite invasions (Ryan et al., 1998).

Fruit quality. Phenolic compounds contribute to olive fruit quality in several ways. They are responsible for the colour (*e.g*. cyanidin-3-*O*-rutinoside) and flavour (*e.g.* bitterness of oleuropein), while some are also associated with their preservability and commercial value (Ryan et al., 1998).

Nutritional quality. Olive fruit phenols have mainly attracted attention as powerful food antioxidants; several *in vitro* studies proved their strong antioxidant activities (*e.g.* McDonald et al., 2001), while the *in vivo* assessments confirmed an increased plasma antioxidant potential in humans after the fruits administration (Kountouri et al., 2007).

3.3.2.4 Factors affecting yield & presence

The type and content of phenols found in olive fruits depend on several factors such as varietal, agronomic and environmental ones.

Cultivar and genetics.

The phenolic profile uniqueness of olive fruit cultivars has been the subject of several studies and yet some of them indeed established the correlation with variety and/or its genetic code. For example, Lentisca *cv*. was characterised by a high level of oleuropein and hydroxytyrosol, while Santulhana *cv*. with a high content of oleuropein. Moreover, the cultivar was proved to be the key factor determining the TP variation of olives (63–78%), and a small size fruits were associated with higher content of oleuropein (Goulas et al., 2012; Riachy et al., 2011b).

Agronomic and environmental factors.

Ripening stage. Several authors studied the phenolic profile modification during olive fruits physiological development. Two degrees of maturation were recognized, namely the green and black, and both associated with the biosynthesis and/or degradation of particular phenols. Such is the case of ligstroside and oleuropein showing decline during black maturation, conversely to anthocyanins displaying a marked increase. The drop of oleuropein was also accompanied by a rise of demethyloleuropein, elenolic acid glucoside, hydroxytyrosol, tyrosol and verbascoside, while luteolin-7-*O*-glucoside, rutin and luteolin showed a consistent rise with fruits maturation (Goulas et al., 2012; Riachy et al., 2011b; Ryan et al., 1998).

Cultivation zone. Another factor influencing the fruit phenolic profile is a cultivation zone and/or its related pedo-climatic conditions. For example, olives from higher altitudes had higher TP yields than those from lower altitudes and the frost-damaged fruits had a lower content of secoiridoids (Goulas et al., 2012; Riachy et al., 2011b).

Water availability. A negative relationship was established between the fruits TP yields and the water availability, most likely due to suppression of enzymes responsible for the phenolic biosynthesis (Goulas et al., 2012; Riachy et al., 2011b).

Sanitary state. A recent study demonstrated that olive fly attack may decrease the TP yields in olive drupes, which has been ascribed to an advanced enzymatic oxidations (Goulas et al., 2012).

3.3.3 Olive oil phenols

Phenolic compounds are peculiar to virgin olive oil and are not present in any other vegetable oils (Ramírez-Tortosa et al., 2006).

3.3.3.1 Main representatives

Olive oil comprises phenols from all seven classes; cinnamic acids are typically represented by caffeic, sinapic, *p*- and *o*-coumaric acids, while benzoic acids by vanillin, vanillic, syringic, protocatechuic, gallic and *p*-hydroxybenzoic acids. Among simple phenols, hydroxytyrosol, tyrosol and their acetates are typically found in oil, whereas flavonoids are characterised by the two aglycones, *i.e.* luteolin and apigenin. The most abounded group in olive oil is the class of secoiridods encompassing aglycones of oleuropein (3,4-DHPEA-EA) and ligstroside (*p*-HPEA-EA) or their decarboxymethylated forms; 3,4-DHPEA-EDA and *p*-HPEA-EDA. Another major class are lignans (*e.g.* pinoresinol and acetoxypinoresinol), while the incidence of hydroxy-isochromans has been rarely reported (Servili et al., 2009; Bianco et al., 2001). Other representatives of olive oil phenols are listed in **Table 5**.

3.3.3.2 Functions

Sensory quality. Phenols, together with volatiles, are the main responsible factors for sensory attributes of olive oils, providing a delicate and unique flavour highly appreciated by the consumers. For example, 3,4-DHPEA-EDA and oleuropein aglycone were associated with bitterness, while *p*-HPEA-EDA with pungency. Other phenols affecting sensorial characteristics are tyrosol, caffeic, *p*-coumaric and *p*-hydroxybenzoic acids (Riachy et al., 2012b; Servili et al., 2009). However, phenols may also contribute to a flavour in a negative sense like ethyl ester of cinnamic acid and 4-vinylphenol (Ryan et al., 1998).

Oxidative stability. Phenols are fundamental also for the shelf-life and oxidative stability of virgin olive oils. They combat a lipid oxidation already at initial stage *via* mechanisms such as radical scavenging, hydrogen atom transfer and/or metal-chelating abilities. Phenols endowed with high antioxidant activities (*e.g.* hydroxytyrosol and 3,4-DHPEA-EDA) are the most responsible for oxidative stability of olive oils (Riachy et al., 2012a).

Nutritional quality. The presence of phenols with high antioxidant activities increases the nutritional value of olive oils. However, *o*-diphenols such as hydroxytyrosol, 3,4-DHPEA-EDA and oleuropein aglycone are, in this order, the most potent antioxidants, while the contribution of monohydroxylated phenols (*e.g.* tyrosol) is typically lower and less frequently investigated. Interestingly, among flavonoids luteolin displayed the highest antioxidant activity comparable to hydroxytyrosol, whereas apigenin did not show any antiradical activity (Riachy et al., 2012a).

Health benefits of olive oil phenols. The beneficial effects of olive oil phenols have been the focus of several investigations. In addition to their widely documented antioxidant activities, they seem to exert also others such as antithrombotic and antihypertensive effects. Moreover, they were associated with protective effects against certain types of cancer, neurodegenerative and cardiovascular diseases, in addition to the anti-aging protection. Their wide range of health benefits are in depth reviewed elsewhere (Servili et al., 2009; Covas, 2007; Fitó et al., 2007; Covas, 2006; Vissers et al., 2004).

3.3.3.3 Factors affecting yield & presence

The type and amount of olive oil phenols depend on several factors, encompassing those that influence their yields in fruits (Section 3.3.2.4) and those influencing their transfer, transformation and partition trail during olive oil production (Section 3.3.5), in addition to the storage conditions.

3.3.4 Olive mill waste phenols

Olive mill waste phenols display a great complexity in structures and concentrations, in depth reviewed recently by Obied (2010).

3.3.4.1 Main representatives

More than 30 different phenols have already been identified in olive mill wastes, however, the most representative are hydroxytyrosol, tyrosol, verbascoside, vanillic acid, caffeic acid, rutin, luteolin-7-*O*-glucoside, catechol and some others (Obied et al., 2005).

Phenolic compounds of wastewater are classified into two groups; the first is represented by simple phenols, non-oxidised tannins of low MW and flavonoids, while the second contains darkly coloured polymers as a result of polymerisation and auto-oxidation of the first group phenols (Niaounakis et al., 2006).

3.3.4.2 Functions

A diverse range of bioactivities were reported for the olive mill waste phenols, including antioxidant, anti-inflammatory, antimicrobial, antiviral antiatherogenic, molluscicidal, chemo- and cardioprotective activities, in addition to hypoglycemic, cytostatic activities and others summarised by Obied et al. (2005).

3.3.4.3 Factors affecting yield & presence

The profiles of olive mill wastes demonstrates a large diversity in quali- and quantitative phenolic composition. Factors contributing to a such variability include those affecting the fruits (Section 3.3.2.4) up to those directly influencing the wastes such as the type of technology, the storage conditions and the sample preparation (Obied et al., 2005).

3.3.5 Olive phenols in olive oil processing

Olive phenols entailed in olive oil processing encompasses those originally present in the fruits and those newly formed *via* various chemical and/or enzymatic reactions. The initial fruit phenols derived from either peel, pulp and/or stone present the available pool of phenols that could end-up in one or all of the final products, however, the form and extent in which they reach them is yet poorly understood as the chemistry behind is very complex, arising from all – the transfer, transformation and partition phenomena. The studies covering this topic are scarce and limited in many aspects, but those available were somewhat grouped and presented below according to the issue relevant for the field.

Phenols transfer. The aspect of matter transfer during olive oil production has been the subject of an excellent review monograph provided by Herrera (2007), which to authors' best knowledge is the only considering this topic. There are three important factors, which affect each of matter transfer; i) the compounds nature, ii) the initial

fruits status, and iii) the process operative conditions. Crushing, which results in the formation of olive paste, disrupts the equilibrium between the physiological parts of fruit and releases constituents (*e.g.* phenols) which are mixed and distributed among the systems integrating a newly formed paste, *i.e.* oil, pulp, vegetative water and fragments of stone, according to their affinities and concentrations. The magnitude of these transfers depend on the characteristics of interfacial regions between the systems as well as on the operative conditions, which regulate the rate of enzymatic and chemical processes, resulting in various transformations.

Phenols transformation. The degradative mechanism of fruits native phenol representatives during olive oil extraction, leading to their new respective derivatives, is still much in its infancy at present, though imperative from both, the applied and fundamental perspectives. Such knowledge could open the possibility to manipulate their levels and occurrence in the final products, improving the nutritional value of olive oils as well as mitigate the environmental side effects. As already stated by Servili et al. (2004) "One of the most important aspects is to define the biochemical mechanisms that would explain the occurrence of phenols in olive oil; the mechanisms that are largely unknown".

Among all phenol classes, only secoiridoids have been the subject of more intensive investigations, while biochemical transformations of others have been sparingly discussed in the existing literature. However, only two studies best to our knowledge has assigned the biodegradation pathway of fruit main secoiridoid glucosides; Servili et al. (2004) demonstrating the evolution of oleuropein and ligstroside aglycones and their respective decarboxymethylated forms (**Figure 3**) and Obied et al. (2007a) revealing their further isomerisation and equilibria patterns (**Figure 4)**. The interconversions of other native fruit secoiridoids such as nüzhenides yet remains unknown, similarly as for other classes' representatives.

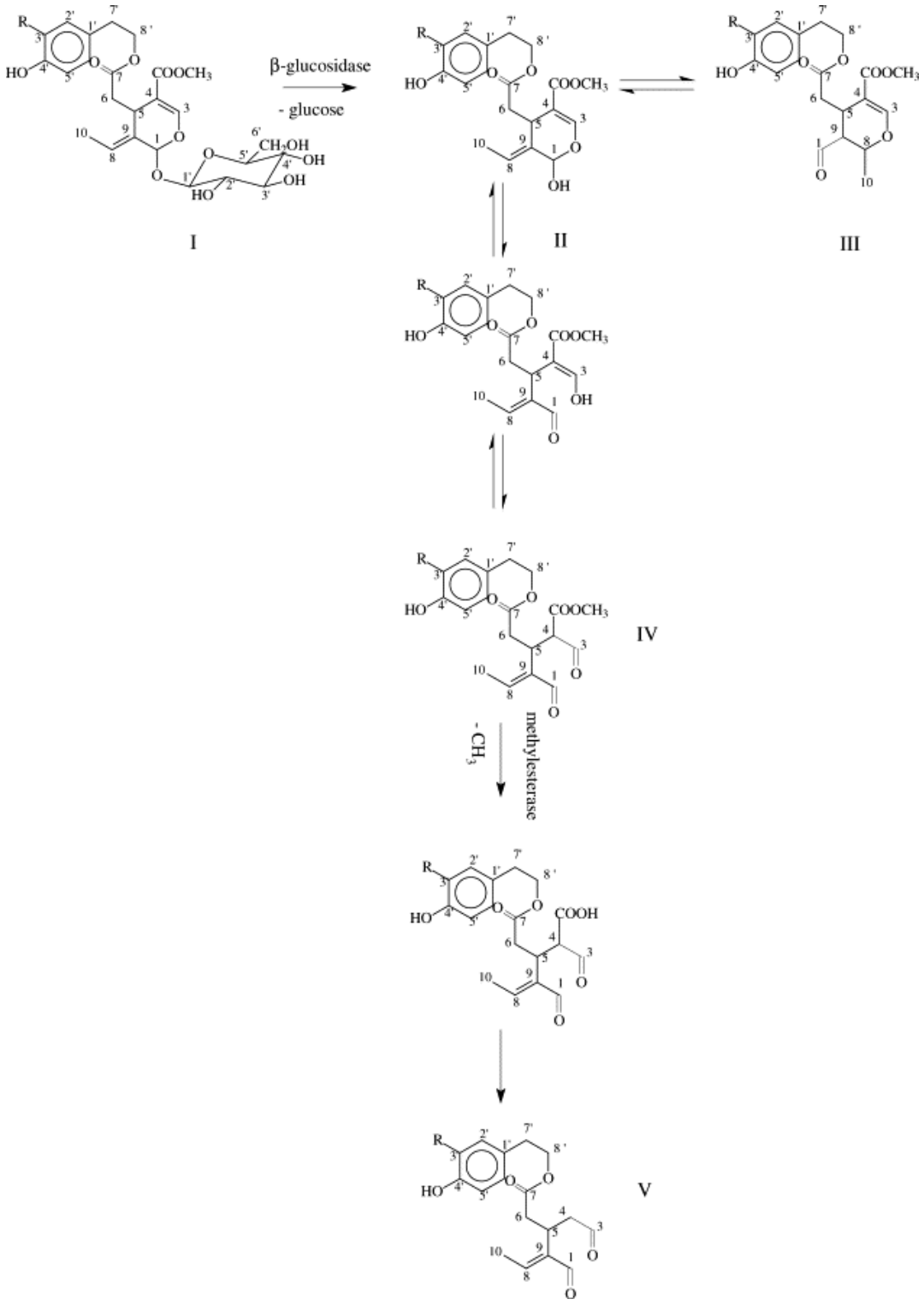


Figure 3 Proposed biochemical transformation of selected secoiridoids (Adapted by Servili et al., 2004)

(Compounds are identified as follows; (**I**) R = H: ligstroside; R = OH: oleuropein; (**II**) R = H: ligstroside aglycon; (**III**) R = OH: 3,4-DHPEA-EA; (**IV**) R = H: dialdehydic form of ligstroside aglycon; R = OH: dialdehydic form of oleuropein aglycon; (**V**) R = H: *p*-HPEA-EDA; R = OH: 3,4-DHPEA-EDA).

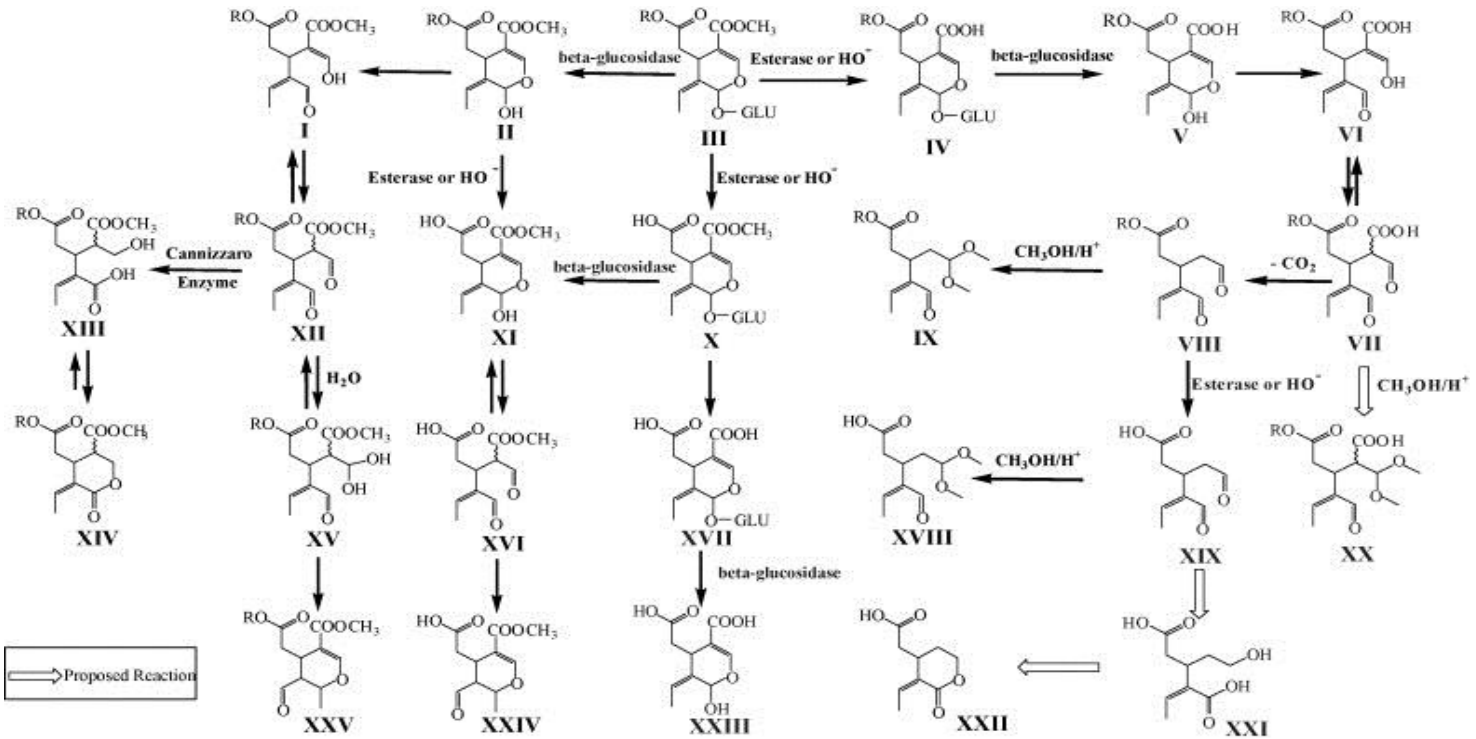


Figure 4 Transformations of oleuropein and ligstroside during maturation, processing and/or sample handling (Adapted by Obied et al., 2007a)

(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) and (IX) 3,4-DHPEA-DEDA acetal (366), (**X**) oleoside methyl ester (404), (**XI**) elenolic acid (242), (**XII**) oleuropeindial (keto form) (378), (**XIII**) Cannizzaro-like product of oleuropeindial (396), (**XIV**) lactone of **XIII** (378); (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**, (**XXIII**) demethyl elenolic acid (228), (**XXIV**) elenolic acid mono-aldehyde (rearrangement product), (**XXV**) hydroxytyrosol elenolate (oleuropein aglycone aldehyde form or 3,4-DHPEA-EA) (378)).

Phenols partition. The study of Rodis et al. (2002) is the only report that solely addressed the subject of phenols partition phenomena during olive oil extraction. Olive phenols are amphiphilic in nature with a higher solubility in water than in oil phase, which affect their distribution when pressed to olive oil. The partition of phenols in oil/water mixtures is characterized by the partition coefficient (K_p) defined as the equilibrium of phenols concentration in oil [C_{oil}] and water [C_{water}] (K_p = [C_{oil}]/[C_{water}]), and differs for each individual; ranging from 6×10^{-4} (oleuropein) to 1.5 (oleuropein aglycone). This also explains why some of them cannot be found in the oil (*e.g.* oleuropein), while others (*e.g.* oleuropein aglycones) are present in large quantities. Moreover, the experimental study also showed that majority of phenols has low K_p and will thus be lost with the wastewater during processing. However, the proportions of phenols residing in the final products also depend on their composition, relative amounts and the operative conditions.

A detailed literature state-of-the-art review was further conducted as regards to operative conditions considered within the doctoral study. All existing reports evaluating the impact of crushing step and/or malaxation along with operative parameters of malaxing time/temperature, lukewarm water, NaCl and talc additions on phenols appearance, yield and/or behaviour during olive oil extraction were reviewed with aim to elucidate their role, some drawbacks and some forward potentials.

Crushing. Though crushing has been recognized as one of the most critical steps in the olive oil processing, the studies covering this topic accounts for a limited data restricted in many aspects. To our best knowledge only four studies (Inarejos-García et al., 2011; Gómez-Rico et al., 2009; Montedoro et al., 2002; Servili et al., 1999b) have evaluated, in a comparative way, the impact of crushing on phenols quali- and quantitative changes by correlating the paste's profile with that of initial fruit composition. Interestingly, none of them has quantified their total loss, neither discussed and/or proposed any of their new interconversion relationships. This is somewhat expected as the range of phenols followed was very limited; for example Gómez-Rico et al. (2009) reported the results only for twelve phenols, while others even less, *i.e.* ten (Servili et al., 1999b), seven (Montedoro et al., 2002) and six (Inarejos-Garcia et al., 2011). With a slight difference, the impact of crushing has

also been studied as a function of crushers type on oils' TP yields (Inarejos-Garcia et al., 2011; Preziuso et al., 2010; Di Giovacchino et al., 2002a; Caponio et al., 1999), but the profiles of pastes were not provided (except in a former study). Even so, the TP yield behaviour of olive oils was correlated with the behaviour of olive paste, postulating to its higher release and/or altered partition pattern between its aqueous and oil phases.

Impact of malaxation time-temperature. These two parameters are the most widely studied among various operative conditions influencing phenol yields, evaluated as either sole variables or in a combination with others such as cultivar and the type and scale of extraction system. However, in spite of intense investigations, only few have in addition evaluated their impacts on the quantity of final products formed.

Impact of time

The majority of studies evaluated the impact of malaxation time on olive oil phenol yields, mainly total (Youssef et al., 2013; Inarejos-García et al., 2009; Di Giovacchino et al., 2002b; Angerosa et al., 2001) and less on individuals (Stefanoudaki et al., 2011; Kalua et al., 2006; Ranalli et al., 2003; Servili et al., 1999b). However, contradictive results have been reported, demonstrating more or less substantial rises and/or drops depending on the cultivar and time ranges investigated. While increases were mainly linked with higher TP releases from paste and improved partition to oily phase, the TP yield decreases were typically ascribed to an advanced enzymatic oxidations. Interestingly, only one study (Inarejos-García et al., 2009) in addition proposed an altered TP partition pattern between the oily and aqueous phases during paste malaxation, but unfortunately, the wastewater TP yields have not been analysed to confirm nor reject the notion established.

The impact of malaxing time has also been studied twice as a function of paste's TP yield behaviour during course of malaxation (Gómez-Rico et al., 2009; Artajo et al., 2007). However, in both studies a significant drop of secoiridoid glucosides and of 3,4-DHPEA-EDA was observed, while other secoiridoid derivatives showed a less consistent behaviour, similarly as flavonoids, verbascoside and phenolic acids remaining rather constant. By contrast, phenolic alcohols displayed a distinct

behaviour, showing rise in the study of Gómez-Rico et al. (2009) and a drop in that of Artajo et al. (2007).

Best to our knowledge, only two studies have investigated the impact of malaxing time on phenol yields in olive waste matrices, *i.e.* one in a 2-phase pomace (Obied et al. 2008b) and one in the wastewater (Di Giovacchino et al., 2002b). While the time increase (30–60 min) has clearly raised the TP yields in pomace, the majority of individuals followed (five in total) remained unaffected. Likewise, the TP yields of wastewater showed an increase, though limited up to 45 min, while the behaviour of individual phenols has not been monitored. Interestingly, the corresponding 3-phase pomace has not been analysed or the results were simply omitted.

Impact of temperature

The problem of temperature management during paste malaxation has been the focus of many research investigations, but yet remains a controversial issue. The majority of studies has almost exclusively quantified their impacts on olive oil phenol yields (mainly Folin-Ciocalteu TP based) and barely in any others; best to our knowledge only once in paste (Gómez-Rico et al., 2009) and once in a 2-phase pomace (Obied et al., 2008b). However, a limited rise up to 27 and 30 °C (Parenti et al., 2008; Ranalli et al., 2001) or a non-limited one up to 40 and 42 °C (Stefanoudaki et al., 2011; Inarejos-García et al., 2009) has been reported for oils, whereas a marked drop in pomace upon temperature rise (15–30 °C). Many premises were established explaining such behaviour; while the TP yield rises in olive oils were linked to a higher TP releases from paste (Parenti et al., 2008; Ranalli et al., 2001), increased solubility in oily phase (Rodis et al., 2002) and increased partition between the oily and aqueous phases (Inarejos-García et al., 2009), the TP drops have always been ascribed to advanced oxidative degradations. Interestingly, the same TP drop in pomace was attributed to an increased TP partition to olive oil (Obied et al., 2008b), but the yields in latter have unfortunately not been analysed.

Impact of lukewarm water addition. The addition of lukewarm water to olive paste during malaxation to improve the olive oil extractability has been suggested several years ago in 1975 (Clodoveo, 2012). Since then, several investigations were carried

out reporting both, its yield improvement or reduction depending on individual characteristics of olive cultivar. At the same time, these paste dilutions have been largely reported to wash out the olive oil phenols as verified by a number of representative reports (Carrapiso et al., 2013; Ben-Bavid et al., 2010; Issaoui et al., 2009; Salvador et al., 2004; Di Giovacchino et al., 2002a; Gimeno et al., 2002; De Stefano et al., 1999; Angerosa et al., 1996), though being essential in some of extraction systems (*e.g.* in 3-phase centrifuge). However, most of these studies have quantified their impacts on olive oils TP yields using Folin-Ciocalteu analysis and rarely on individuals, and yet with a limited range of representatives (Stefanoudaki et al., 2011; De Stefano et al., 1999; Angerosa et al., 1996). Moreover, there is scarce information available about their impacts on phenol yields behaviour in the corresponding waste matrices. To our best knowledge, only two studies have evaluated the impact of water addition on wastes' phenol yields (Obied et al., 2008b; Lesage-Meessen et al., 2001), however, only on solids, *i.e.* pomace, while none of the studies have quantified their impacts on phenol yields in all matrices in a single study.

Impact of talc and NaCl addition. Talc, next to calcium carbonate, is the only legal co-adjuvant allowed in the olive oil processing under current EU regulation due to its exclusively physical action. Chemically, it is a hydrated magnesium silicate of particle size lower than 40 μm, typically added to difficult paste to facilitate the extraction of bonded oil (Clodoveo, 2012). Its ability to improve the physical extractability of olive oil has been verified by a number of examples, while on the other side, it has been rarely investigated for the purpose of phenols oil enrichment – four times and yet mostly evaluated with Folin-Ciocalteu TP based analysis (Carrapiso et al., 2013; Ben-David et al., 2010; Artajo et al., 2006b; Cert et al., 1996). Recently, NaCl was proposed as a timid alternative to talc due to its strong demulsifying ability, which increases the extraction of olive oils (Chumsantea et al., 2012; Cruz et al., 2007). However, its potential to alter phenols solubility in aqueous solution has already been modelled before (Noubigh et al., 2007), while experimentally during course of olive oil production has not been yet established. The only reported example to authors' best knowledge has investigated the impact of NaCl + talc addition on phenol yields in olive oil (Pérez et al., 2008), where the yield rises were attributed to an altered TP partition equilibrium between the oily and

aqueous phases without investigating their yields in the wastewater sample. However, what is of interest is the fact that none of the studies reported the extraction yield behaviour for the corresponding waste matrices, imperative from environmental perspective. Similarly, the same data were omitted in all talc-addition investigations.

Based on this extensive literature review, some of the main issues could be summarised as follows. None of the studies yet have systematically followed the fate olive fruit phenols through all operative steps, *i.e.* from fruits to paste and its final products – oil and wastes, in respect to different operative conditions using a common 3-phase centrifugal system. Best to our knowledge, only one have trailed their fate using a 3-phase (Servili et al., 1999b) and a 2-phase extraction lines (Gómez-Rico et al., 2009), but both with a limited data compilation. Three studies have followed the olive phenols partition trail from paste, and yet not in relation to operative conditions, but instead to fruits ripening stage (Artajo et al., 2006c), harvest period (Artajo et al., 2007) and olive trees irrigation (Artajo et al., 2006b).

The major drawback of all existing studies is the restricted analysis of olive matrices entailed in olive oil processing, typically focused solely on olive oil and barely on any others. The absence of quantified impacts on all the process-derived matrices from a single experiment reveals no mechanisms behind the effects of individual technological variables, remaining many questions as regard to olive fruit phenols transfer/partition trail yet unanswered. The lack of process mass balance data is another major failure of existing studies, exploring variables potential without examining their impacts on the quantity of products, *i.e.* oil and wastes, produced. Moreover, a commonly employed Folin-Ciocalteu TP based analysis has not only restricted an insight into olive fruit phenols qualitative transformation trail, but also prevented to determine the uniqueness of its pattern. Similarly, a limited range of individual phenols did not permit an adequate study of fruit phenols degradation pathway nor to follow the evolution of their respective derivatives. Furthermore, no conceptual distinction has yet been made as regard to olive phenols origin as none of the studies has separately followed their trail from the two main sources *i.e.* stone and the rest of fruit compartment (peel and pulp), neither evaluate their further

behaviour through all operative steps. Hence, some of these missing issues were the subject of present doctoral investigation.

3.3.6 Olive phenol analysis

Several analytical methods have been proposed in the phenol analysis of olives and their process-derived matrices comprising various sample preparation and detection methods reviewed in detail elsewhere (Bendini et al., 2007; Carrasco-Pancorbo et al., 2005; Antolovich et al., 2000; Ryan et al., 1998). Here, only those used in the doctoral thesis are briefly introduced below in order to justify the choice of their selection.

Most of existing analytical methods are based on a three-step analysis comprised of extraction, separation and quantification of phenolic compounds.

Extraction. Solid samples are the most challenging, as they require a quantitative and representative collection of analytes in the liquid solvent with as low interfering components possible. Methods applied for olive matrices typically utilises a solid-phase (SPE), liquid-liquid (LLE) or solid-liquid extraction using different organic solvents and conventional methods of manual or mechanical agitation. In recent years, ultrasound (US) has gained significance in the extraction assistance of phenols, mainly due to advantages such as higher efficiency, lower solvent consumption and a faster extraction. Such assistance is typically provided by high-power US baths or probe-type sonicators (100–450 W) whose powerful stirring, mixing and agitating abilities has also been tested for olive phenols isolation in our samples, *i.e.* fruits, wastewater and oil, and of which results are presented in the form of published papers (**Annex B1–B3**).

Detection. While total phenols (TP) are typically quantified based on the colorimetric detection at 765 nm using Folin-Ciocalteu reagent, the separation and detection of individual phenols in olive extracts is carried out by reverse phase high performance liquid chromatography (RP-LC) system coupled with different detectors such as UV/VIS (DAD), fluorescence (FLD) and mass spectrometer (MS).

Diode-array (DAD) detection. All phenolic compounds exhibit a strong absorption in the UV, and some also in the Vis region, which makes them perfect candidates for spectroscopic detection. Their characteristic UV-Vis spectral shapes often allow their classification and sometimes provides additional structural information, but entails a good chromatographic separation and is far less sensitive than MS (Obied et al., 2007a).

Mass spectrometry (MS) detection. LC-MS analysis is one of the most preferable methods used in the phenol profiling of olive matrices as reflected by a large of number of reports provided in **Table 5**. MS detector is much more selective and sensitive than DAD, and provides important structural information based on the compound's molecular weight and its fragmentation profile (Obied et al., 2007a). Most recently, a high resolution accurate mass spectrometry (HR-MS) has been adopted in the analysis of several complex matrices benefiting in many aspects, but most importantly in a higher mass accuracy. This detector allows to identify the compound based on accurate mass measurements of molecular and fragment ions, providing elemental composition of known and novel constituents with a high mass accuracy, typically below 10 ppm (Fu et al., 2009a; Fu et al., 2009b).

Phenols found in *Olea europeae* L. – a literature review. Numerous papers have been published defining the phenol profile of different *O. europaea* L. matrices, however, the majority has been focused on olive oil, followed by the fruits and wastes such as leaves, wastewater and pomace, whereas other matrices, *i.e.* paste, stones and/or seeds have only been sparingly investigated.

As the main strategy of olive phenols identification used in the doctoral thesis was based on MS detection, the literature review of previously identified phenols was done based on existing LC-MS data reports. Interestingly, there is no timely review yet available in the existing literature combining all matrices entailed in olive oil processing. **Table 5** hence reviews all of them, *i.e.* the fruits, stone, paste, pomace, wastewater and oil, with aim of creating the platform for further literature data comparison with our results (discussed elsewhere, Section 5.1.1). However, in this table, olive phenols are organised into seven main classes with representatives listed

according to their MW (in a decreasing order) and assigned with the most relevant references.

Collectively, more than hundred olive phenols were united in a single table, revealing some of the inconsistencies among existing data reports; for example, the same name was assigned for a different phenol and *vice versa*. A very recent study of Peralbo-Molina et al. (2012) reported the presence of secologanoside in pomace, providing incorrect molecular formula ($C_{25}H_{32}O_{14}$) though its chemical structure is well known ($C_{16}H_{22}O_{11}$) from several other reports (*e.g.* Obied et al., 2007a; Obied et al., 2008a). Likewise, the use of several synonyms and abbreviations can easily lead to identity miss-assignment of various phenolic representatives. One such example is a commonly reported phenol known as dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol. Most studies refer to this compound by using abbreviated term, *i.e.* 3,4-DHPE-EDA (Servili et al., 2004), while others uses; 3,4-DHPEA-DEDA (Obied et al., 2007a), DOA (Ouni et al., 2011), decarboxymethyl oleuropein aglycon (Dierkes et al., 2012) or deacetoxy oleuropein aglycon oleuropein (Bonoli, 2004). Nevertheless, consideration should also be given to some of the chemical structures provided in the literature.

Table 5 Identified phenols in different olive matrices; fruit, stone, paste, pomace, wastewater and oil

Class/Phenolic compound	MW[A]	IM[B]	Major signals	Olive matrix					
				Olive fruit	**Stone**	**Paste**	**Pomace**	**Wastewater**	**Oil**
Simple phenols									
Hyroxytyrosol rhamnoside	482	-	481, 265, 153				Peralbo-Molina, 2012		
Hyroxytyrosol diglucoside	476	-	475, 245, 153				Peralbo-Molina, 2012		
4-[β-D-xylopyranosyl-(1→6)]-β-D-glucopyranosyl-1,4-dihydroxy-2-methoxybenzene	434			Obied, 2007a			Obied, 2007a		
Hydroxytyrosol glucoside	316	-	315, 153, 123	Savarese, 2007	Ryan, 2002b	Kanakis, 2013	Cardoso, 2005	Artajo et al., 2006b	Bianco, 1998
Tyrosol glucoside	300	-	299, 179, 137, 119	Romero, 2002a	Maestro-Durán, 1994	Kanakis, 2013	Peralbo-Molina, 2012		
D (+)-Erythro-1-(4-hydroxy-3-methoxy)-phenyl-1,2,3-propantriol	214	-	213, 195, 151	Obied, 2007a			Obied, 2007a		Obied, 2007a
Hydroxytyrosol	154	-	153, 123	Savarese, 2007	Ryan, 2002b	Silva, 2006	Lozano-Sánchez, 2011	Lozano-Sánchez, 2011	Dierkes, 2012
Hydroxytyrosol acetate	196	-	195, 153, 137, 123, 77, 68	Morelló, 2004				Artajo, 2006b	Brenes, 1999
Tyrosol acetate	180								Mateos, 2001
Tyrosol	138	-	137, 93	Romero, 2002a	Ryan, 2003	Montedoro, 2002	Lozano-Sánchez, 2011	Lozano-Sánchez, 2011	Suárez, 2008
Catechol	110			Romero, 2002a					Brenes, 2004
Benzoic acids									
Syringic acid	198	-	197	Ryan, 1999	Fernández-Bolaños, 1998		Alu'datt, 2010		Cioffi, 2010
Quinic acid	192	-	191, 103				Lozano-Sánchez, 2011	Lozano-Sánchez, 2011	
Homovanillic acid	182	-	181	Ryan, 2002b	Ryan, 2002b	Artajo, 2007		Artajo, 2006b	
4-Hydroxy-3-methoxy-phenylacetic acid	182								Bendini, 2007
Syringaldehyde	182				Fernández-Bolaños, 1998				
3-(3,4-Dihydroxyphenyl)propanoic acid	182								Bemdini, 2007
Gallic acid	170	-	169, 125	Mcdonald, 2001			Peralbo-Molina, 2012		Cioffi, 2010

Table 5 *(Cont)*

Class/Phenolic compound	MW	IM	Major signals	Olive matrix					
				Olive fruit	**Stone**	**Paste**	**Pomace**	**Wastewater**	**Oil**
Vanillic acid	168	-	167, 123, 108	Romero, 2002a	Fernández-Bolaños, 1998	Artajo, 2007	Peralbo-Molina, 2012	De Marco, 2007	De La Torre-Carbot, 2005
3,4-Dihydroxyphenylacetic acid	168			Boskou, 2006	Boskou, 2006				Bendini, 2007
Protocatechuic acid	154	-	153, 109	Boskou, 2006	Boskou, 2006		Alu'datt, 2010		
Gentisic acid	154								Carrasco-Pancorbo, 2007
2,4-Dihydroxybenzoic acid	154			Mcdonald, 2001					Bendini, 2005
4-Hydroxyphenylacetic acid	152	-	151, 123, 108	Boskou, 2006	Boskou, 2006		Cardoso, 2005		Caponio, 1999
Vanillin	152	-	151, 123	Morelló, 2004	Fernández-Bolaños, 1998	Artajo, 2007	Lozano-Sínchez, 2011	Lozano-Sánchez, 2011	De La Torre-Carbot, 2005
p-Hydroxybenzoic acid	138			Boskou, 2006	Boskou, 2006		Alu'datt, 2010		Caponio, 1999
Cinnamic acids									
β-Ethyl-OH-verbascoside	668	-	667, 621, 487, 459, 179, 161				Innocenti, 2006		
β-OH-verbascoside	640	-	639, 621, 459, 179, 161	Kanakis, 2013		Kanakis, 2013	Innocenti, 2006		
Verbascoside derivative	638	-	637, 461, 315, 193, 175				Innocenti, 2006		
Verbascoside / acteoside	624	-	623, 461, 161	Rigane, 2011	Ryan, 2003	Gómez-Rico, 2009	Cardoso, 2005	Mulinacci, 2001	
Isoverbascoside / isoacteoside	624	-	623, 461, 161	Obied, 2007a			Innocenti, 2006		
Chlorogenic acid	354	-	353, 191, 179, 161	Ryan, 2003	Ryan, 2002b		Cardoso, 2005		
Sinapic acid	224			Ryan, 1999			Alu'datt, 2010		
Ferulic acid	194	-	193, 134	Boskou, 2006	Boskou, 2006		Peralbo-Molina, 2012		Cioffi, 2010
Caffeic acid	180	-	179, 135	Savarese, 2007	Ryan, 2002	Montedoro, 2002	Obied, 2007	Mulinacci, 2001	Mateos, 2001
Shikimic acid	174	-	173				Peralbo-Molina, 2012		
Phenylalanine	165	-	164				Peralbo-Molina, 2012		
p-Coumaric acid	164	-	163, 135	Ryan, 2002b	Boskou, 2006		Peralbo-Molina, 2012	Suárez, 2010	De La Torre-Carbot, 2005
o-Coumaric acid	164	-	163	Mcdonald, 2001					Mateos, 2001
Cinnamic acid	148	-	147	Boskou, 2006	Boskou, 2006		Peralbo-Molina, 2012		Mateos, 2001

Table 5 *(Cont)*

Class/Phenolic compound	MW	IM	Major signals	Olive matrix					
				Olive fruit	**Stone**	**Paste**	**Pomace**	**Wastewater**	**Oil**
Flavonoids									
Hesperidin	610	-	609, 463, 377, 361	Kalua, 2005			Alu'datt, 2010		
Rutin	610	-	609, 301, 179	Savarese, 2007	Rovellini, 1997	Gómez-Rico, 2009	Cardoso, 2005	Mulinacci, 2001	
Cyanidin-3-*O*-rutinoside	595			Romero, 2002a					
Luteolin-7-*O*-rutinoside	594	-	593, 447, 285	Bouaziz, 2010	Rovellini, 1997	Kanakis, 2013	Cardoso, 2005		
Luteolin-4'-*O*-rutinoside	594						Obied, 2007a		
Apigenin 6,8-di-*C*-glucoside	594	+	595	Bouaziz, 2005					
Apigenin-7-*O*-rutinoside	578	-	577, 431, 269	Romero, 2002a			Obied, 2007a		
Quercetin-3-O-glucoside	464	+	465	Bouaziz, 2005					
Chrysoeriol-7-*O*-glucoside	462	+	463	Bouaziz, 2005					
Cyanidin-3-*O*-glucoside	449			Romero, 2002a					
Luteolin-7-*O*-glucoside	448	-	447, 285	Savarese, 2007	Ryan, 2002b	Silva, 2006	Cardoso, 2005	Mulinacci, 2001	Yorulmaz, 2011
Luteolin-4'-*O*-glucoside	448	-	447, 285	Savarese, 2007		Silva, 2006	Cardoso, 2005		
Luteolin-6-*C*-glucoside	448			Bouaziz, 2005					
Quercitrin	448	-	447, 301, 300	Savarese, 2007			Obied, 2007a		
Apigenin-7-*O*-glucoside	432	-	431, 265	Obied, 2007a		Gómez-Rico, 2009	Peralbo-Molina, 2012	Suárez, 2010	
Taxifolin	304	-	303				Peralbo-Molina, 2012		Bendini, 2007
Quercetin	302			Rigane, 2011			Alu'datt, 2010		
Chrysoeriol	300	-	299	Bouaziz, 2005					Dierkes, 2012
Methoxyluteolin	300	-	299, 227, 199, 191						De La Torre-Carbot, 2005
Cyanidin	287			Ryan, 1999					
Luteolin	286	-	285	Ryan, 2002b	Rovellini, 1997	Artajo, 2007	Lozano-Sánchez, 2011	Lozano-Sánchez, 2011	Fu, 2009a
Apigenin	270	-	269	Bouaziz, 2005		Artajo, 2007	Lozano-Sánchez, 2011	Lozano-Sánchez, 2011	Fu, 2009a
Lignans									
Syringaresinol	418	-	417						García-Villalba, 2010

Table 5 *(Cont)*

Class/Phenolic compound	MW	IM	Major signals	Olive matrix					
				Olive fruit	**Stone**	**Paste**	**Pomace**	**Wastewater**	**Oil**
Acetoxypinoresinol	416	-	415	López, 2008	López, 2008	Servili, 2007	Lozano-Sánchez, 2011	Suárez, 2010	Fu, 2009a
Pinoresinol	358	-	357, 151	Bonoli, 2004	López, 2008	Servili, 2007	Suárez, 2010	Suárez, 2010	Fu, 2009a
Hydroxypinoresinol	374	-	373		López, 2008				Obied, 2007a
Acetylhydroxypinoresinol	432	-	431						Obied, 2007a
Hydroxy-isochromans									
1-(3'-Methoxy-4'-hydroxy)-phenyl-6,7-dihydroxyisochroman	288	-	287, 257, 242						Bianco, 2001
1-Phenyl-6,7-dihydroxyisochroman	242	-	241, 211, 193						Bianco, 2001
Secoiridoids									
Oleuropein pentamer	2692	-	2691	Cardoso, 2006			Cardoso , 2006		
Oleuropein tetramer	2154	-	2153	Cardoso, 2006			Cardoso , 2006		
Nüzhenide tri(11-Methyl oleoside)	1844	-	1843, 1071, 685, 771		Silva, 2010				
Oleuropein trimer	1616	-	1615	Cardoso, 2006			Cardoso , 2006		
Nüzhenide di(11-Methyl oleoside)	1458	-	1457		Silva, 2010				
Oleuropein dimer	1076	-	1075	Cardoso, 2006			Cardoso , 2006		
Nüzhenide 11-Methyl oleoside	1072	-	1071		Silva, 2010	Kanakis, 2013			
Oleuropein diglucoside	702	-	701, 539, 377, 307, 285	Bouaziz, 2010	Servili, 1999a		Cardoso, 2005		
Neo-nüzhenide	702	-	541, 523, 505, 387, 369	Di Donna, 2007b					
Nüzhenide	686	-	685, 523, 453, 421, 403, 299	Bouaziz, 2010	Silva, 2010	Kanakis, 2013	Obied, 2007a		
Loganin glucoside	570	-	569, 389, 313				Peralbo-Molina, 2012		
10-Hydroxyoleuropein	556	-	555, 537, 376	Cardoso, 2005			Peralbo-Molina, 2012		
2''-Hydroxyoleuropein	556	-	539, 359, 377, 225, 234, 153	Di Donna, 2007b					
Ligstroside derivative	554	-	553, 341, 257, 181, 137, 109						De La Torre-Carbot, 2005
Caffeoyl-6'-secologanoside	552	-	551, 507, 389, 385, 341, 303, 281, 251, 179, 161	Obied, 2007a		Kanakis, 2013	Obied, 2007a	Obied, 2007a	
6'-β-Hexopyranosly oleoside	552	-	551, 507, 389, 341	Savarese, 2007			Cardoso, 2005		
Oleoside glucoside	552	-	551, 507, 239, 209				Peralbo-Molina, 2012		

Table 5 *(Cont)*

Class/Phenolic compound	MW	IM	Major signals	Olive matrix					
				Olive fruit	**Stone**	**Paste**	**Pomace**	**Wastewater**	**Oil**
Dihydro-oleuropein	544	-	543, 377	Obied, 2008a		Kanakis, 2013	Peralbo-Molina, 2012		
Oleuropein	540	-	539, 377, 307, 275, 225	Savarese, 2007	Ryan, 2002b	Gómez-Rico, 2009	Cardoso, 2005	Suárez, 2010	Bianco, 1998
Oleuroside	540	-	539, 377, 307, 275	Savarese, 2007	Ryan, 2002b	Kanakis, 2013	Obied, 2007a		
Loganin acid glucoside	538	-	537, 375, 179				Peralbo-Molina, 2012		
Comselogoside	536	-	535, 491, 389, 345, 265, 163, 145	Obied, 2007a		Kanakis, 2013	Obied, 2007a		
6-deoxyhexopyranosyl-oleoside	536	-	535, 517, 491, 390, 345, 325, 307, 285	Bouaziz, 2010			Cardoso, 2005		
Oleuropein derivative 2	528	-	527, 377				Peralbo-Molina, 2012		
Demethyloleuropein	526	-	525	Savarese, 2007	Servilli, 2007	Montedoro, 2002			Bianco, 1998
Ligstroside	524	-	523, 361, 291, 259, 101	Savarese, 2007	Ryan, 2002b	Silva, 2006		De Marco, 2007	
Demethylligstroside	510	-	509	Sivakumar, 2005		Artajo, 2007			
Oleoside riboside	506	-	505, 389, 345				Peralbo-Molina, 2012		
Oleoside dimethylester	418	-	417				Peralbo-Molina, 2012		
Methyl oleuropein aglycon	410	-	409, 377, 275						Suárez, 2008
Elenolic acid glucoside	404	-	403, 223, 179, 119, 101	Savarese, 2007	Ryan, 2002b	Kanakis, 2013	Cardoso, 2005		
Secologanic acid	402	-	401				Peralbo-Molina, 2012		
Deoxyloganic acid lauryl ester	394	-	393, 349, 331, 225				Rigane, 2011		
10-hydroxy oleuropein aglycon	394	-	393						García-Villalba, 2010
Methyl oleuropein aglycon	392	-	391, 345, 275				Suárez, 2010		García-Villalba, 2010
Oleoside	390	-	389, 345, 209, 121, 101	Bouaziz, 2010		Kanakis, 2013	Cardoso, 2005		Fu, 2009a
Secologanoside	390	-	389			Kanakis, 2013			Fu, 2009a
Secologanol	390	-	389						Fu, 2009a
Loganin	390	-	389, 151, 113				Peralbo-Molina, 2012		

Table 5 *(Cont)*

Class/Phenolic compound	MW	IM	Major signals	Olive matrix					
				Olive fruit	**Stone**	**Paste**	**Pomace**	**Wastewater**	**Oil**
Secologanin	388	-	387				Peralbo-Molina, 2012		
Hydroxytyrosol acyclodihydroelenolate	382	-	381, 363, 349, 245, 227, 153	Obied, 2007a			Obied, 2007a		
3,4-DHPEA-EA	378	-	377, 345, 327, 307, 275, 149, 139	Savarese, 2007		Gómez-Rico, 2009	Cardoso, 2005	Suárez, 2010	Fu, 2009b
Loganin acid	376	-	375, 151, 113				Peralbo-Molina, 2012		
Dehydro oleuropein aglycon	376	-	375, 239, 195, 179, 137						Dierkes, 2012
Acetal of 3,4-DHPEA-EDA	366	-	365, 229, 153, 138	Obied, 2007a					De La Torre-Carbot, 2005
p-HPEA-EA	362	-	361, 291, 259			Gómez-Rico, 2009	Peralbo-Molina, 2012	Suárez, 2010	Fu, 2009a
7-Deoxyloganic acid	360	-	359				Peralbo-Molina, 2012		
Decarboxymethyl 10-OH-oleuropein aglycon	336	-	335, 199				Lozano-Sanchez, 2011		Dierkes, 2012
Hemiacetal of ligstroside	336	-	335, 275, 377						Kalua, 2005
Ligstroside derivate	336	-	335, 199, 155				Suárez, 2010	Suárez, 2010	Suárez, 2008
3,4-DHPEA-EDA	320	-	319, 249, 195, 183, 139, 95, 69	Savarese, 2007	Ryan, 2003	Gómez-Rico, 2009	Peralbo-Molina, 2012	Suárez, 2010	Dierkes, 2012
Hydroxy-D-ligstroside aglycon	320	-	319, 199						García-Villalba, 2010
p-HPEA-EDA	304	-	303, 285, 183, 179, 165, 59	Obied, 2007a		Gómez-Rico, 2009	Peralbo-Molina, 2012	Suárez, 2010	Dierkes, 2012
Elenolic acid	242	-	241, 165, 139, 127, 121, 101, 95	Savarese, 2007			Peralbo-Molina, 2012	Mulinacci, 2001	Dierkes,2012
Hydroxylated form of elenolic acid	258	-	257, 213, 181, 137				Lozano-Sánchez, 2011		García-Villalba, 2010
Elenolic acid metylester	256	-	255, 223, 179, 147, 101, 69						Dierkes, 2012
Desoxy elenolic acid	226	-	225, 123, 101						Dierkes, 2012
Dialdehydic form of decarboxymethyl elenolic acid	184	-	183, 139				Lozano-Sánchez, 2011	Lozano-Sánchez, 2011	

[A]Molecular weight (Da). [B]Ionisation mode.

4 EXPERIMENTAL

4.1 Materials

4.1.1 Sampling and samples pre-treatment

Olive fruits – seasonal and geographical phenol profile variation

Olive fruits of Istrska belica *cv*. were sampled from four olive orchard locations in the north (Goriška Brda), middle (Vipavska dolina) and southern part of Slovenia (Slovenian Istria) (**Figure 5**). The healthy fruit samples were randomly collected (app. 1 kg) at optimum ripening stage (one day prior being processed to olive oil) over two crop seasons (2009–2010) between October and November, depending on region's harvest time. All fruits were immediately frozen with liquid nitrogen (liq. N_2) and freeze-dried in a Kambič LIO-5P lyophilisator (Semič, Slovenia). The pulp was separated from the stone, ground into homogeneous powder with the aid of liq. N_2 and stored at –25 °C until analysis (Jerman et al., 2010).

Figure 5 Sampling locations of Istrska belica *cv*. fruits

Olive fruit, stone, paste, pomace, wastewater and oil – olive oil processing trial

Olive fruits of Istrska belica *cv.* were harvested from olive grove of Gradno (Goriška Brda, Slovenia) and stored overnight at 4 °C until being processed the next day using an Abencor system. Prior to that, a lot of fruits were randomly chosen as a representative starting fruit material, balanced and frozen with liq. N_2 before subjected to freeze-drying process. Then, fruits were manually de-stoned and re-balanced in order to obtain the pulp/stone average mass ratio. Both of constituents were ground separately into homogeneous powder with the aid of liq. N_2 and stored

at −25 °C until analysis. Other process-derived matrices, *i.e.* pomace, wastewater and oil were sampled at the end of processing trial with an exception of paste, which was collected immediately after fruits crushing. All the samples were prepared and stored as previously described (Jerman Klen et al., 2012b; Jerman Klen et al., 2011; Jerman et al., 2010), where paste, stone and pomace were frozen with liquid nitrogen, freeze-dried and stored in the dark-glass containers at −25 °C, while the wastewater was primarily acidified (HCl, pH = 2.0) and defatted with *n*-hexane. The olive oil samples were solely stored in the dark glass containers at −25 °C until analysis.

Commercial olive mill wastes

Olive wastes from industrial-scale olive oil production were collected in the harvest season 2010; two wastewater samples and their corresponding pomaces from 3-phase centrifuges located in Dobrovo (Goriška Brda) and Šmarje (Slovenian Istria), and two semi-solid pomaces from 2-phase centrifuges in Pobegi and Šalara (Slovenian Istria). The fresh samples were immediately transferred to the laboratory, where they were prepared and stored as described above.

4.1.2 Solvents, chemicals & preparation

Glacial acetic and hydrochloric acid (35%) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), while methanol (HPLC grade) from Merck (Darmstadt, Germany). The double deionised water (DI) used in the experiments was purified on a Millipore Milli Q Plus Ultra-pure water system (Billerica, MA, USA).

Phenolic standards of hydroxytyrosol, tyrosol, oleuropein, luteolin, luteolin-7-*O*-glucoside, luteolin-4'-*O*-glucoside, apigenin, apigenin-7-*O*-glucoside, verbascoside, quercitrin and rutin were obtained from Extrasynthese (Genay, France). Others; vanillin, vanillic, caffeic and *p*-coumaric acid were purchased from Sigma-Aldrich Co. (Steinheim, Germany), while pinoresinol from ArboNova (Turku, Finland). The standard stock solutions were prepared by dissolving standards in pure methanol from which the calibration curves were prepared as follows – the aliquots of individual stock solutions were evaporated by rotary evaporation at 35 °C and in turn re-dissolved in a mixture of HPLC eluents A (H_2O/CH_3COOH, 95:5, v/v) and B (methanol) (90:10), respectively.

4.2 Methods

4.2.1 Olive oil processing trial

Technological assay comprised of 26 olive oil processing trials (13 treatments × 2 replicates) was performed with Abencor laboratory-scale olive mill (Seville, Spain) schematically shown in **Figure 6**. The system is composed of three essential elements, *i.e.* the hammer mill (a), the thermo-malaxer (b) and the centrifuge (c), imitating the industrial-scale olive oil production. This system is advantageous due to reduced amount of fruits needed for the statistical reliable results and a convenient control of operative parameters (Ben-David et al., 2010).

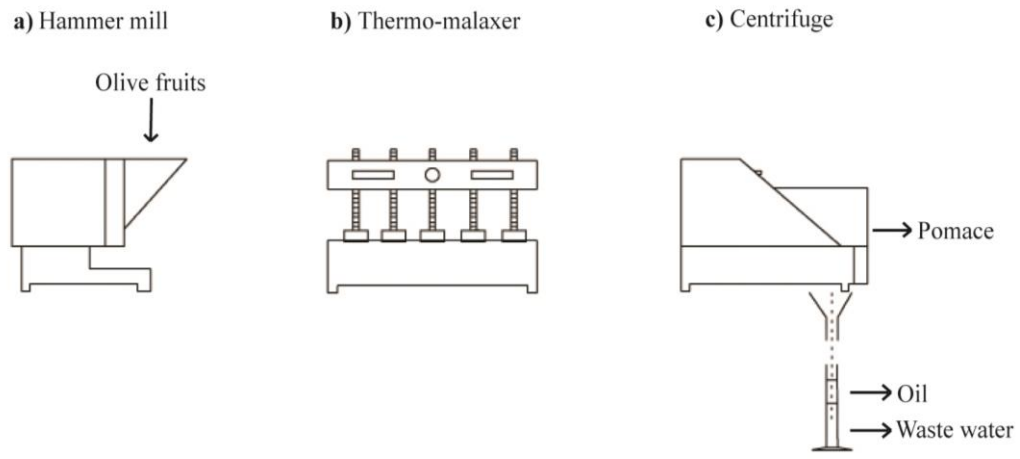


Figure 6 Schematic presentation of Abencor olive oil extraction system

First, olives were ground to a paste by the hammer mill and carefully homogenised in order to reduce the potent differences in the starting fruit material. For each treatment, approximately 700 g of paste was put into a metallic pitcher and 100 mL of water (25 °C) added to improve its rheology. Then, the paste was malaxed in thermo-malaxer for different times and temperatures, with or without addition of lukewarm water, NaCl and talc, running in total 13 trials in detail described below. Afterword, each paste was centrifuged (1400 g, 1 min) obtaining the three final products – oil and two wastes (wastewater and pomace). While the yield of pomace, which remained in the centrifuge was calculated, the masses of two liquid phases (oil

+ wastewater) separated by decantation were balanced in order to assess the process mass balance approach. All the trials were performed in duplicates.

Impact of malaxation time and temperature. Two malaxation times of 30 and 60 min combined with three malaxation temperatures of 25, 35 and 45°C were studied under the controlled conditions provided by thermo-malaxer.

Impact of lukewarm water addition. Two amounts (200 and 300 mL) of lukewarm water (40 °C) were subsequently added to a paste after 20 min malaxation, corresponding to 29% and 43% of paste´s fresh weight (FW, w/w). Afterword, the paste was re-malaxed for a further 10 or 40 min, reaching a total malaxation time of 30 or 60 min, respectively.

Impact of talc and NaCl addition. Three treatments involving addition of two co-adjuvants were tested by adding each to 700 g paste pitcher prior malaxation as follows; talc (10 g), NaCl (10 g) and talc + NaCl (5g + 5g) corresponding to 1.4% of paste´s FW (w/w).

The dry weight (DW) measurements of freeze-dried samples (fruits, stone, paste, wastewater and pomace) were performed gravimetrically according to Lesage-Meessen et al. (2001), providing the basis for phenols partition rate calculations.

4.2.2 Extraction of phenols

4.2.2.1 De-stoned fruit, stone, paste, pomace and wastewater

Phenols were extracted according to a previously published ultrasound-assisted solid liquid extraction (USLE) method (Jerman Klen et al., 2011; Jerman et al., 2010), where a freeze-dried sample (1.5 g) was sonicated (3 × 20 min) with 25 mL of methanol. The homogenates of each extraction step were centrifuged (9000 rpm, 5 min) and combined supernatants diluted with methanol to 100 mL, further stored in the screw-capped dark glass containers at –25 °C until analysis.

4.2.2.2 Olive oil

Extraction was carried using ultrasound-assisted liquid liquid extraction (US-LLE) method previously described (Jerman Klen et al., 2012b), where a 10 g of olive oil was dissolved in *n*-hexane (10 mL) and sonicated (3 × 10 min) with pure methanol (5 mL). The homogenates of each extraction step were centrifuged (9000 rpm, 2 min), combined and defatted with a freeze-based fat precipitation (20 min, −80 °C). Afterward, the remaining extract was reconstituted to 25 mL with methanol and stored in the screw-capped dark glass containers at −25 °C until analysis.

4.2.3 High performance liquid chromatography (HPLC) analysis

The aliquots of methanolic extracts were concentrated 10-times using a rotary evaporation at 35 °C. After successive methanol evaporation, the dry residue was re-dissolved in a mixture of HPLC eluents A (H_2O/CH_3COOH, 95:5, v/v) and B (methanol) (90:10), filtered through 0.22 μm/13 mm PVDF filters from Carl Roth GmbH+Co (Dűren, Germany) and immediately analysed by U(H)PLC analysis.

4.2.3.1 Qualitative analysis using UPLC-DAD-ESI-QTOF-HRMS

Phenols were identified by using ultra high pressure liquid chromatography system coupled with diode array and electrospray ionisation time-of-flight high resolution mass spectrometry detections (UPLC-DAD-ESI-QTOF-HRMS). Analyses were performed with UPLC liquid chromatograph equipped with DAD and HRMS-QTOF Synapt MS with electrospray ionization (ESI) system from Waters Corporation (Milford, MA, USA) under the same conditions described for the quantitative analysis (Section 4.2.3.2). MS scans were performed after 10 μL injection of extracts in both positive (PIM) and negative (NIM) ion modes, scanning from *m/z* 50 to *m/z* 3000 Da under the following conditions: capillary voltage 3 kV, sampling cone 40 V, extraction cone 3 V, source temperature 100 °C, desolvation temperature 350 °C, cone gas flow (N_2) 50 L/h, desolvation gas flow (N_2) 800 L/h. MS was calibrated using sodium formate and leucine encephalin was used as the lock mass. Under these conditions the instrument is expected to provide experimental data with accuracy within ± 3 ppm. The accurate mass data of molecular and fragments ions were processed through the MassLynx software 4.1 (Waters Corp., Milford, MA, USA).

4.2.3.2 Quantitative analysis using U(H)PLC-DAD

The quantitative phenol analysis was performed with a Dionex Ultimate 3000 U(H)PLC-DAD system (Thermoscientific, CA, USA). Chromatographic separation was achieved by gradient elution on Kinetex PFP column (2.6 μm, 100 mm × 4.6 mm) attached to a PFP security guard (2.1 mm × 4.6 mm) both from Phenomenex (Sydney, Australia). Analysis conditions were similar to our previous HPLC-DAD reports (Jerman Klen et al., 2012b; Jerman Klen et al., 2011; Jerman et al., 2010) using the same solvent system composed of water-acetic acid (95:5, v/v) (A) and HPLC grade methanol (B) at flow rate 1 mL/min. A slight modification in the gradient program was made to achieve better resolution and a shorter analysis time; a 65 min total run time (including the post-run equilibration) was sufficient for the fruit analysis and 88 min for the other olive matrices. The injection volume for fruits, paste, pomace and wastewater analysis was 10 μL and the gradient elution involved a nine-step program: 1% B (0–7 min), 25% B (10 min), 36.2% B (35 min), 36.5% B (35.5 min), 43.7% B (52 min), 45% B (53 min), 50% B (66 min), 100% B (72–76 min) and 1% B (79–88 min). For olive oil, a 20 μL injection volume was used and the gradient elution program employed was as follows: 1% B (0–7 min), 25% B (10 min), 29.4% B (20 min), 29.9% B (20.1 min), 40% B (21.1–21.5 min), 30% B (22.5 min), 28% B (23–25 min), 30% B (26 min), 31.8% B (26.5 min), 32.8% B (27 min), 40% B (27.5 min), 41% B (28 min), 33% B (28.5min), 36% B (34 min), 36.8% B (35 min), 40% B (35.5 min), 36% B (36 min), 50% B (66 min), 100% B (72–76 min) and 1% B (79–88 min).

Phenols were quantified based on calibration curves of their authentic standards when available, while others were expressed as equivalents; hydroxytyrosol glucosides and hydroxytyrosol acetate as hydroxytyrosol, tyrosol glucoside as tyrosol, all verbascoside derivatives as verbascoside, luteolin rutinosides as luteolin-7-*O*-glucoside, luteolin-3'-*O*-glucoside and luteolin-4',7-*O*-diglucoside as luteolin-4'-*O*-glucoside, and all secoiridoids as oleuropein with an exception of caffeoyl-6'-secologanoside, which was expressed as caffeic acid, and unknown A, unknowns of 408 MW and comselogoside expressed as *p*-coumaric acid equivalents.

4.2.4 Method validation

System suitability. Prior to quantification, the suitability of U(H)PLC-DAD system was checked in terms of injector's reproducibility and linearity using four commercially available standards provided by a local supplier.

Linearity and range. The linearity of method was evaluated by serial dilution of standard stock solutions over the broad concentration ranges using ten-point calibration curves.

Extraction efficiency – N° extraction steps. Extraction efficiencies of USLE and US-LLE methods were checked prior to application to olive matrices in terms of phenols yield recoveries obtained by a consecutive five-step extraction. The extracts of de-stoned fruits, stone, paste, pomace and wastewater were submitted to a complete USLE method (3 × 20 min) using 25 mL of pure methanol. Then, the remaining solid residue was re-extracted twice and analysed by U(H)PLC for a potent presence of phenols in the 4^{th} and 5^{th} extraction steps, where a summation of phenol yields from a five-step extraction was considered as 100%. Analogously, the efficiency of US-LLE method (3 × 10 min, 5 mL 100% methanol) has been checked for the olive oil sample.

Sensitivity. The LODs and LOQs were calculated from y-intercept standard deviations (S_b) and slopes (a) of calibration curves using signal-to-noise ratio criteria of 3.3 (LOD = 3.3 x Sb/a) and 10 (LOQ = 10 x Sb/a) in the concentration ranges close to LOQs expected for each phenolic compound in extracts (μg/mL): 0.33–1.67 (vanillin), 0.35–1.73 (vanillic acid), 0.33–1.65 (*p*-coumaric acid), 1.24–7.44 (caffeic acid), 1.44–5.76 (hydroxytyrosol), 1.40–3.93 (tyrosol), 1.00–2.99 (oleuropein), 0.39–0.97 (verbascoside), 0.24–1.20 (rutin), 0.23–1.17 (quercitrin), 0.25–1.23 (luteolin-4'-*O*-glucoside), 1.62–5.85 (apigenin), 0.48–2.87 (apigenin-7-*O*-glucoside), 0.56–4.49 (luteolin), 1.26–3.78 (luteolin-7-*O*-glucoside) and 0.79–4.76 (pinoresinol).

4.2.5 Phenols partition rate calculation

The percentage of phenols transfer/partition rate from fruits to paste and its final products, *i.e*. pomace, wastewater and oil, was calculated considering the mass balance of each trial according to the equation (1):

$$\%P_{matrix} = (P_{matrix}/P_{fruit)} \times (m_{matrix}/m_{fruit}) \times 100 \quad \text{(Eqs 1)}$$

where P is the concentration of individual phenolic compound in particular matrix (mg/kg per fruits FW), and its content in the fruit (composed of de-stoned fruit + stone) is considered as 100% of the available input.

Additionally, the percentage of phenols increase/decrease during crushing and malaxation was calculated using equations (2) and (3):

%P increase/decrease during crushing;

$$(\Delta c) = P_{paste} \times 100 / P_{fruit} \quad \text{(Eqs 2)}$$

%P increase/decrease during malaxation;

$$(\Delta c) = (P_{pomace} + P_{wastewater} + P_{oil}) \times 100 / P_{paste} \quad \text{(Eqs 3)}$$

where expression $P_{pomace} + P_{wastewater} + P_{oil}$ presents the sum of phenols quantified in the final products at the end of process also referred as TP pool when discussed further on.

The same equations were also used for TP transfer/partition rate calculations, where the term TP refers to a sum of all HPLC-DAD quantified phenols.

4.2.6 Statistical analysis

The results were expressed as means ± SD obtained from at least triplicate analyses and tested by ANOVA or *t*-test using Costat Statistics Software 6.4 (CoHort Software, CA, USA). A Duncan's multiple range test was used to discriminate among the means at 95% confidence level, where *P* values < 0.05 were regarded as significant (*), *P* values < 0.01 as very significant (**), *P* values < 0.001 as extremely significant (***) and *P* values > 0.05 as not significant (ns).

5 RESULTS AND DISCUSSION

The results of doctoral thesis are organized into four main sub-chapters and are together with the discussion presented in the following order:

- Results of olive phenol analysis
- Results of olive oil processing trial
- Results of Istrska belica *cv*. fruits seasonal and geographical phenol profile variation
- Results of Slovenian commercial olive mill wastes phenolic composition

The concluding remarks from all are given in the conclusions.

5.1 Olive phenol analysis

The first part of doctoral study was dedicated to the quali- and quantitative phenol analysis of different olive matrices.

5.1.1 Qualitative analysis (UV-Vis and MS spectroscopic study)

Two screening strategies were used in the phenol profiling study of extracts from olive oil processing trial composed of de-stoned fruit, stone and their process-derived matrices; paste, pomace, wastewater and oil. First, the extracts were screened for the range of phenols previously reported in *O. europaea* L. matrices and their identification confirmed based on accurate masses and MS fragmentation profiles comparison with literature data. In addition, the UV-Vis spectra from DAD were generated allowing their classification and (if) some structure/spectra relationship assessment. When the reference compounds were available, phenols were also compared with standards in terms of R_t, UV-Vis and MS spectral characteristics. The second screening strategy was used for the novel compounds and was based on the generation of UV-Vis and MS spectra from DAD and TIC chromatograms scanned in both, the NIM and PIM ion modes, at soft and strong ionisations, obtaining major ESI^- and ESI^+ signals. Both profiling methods were also used for Istrska belica *cv*. fruits seasonal and geographical phenol profile variation (Section 5.3) as well as for the commercial Slovenian olive mill wastes phenolic composition assessment (Section 5.4), but the results due to common phenols found have not been separately presented.

In spite of the predominant use of LC-MS analysis in the phenol profiling of olive matrices, only few studies have adopted a high-resolution MS spectrometry for their identity assignment. Best to our knowledge this type of analysis has been only few times adopted for oil (Dierkes et al., 2012; García-Villalba et al., 2010; Fu et al., 2009a; Fu et al., 2009b), leaf (Quirantes-Piné et al., 2013; Fu et al., 2010; Di Donna et al., 2007a) and wastes (Peralbo-Molina et al., 2012; Lozano-Sánchez et al., 2011), once for the fruit (Di Donna et al., 2007b) and paste (Kanakis et al., 2013) and never for the stone. Interestingly, only once this detector has also been coupled to DAD (Lozano-Sánchez et al., 2011).

Table 6 summarises the MS and UV-Vis spectral data of identified phenolic compounds found in de-stoned fruit, its stone and their process-derived matrices (paste, pomace, wastewater and oil). It also provides the information about their elution time, molecular formula, theoretical and experimental *m/z* of deprotonated molecular ions in NIM mode, and the calculated error between them. The accurate mass data for each molecular ion is provided for the low ionisation conditions, where a limited fragmentation took place and $[M-H]^-$ was clearly the predominant ion. The final results were organised in a way that allow to follow the fate of olive fruit phenols through all operative steps, *i.e.* from input (peel and pulp/stone), intermediate (paste) and output products (pomace, wastewater and oil). Moreover, such data presentation also permits to infer; i) the difference between the detectors ability to assign the presence of individual phenolic compound, and ii) to track its origin, evolution and disappearance during processing. The U(H)PLC-DAD chromatograms of all matrices were placed immediately below (**Figures 7–12)**, while the MS and UV-Vis spectra of individual phenols are collectively presented in the appendix (**Annex A**).

The following discussion was limited to those phenols that best illustrates the novelty of study and to those for which the lack of standards was limitative for their unequivocal identification. Their potent interconversion relationships as a result of technological process are discussed elsewhere (Section 5.2).

Table 6 Phenols identified in de-stoned olive fruit, its stone, paste, pomace, wastewater and oil by UPLC-DAD-ESI-QTOF-HRMS analysis

N°	Class/ Phenolic compound	R_t (min)	Formula *m/z* theor[C]	Olive matrix																
				Input						*Intermediate*			*Output*							
				De-stoned fruit			**Stone**			**Paste**			**Pomace**			**Wastewater**			**Oil**		
				m/z exp[D]	Δm[E]	λ_{max}	*m/z* exp	Δm	λ_{max}	*m/z* exp	Δm	λ_{max}	*m/z* exp	Δm	λ_{max}	*m/z* exp	Δm	λ_{max}	*m/z* exp	Δm	λ_{max}
Simple phenols																				
1[G]	Hydroxytyrosol glucoside[A]	3.2	$C_{14}H_{20}O_8$ *315.1080*	315.1085	*1.6*	235 276	315.1090	*3.2*	230 276	315.1102	*7.0*	236 276	315.1076	*-1.3*	236 276	315.1059	*-6.7*	236 276	315.1079	*-0.3*	233 276
2	Hydroxytyrosol-1-β-glucoside[A]	3.3	$C_{14}H_{20}O_8$ *315.1080*	315.1074	*-1.9*	236 279	315.1102	*7.0*	235 277	315.1071	*-2.9*	236 279	315.1060	*-6.3*	236 279	315.1075	*-1.6*	236 279	315.1088	*2.5*	233 280
3	Hydroxytyrosol[B]	3.5	$C_8H_{10}O_3$ *153.0552*	153.0547	*-3.3*	238 279	153.0550	*-1.3*	236 279	153.0559	*4.6*	236 280	153.0545	*-4.6*	236 280	153.0558	*3.9*	236 280	153.0550	*-1.3*	235 280
6	Tyrosol glucoside[A]	5.3	$C_{14}H_{20}O_7$ *299.1131*	299.1118	*-4.3*	238 275	299.1121	*-3.3*	233 275	299.1132	*0.3*	236 275	299.1124	*-2.3*	237 275	299.1130	*-0.3*	237 275	299.1131	*0.0*	237 275
8	Tyrosol[B]	5.8	$C_8H_{10}O_2$ *137.0603*	li	–	236 277	li	–	235 275	li	–	235 275	li	–	234 275	li		237 275	li	–	234 275
19	Hydroxytyrosol acetate[A]	13.4	$C_{10}H_{12}O_4$ *195.0657*	195.0646	*-5.6*	235 281	nd	–	nd	195.0651	*-3.1*	*	195.0653	*-2.1*	*	195.0654	*-1.5*	*	195.0657	*0.0*	234 280
Benzoic acids																				
13	Vanillic acid[B]	10.3	$C_8H_8O_4$ *167.0344*	li	–	nd	nd	–	nd	li	–	246 293	li	–	250 293	li	–	245 293	li	–	258 293
15	Vanillin[B]	12.4	$C_8H_8O_3$ *151.0395*	nd	–	nd	151.0405	*6.6*	235 278 308	nd	–	nd	nd	–	nd	nd	–	nd	151.0397	*1.3*	235 279 308
Cinnamic acids																				
16	*p*-Coumaric acid[B]	13.1	$C_9H_8O_3$ *163.0395*	163.0398	*1.8*	*	nd	–	nd	163.0393	*-1.2*	235 309	163.0390	*-3.1*	236 310	163.0386	*-5.5*	236 310	163.0391	*-2.5*	236 308
	β-OH-verbascoside																			
17	isomer 1[A]	13.2	$C_{29}H_{36}O_{16}$ *639.1925*	639.1890	*-5.5*	251 281sh 329	639.1931	*0.9*	253 279sh 329	639.1900	*-3.9*	246 284sh 329	639.1929	*0.6*	246 285sh 329	639.1891	*-5.3*	245 285sh 329	nd	–	nd
18	isomer 2[A]	13.4	$C_{29}H_{36}O_{16}$ *639.1925*	639.1934	*1.4*	253 281sh 328	639.1902	*-3.6*	236 281sh 328	639.1888	*-5.8*	249 285sh 329	639.1909	*-2.5*	246 286sh 329	639.1926	*0.2*	246 285sh 329	nd	–	nd

Table 6 *(Cont)*

N°	Class/ Phenolic compound	R_t (min)	Formula *m/z* theor	Olive matrix																
				Input						*Intermediate*			*Output*							
				De-stoned fruit			**Stone**			**Paste**			**Pomace**			**Wastewater**			**Oil**		
				m/z exp	Δm	λ_{max}	*m/z* exp	Δm	λ_{max}	*m/z* exp	Δm	λ_{max}	*m/z* exp	Δm	λ_{max}	*m/z* exp	Δm	λ_{max}	*m/z* exp	Δm	λ_{max}
26	Verbascoside[B]	17.1	$C_{29}H_{36}O_{15}$ *623.1976*	623.1976	*0.0*	245 284sh 330	623.2009	*5.3*	249 284sh 330	623.2003	*4.3*	248 285sh 330	623.1994	*2.9*	247 285sh 330	623.2017	*6.6*	245 285sh 330	nd	–	nd
Flavonoids																				
37	Rutin[B]	21.1	$C_{27}H_{30}O_{16}$ *609.1456*	609.1473	*2.8*	255 354	609.1442	*-2.3*	*	609.1446	*-1.6*	252 354	609.1459	*0.5*	252 355	609.1419	*-6.1*	248 353	609.1465	*1.5*	*
	Luteolin glucoside																			
22	Luteolin-4',7-*O*-diglucoside[A]	15.9	$C_{27}H_{30}O_{16}$ *609.1456*	609.1425	*-5.1*	268 338	nd	–	nd	609.1469	*2.1*	*	nd	–	nd	nd	–	nd	nd	–	nd
41	Luteolin-7-*O*-glucoside[B]	24.1	$C_{21}H_{20}O_{11}$ *447.0927*	447.0942	*3.4*	254 349	nd	–	nd	447.0948	*4.7*	254 350	447.0932	*1.1*	256 349	447.0913	*-3.1*	257 349	nd	–	nd
59	Luteolin-4'-*O*-glucoside[B]	36.6	$C_{21}H_{20}O_{11}$ *447.0927*	447.0931	*0.9*	268 338	nd	–	nd	447.0917	*-2.2*	268 339	447.0907	*-4.5*	270 339	447.0913	*-3.1*	*	nd	–	nd
61	Luteolin-3'-*O*-glucoside[A]	41.7	$C_{21}H_{20}O_{11}$ *447.0927*	447.0947	*4.5*	268 340	nd	–	nd	447.0924	*-0.7*	269 340	447.0916	*-2.5*	270 340	447.0908	*-4.2*	268 340	nd	–	nd
	Luteolin rutinoside																			
42	isomer 1[A]	24.3	$C_{27}H_{30}O_{15}$ *593.1506*	593.1505	*-0.2*	254 343	nd	–	nd	593.1469	*-6.2*	254 343	593.1505	*-0.2*	254 343	593.1486	*-3.4*	251 343	nd	–	nd
44	isomer 2[A]	25.5	$C_{27}H_{30}O_{15}$ *593.1506*	593.1487	*-3.2*	254 343	nd	–	nd	593.1497	*-1.5*	253 342	593.1497	*-1.5*	251 342	593.1498	*-1.3*	250 342	nd	–	nd
VII	isomer 3[A]	26.6	$C_{27}H_{30}O_{15}$ *593.1506*	593.1473	*-5.6*	nd	nd	–	nd	593.1519	*2.2*	nd	593.1527	*3.5*	nd	nd	–	nd	nd	–	nd
45	Quercitrin[B]	26.3	$C_{21}H_{20}O_{11}$ *447.0927*	447.0937	*2.2*	256 343	nd	–	nd	447.0925	*-0.4*	255 342	447.0914	*-2.9*	254 342	447.0926	*-0.2*	251 342	nd	–	nd
	Apigenin glucoside																			
51	Apigenin-7-*O*-glucoside[B]	30.3	$C_{21}H_{20}O_{10}$ *431.0978*	431.0958	*-4.6*	234 266 337	nd	–	nd	431.0988	*2.3*	236 267 340	431.0957	*-4.9*	237 267 340	431.0965	*-3.0*	231 268 340	nd	–	nd
VIII	isomer 1[A]	35.1	$C_{21}H_{20}O_{10}$ *431.0978*	431.0947	*-7.2*	nd	nd	–	nd	431.0963	*-3.5*	nd	431.1013	*8.1*	nd	nd		nd	nd	–	nd

Table 6 *(Cont)*

N°	Class/ Phenolic compound	R_t (min)	Formula *m/z* theor	Olive matrix																	
				Input						*Intermediate*			*Output*								
				De-stoned fruit			**Stone**			**Paste**			**Pomace**			**Wastewater**			**Oil**		
				m/z exp	Δm	λ_{max}	*m/z* exp	Δm	λ_{max}	*m/z* exp	Δm	λ_{max}	*m/z* exp	Δm	λ_{max}	*m/z* exp	Δm	λ_{max}	*m/z* exp	Δm	λ_{max}
66	Luteolin[B]	51.4	$C_{15}H_{10}O_6$ *285.0399*	285.0418	*6.7*	nd	nd	–	nd	285.0413	*4.9*	252 350	285.0410	*3.9*	255 353	285.0411	*4.2*	254 350	285.0406	*2.5*	254 349
69	Apigenin[B]	60.9	$C_{15}H_{10}O_5$ *269.0450*	nd	–	nd	nd	–	nd	269.0459	*3.3*	242 267 339	269.0442	*-3.0*	235 268 339	269.0458	*3.0*	236 266 337	269.0444	*-2.2*	234 267 338
Lignans																					
55	Pinoresinol[B]	33.7	$C_{20}H_{22}O_6$ *357.1338*	li	–	nd	li	–	nd	li	–	nd	li	–	nd	li	–	nd	li	–	236 279
58	Acetoxypinoresinol[A]	35.9	$C_{22}H_{24}O_8$ *415.1393*	nd	–	nd	nd	–	nd	nd	–	nd	nd	–	nd	nd	–	nd	415.1375	*-4.3*	238 279
Secoiridoids																					
	Oleoside																				
I	Oleoside[A]	3.5	$C_{16}H_{22}O_{11}$ *389.1084*	389.1091	*1.8*	*	389.1061	*-5.9*	*	389.1071	*-3.3*	*	389.1076	*-2.1*	*	389.1081	*-0.8*	*	nd	–	nd
11	Secologanoside[A]	8.9	$C_{16}H_{22}O_{11}$ *389.1084*	389.1091	*1.8*	236	389.1100	*4.1*	236	389.1071	*-3.3*	236	389.1062	*-5.7*	236	389.1063	*-5.4*	237	389.1062	*-5.7*	236
	Elenolic acid glucoside																				
10	isomer 1[A]	8.6	$C_{17}H_{24}O_{11}$ *403.1240*	403.1240	*0.0*	237	403.1227	*-3.2*	236	403.1241	*0.2*	236	nd	–	nd	nd	–	nd	nd	–	nd
12	isomer 2[A]	9.2	$C_{17}H_{24}O_{11}$ *403.1240*	403.1232	*-2.0*	244	403.1227	*-3.2*	240	403.1227	*-3.2*	*	403.1222	*-4.5*	*	403.1223	*-4.2*	*	403.1242	*0.5*	236
14	isomer 3[A]	10.2	$C_{17}H_{24}O_{11}$ *403.1240*	403.1236	*-1.0*	245	403.1244	*1.0*	242	403.1224	*-4.0*	nd	nd	–	nd	nd	–	nd	nd	–	nd
II	isomer 4[A]	11.9	$C_{17}H_{24}O_{11}$ *403.1240*	403.1255	*3.7*	nd	403.1223	*-4.2*	nd	nd	–	nd	nd	–	nd	nd	–	nd	nd	–	nd
20	Demethyloleuropein[A]	13.7	$C_{24}H_{30}O_{13}$ *525.1608*	525.1609	*0.2*	242 280	525.1605	*-0.6*	*	525.1605	*-0.6*	247 280	525.1584	*-4.6*	247 280	525.1583	*-4.8*	244 280	nd	–	nd
	Dihydro-oleuropein																				
21	isomer 1[A]	15.4	$C_{25}H_{36}O_{13}$ *543.2078*	543.2081	*0.6*	251 275	nd	–	nd	543.2054	*-4.4*	250 278	543.2055	*-4.2*	249 278	543.2047	*-5.7*	247 278	nd	–	nd

Table 6 *(Cont)*

N°	Class/ Phenolic compound	R_t (min)	Formula *m/z* theor	Olive matrix																
				Input						*Intermediate*			*Output*							
				De-stoned fruit			**Stone**			**Paste**			**Pomace**			**Wastewater**			**Oil**		
				m/z exp	Δm	λ_{max}	*m/z* exp	Δm	λ_{max}	*m/z* exp	Δm	λ_{max}	*m/z* exp	Δm	λ_{max}	*m/z* exp	Δm	λ_{max}	*m/z* exp	Δm	λ_{max}
27	isomer 2[A]	17.4	$C_{25}H_{36}O_{13}$ *543.2078*	543.2078	*0.0*	251 281	543.2087	*1.7*	248 276	543.2064	*-2.6*	251 277	543.2047	*-5.7*	250 276	543.2069	*-1.7*	248 277	nd	–	nd
	3,4-DHPEA-EDA																				
23	isomer 1[A]	16.0	$C_{17}H_{20}O_6$ *319.1182*	nd	–	nd	nd	–	nd	319.1182	*0.0*	250 280	319.1172	*-3.1*	246 280	nd		nd	nd	–	nd
34	isomer 2[A]	19.9	$C_{17}H_{20}O_6$ *319.1182*	nd	–	nd	nd	–	nd	319.1199	*5.3*	243 280	319.1198	*5.0*	238 280	319.1201	*6.0*	242 280	319.1176	*-1.9*	243 281
	Oleuropein diglucoside																				
30	isomer 1[A]	18.5	$C_{31}H_{42}O_{18}$ *701.2293*	701.2281	*-1.7*	246 283	701.2309	*2.3*	251 276	701.2289	*-0.6*	250 276	701.2283	*-1.4*	*	701.2260	*-4.7*	*	nd	–	nd
33	isomer 2[A]	19.5	$C_{31}H_{42}O_{18}$ *701.2293*	701.2260	*-4.7*	252 275	701.2280	*-1.9*	nd	701.2318	*3.6*	nd	701.2334	*5.8*	nd	701.2319	*3.7*	nd	nd	–	nd
V	isomer 3[A]	19.8	$C_{31}H_{42}O_{18}$ *701.2293*	701.2275	*-2.6*	nd	701.2244	*-7.0*	nd	nd	–	nd	nd	–	nd	nd	–	nd	nd	–	nd
VI	isomer 4[A]	20.8	$C_{31}H_{42}O_{18}$ *701.2293*	701.2298	*0.7*	nd	701.2274	*-2.7*	nd	nd	–	nd	nd	–	nd	nd	–	nd	nd	–	nd
	Nüzhenide																				
III	isomer 1[A]	16.7	$C_{31}H_{42}O_{17}$ *685.2344*	nd	–	nd	685.2344	*0.0*	*	nd	–	nd	nd	–	nd	nd	–	nd	nd	–	nd
32	isomer 2[A]	18.8	$C_{31}H_{42}O_{17}$ *685.2344*	685.2305	*-5.7*	246 276	685.2354	*1.5*	247 275	685.2390	*6.7*	249 275	685.2346	*0.3*	249 275	685.2323	*-3.1*	245 281	nd	–	nd
35	isomer 3[A]	20.5	$C_{31}H_{42}O_{17}$ *685.2344*	685.2320	*-3.5*	nd	685.2352	*1.2*	253 275	nd	–	nd	nd	–	nd	nd	–	nd	nd	–	nd
39	isomer 4[A]	21.7	$C_{31}H_{42}O_{17}$ *685.2344*	685.2308	*-5.3*	nd	685.2310	*-5.0*	250 275	nd	–	nd	nd	–	nd	nd	–	nd	nd	–	nd
	Oleuropein aglycone																				
38	isomer 1[A]	21.5-22.8	$C_{19}H_{22}O_8$ *377.1236*	377.1247	*2.9*	nd	377.1247	*2.9*	nd	377.1255	*5.0*	244 280	377.1236	*0.0*	244 280	377.1221	*-4.0*	244 280	377.1235	*-0.3*	245 281
60	isomer 2[A]	37.5	$C_{19}H_{22}O_8$ *377.1236*	377.1241	*1.3*	nd	377.1213	*-6.1*	nd	377.1258	*5.8*	251 280	377.1244	*2.1*	250 280	377.1253	*4.5*	249 280	377.1237	*-0.3*	249 280

Table 6 *(Cont)*

N°	Class/ Phenolic compound	R_t (min)	Formula *m/z* theor	Olive matrix																
				Input						*Intermediate*			*Output*							
				De-stoned fruit			**Stone**			**Paste**			**Pomace**			**Wastewater**			**Oil**		
				m/z exp	Δm	λ_{max}	*m/z* exp	Δm	λ_{max}	*m/z* exp	Δm	λ_{max}	*m/z* exp	Δm	λ_{max}	*m/z* exp	Δm	λ_{max}	*m/z* exp	Δm	λ_{max}
65	isomer 3[A]	47.3	$C_{19}H_{22}O_8$ *377.1236*	377.1248	*3.2*	nd	377.1245	*2.4*	nd	377.1249	*3.4*	258 276	377.1242	*1.6*	258 276	377.1234	*-0.5*	256 276	377.1227	*-2.4*	252 276
	Oleuropein																				
40	Oleuropein[B]	22.9	$C_{25}H_{32}O_{13}$ *539.1765*	539.1769	*0.7*	244 280	539.1780	*2.8*	244 280	539.1790	*4.6*	245 280	539.1752	*-2.4*	246 280	539.1763	*-0.4*	nd	nd	–	nd
47	Oleuroside[A]	27.0	$C_{25}H_{32}O_{13}$ *539.1765*	539.1758	*-1.3*	243 280	539.1749	*-3.0*	243 282	539.1742	*-4.3*	nd	nd	–	nd	nd	–	nd	nd	–	nd
43	Caffeoyl-6'-secologanoside[A]	24.8	$C_{25}H_{28}O_{14}$ *551.1401*	551.1394	*-1.3*	237 327	nd	–	nd	551.1370	*-5.6*	237 326	551.1387	*-2.5*	239 326	551.1419	*3.3*	238 326	nd	–	nd
46	*p*-HPEA-EDA[A]	26.4	$C_{17}H_{20}O_5$ 303.1232	nd	–	nd	nd	–	nd	303.1237	*1.6*	nd	303.1248	*5.3*	nd	303.1213	*-6.3*	nd	303.1236	*1.3*	246 275
48	Oleuropein + Ligstroside aglycone 1[A]	28.5	$C_{19}H_{22}O_8$ *377.1236*	nd	–	nd	nd	–	nd	nd	–	nd	nd	–	nd	nd	–	nd	377.1252	*4.2*	252 275
			$C_{19}H_{22}O_7$ *361.1287*	nd	–	nd	nd	–	nd	nd	–	nd	nd	–	nd	nd	–	nd	361.1292	*1.4*	
49	Oleuropein + Ligstroside aglycone 2[A]	29.0	$C_{19}H_{22}O_8$ *377.1236*	nd	–	nd	nd	–	nd	nd	–	nd	nd	–	nd	nd	–	nd	377.1238	*0.5*	251 275
			$C_{19}H_{22}O_7$ *361.1287*	nd	–	nd	nd	–	nd	nd	–	nd	nd	–	nd	nd	–	nd	361.1276	*-3.0*	
52	Ligstroside[A]	30.5	$C_{25}H_{32}O_{12}$ *523.1816*	523.1831	*2.9*	246 275	523.1804	*-2.3*	249 275	523.1790	*-5.0*	nd	523.1812	*-0.8*	nd	523.1803	*-2.5*	nd	nd	–	nd
53	Comselogoside[A]	31.2	$C_{25}H_{28}O_{13}$ *535.1452*	535.1440	*-2.2*	236 313	nd	–	nd	535.1461	*1.7*	236 313	535.1455	*0.6*	236 313	535.1462	*1.9*	236 313	nd	–	nd
57	Acetal of 3,4-DHPEA-EDA[A]	35.3	$C_{19}H_{26}O_7$ *365.1600*	nd	–	nd	365.1589	*-3.0*	nd	365.1611	*3.0*	nd	365.1585	*-4.1*	nd	365.1602	*0.5*	242 281	365.1597	*-0.8*	nd
	Ligstroside aglycone																				
X	isomer 1[A]	39.7	$C_{19}H_{22}O_7$ *361.1287*	nd	–	nd	nd	–	nd	nd	–	nd	nd	–	nd	nd	–	nd	361.1284	*-0.8*	nd
64	isomer 2[A]	47.0	$C_{19}H_{22}O_7$ *361.1287*	nd	–	nd	nd	–	nd	361.1281	*-1.7*	nd	361.1265	*-6.1*	nd	361.1280	*-1.9*	nd	361.1287	*0.0*	254 280

Table 6 *(Cont)*

N°	Class/ Phenolic compound	R_t (min)	Formula *m/z* theor	Olive matrix																	
				Input						*Intermediate*			*Output*								
				De-stoned fruit			**Stone**			**Paste**			**Pomace**			**Wastewater**			**Oil**		
				m/z exp	Δm	λ_{max}	*m/z* exp	Δm	λ_{max}	*m/z* exp	Δm	λ_{max}	*m/z* exp	Δm	λ_{max}	*m/z* exp	Δm	λ_{max}	*m/z* exp	Δm	λ_{max}
	Nüzhenide 11-methyl oleoside																				
54	isomer 1[A]	31.6	$C_{48}H_{64}O_{27}$ *1071.3557*	nd	–	nd	1071.3560	*0.3*	252 275	nd	–	nd	nd	–	nd	nd	–	nd	nd	–	nd
56	isomer 2[A]	35.1	$C_{48}H_{64}O_{27}$ *1071.3557*	nd	–	nd	1071.3546	*-1.0*	258 275	nd	–	nd	nd	–	nd	nd	–	nd	nd	–	nd
IX	isomer 3[A]	38.4	$C_{48}H_{64}O_{27}$ *1071.3557*	nd	–	nd	1071.3542	*-1.4*	nd	nd	–	nd	nd	–	nd	nd	–	nd	nd	–	nd
62	isomer 4[A]	41.8	$C_{48}H_{64}O_{27}$ *1071.3557*	nd	–	nd	1071.3563	*0.6*	246	1071.3571	*1.3*	251 276	1071.3558	*0.1*	252 276	1071.3549	*-0.7*	nd	nd	–	nd
	Nüzhenide di(11-methyl oleoside)																				
67	isomer 1[A]	51.5	$C_{65}H_{86}O_{37}$ *1457.4770*	nd	–	nd	1457.4796	*1.8*	253	1457.4722	*-3.3*	nd	1457.4760	*-0.7*	nd	nd	–	nd	nd	–	nd
68	isomer 2[A]	52.0	$C_{65}H_{86}O_{37}$ *1457.4770*	nd	–	nd	1457.4722	*0.1*	251	1457.4756	*-1.0*	nd	1457.4811	*2.8*	nd	1457.4836	*4.5*	nd	nd	–	nd
XI	isomer 3[A]	58.7	$C_{65}H_{86}O_{37}$ *1457.4770*	nd	–	nd	1457.4767	*-0.2*	nd	nd	–	nd	nd	–	nd	nd	–	nd	nd	–	nd

[A]Tentative identification based on accurate mass and literature data (tolerable range: 10 ppm). [B]Identification also confirmed by the use of standard. [C]Calculated monoisotopic mass $[M–H]^-$. [D]Detected mass. [E]Calculated mass deviation; (*m/z* exp - *m/z* theor*) / m/z* theor $\times 10^6$). [F]Compound is described as: (nd) not detected, (∗) the evidence for its presence is inconclusive, (li) not detected due to low ionisation. [G]Arabic numbers refers to DAD detected/quantified phenols, while romans to only LC-MS detected phenols.

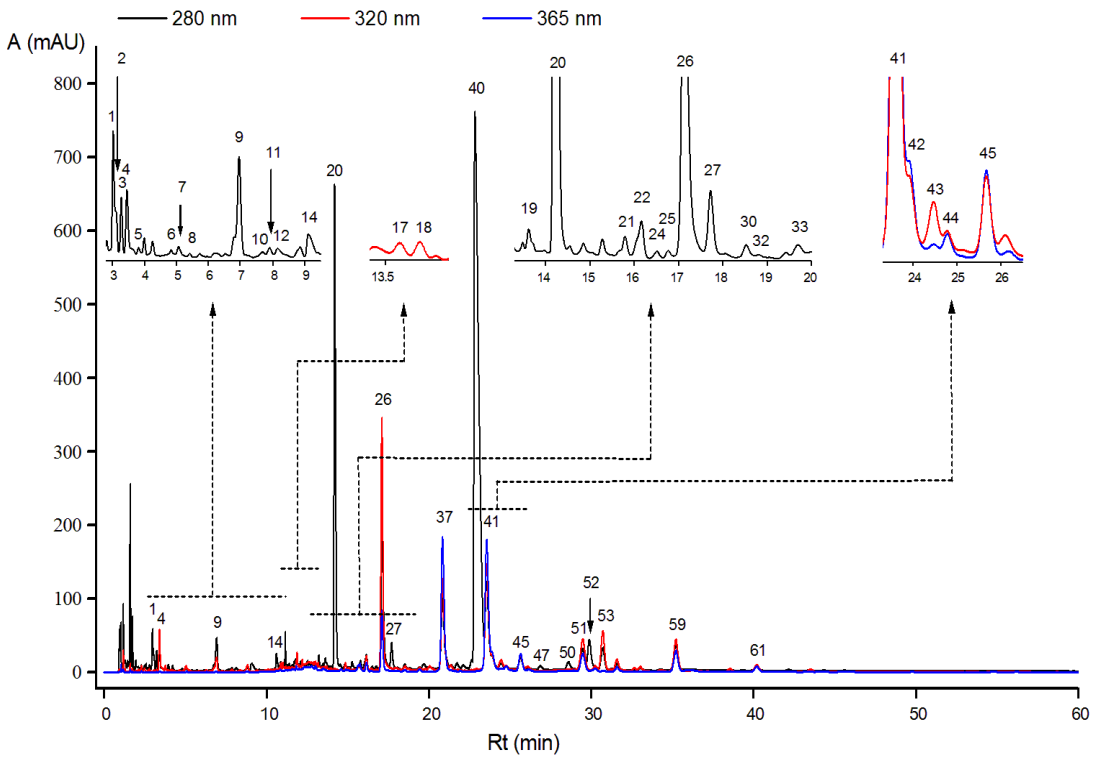


Figure 7 U(H)PLC-DAD phenolic profile of olive de-stoned fruit extract monitored at 280, 320 and 365 nm. Peak assignment refers to **Table 6** and **7**

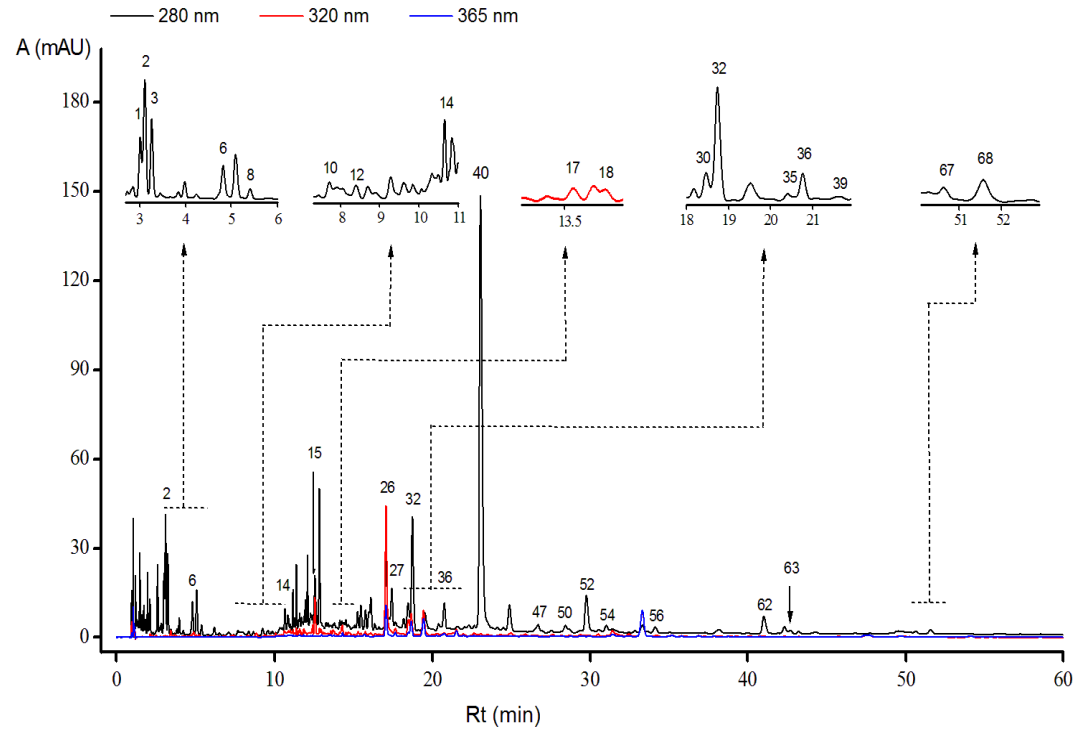


Figure 8 U(H)PLC-DAD phenolic profile of olive stone extract monitored at 280, 320 and 365 nm. Peak assignment refers to **Table 6** and **7**

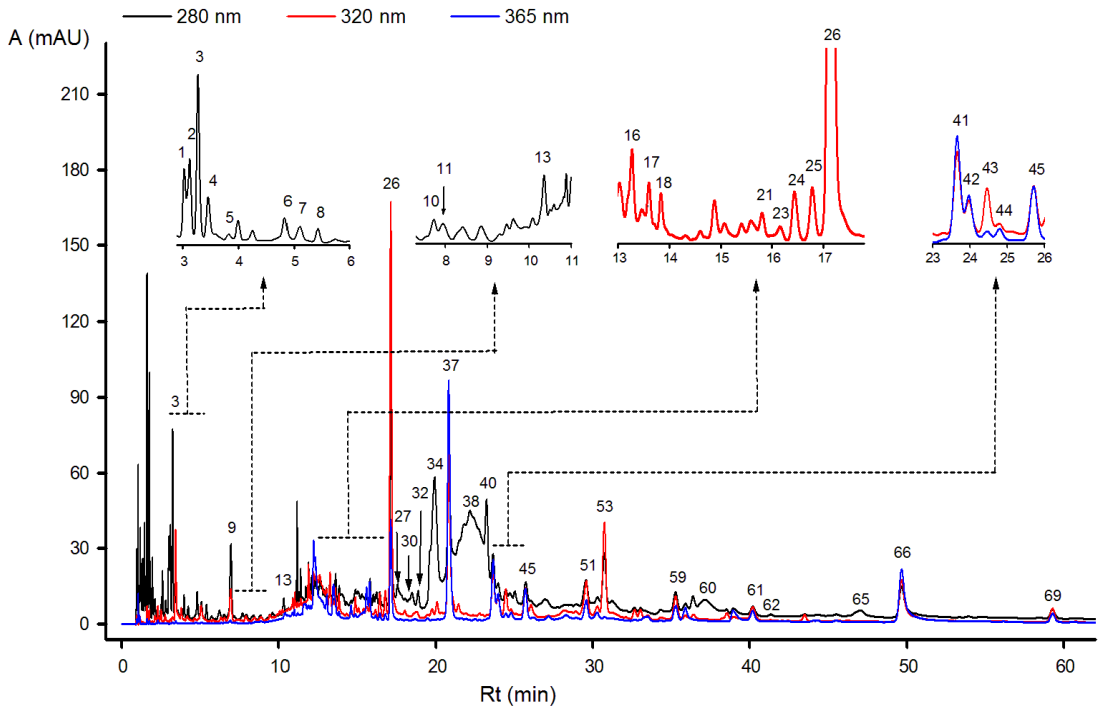


Figure 9 U(H)PLC-DAD phenolic profile of olive paste extract monitored at 280, 320 and 365 nm. Peak assignment refers to **Table 6** and **7**

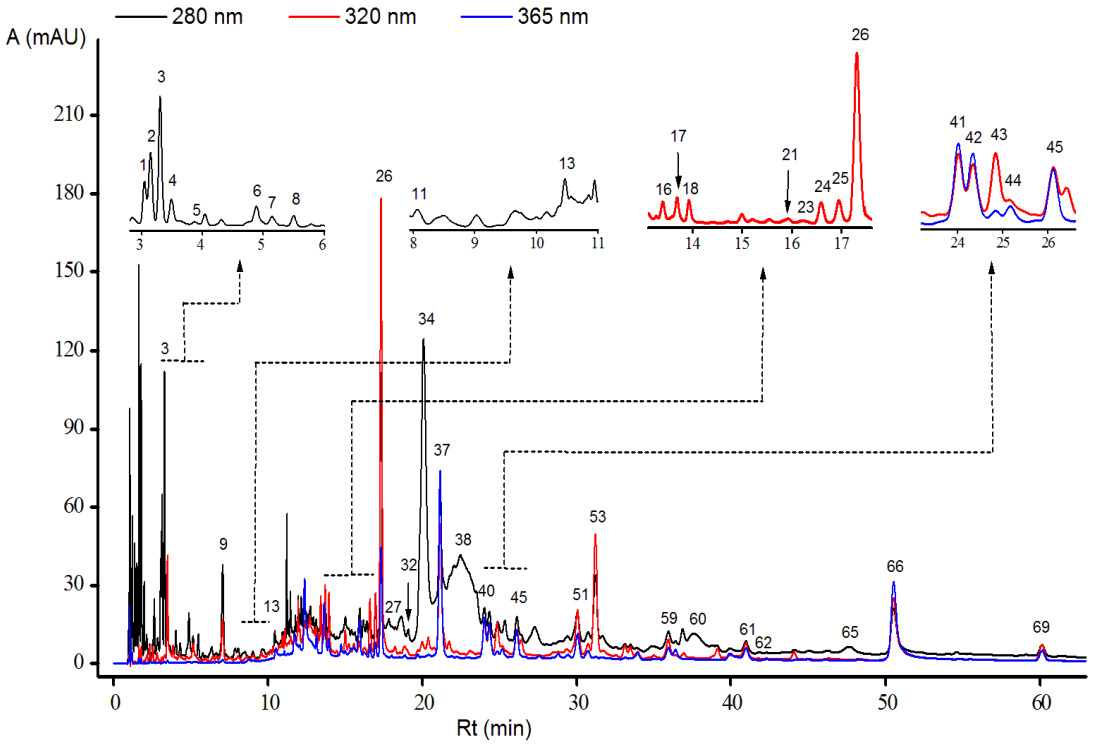


Figure 10 U(H)PLC-DAD phenolic profile of olive pomace extract monitored at 280, 320 and 365 nm. Peak assignment refers to **Table 6** and **7**

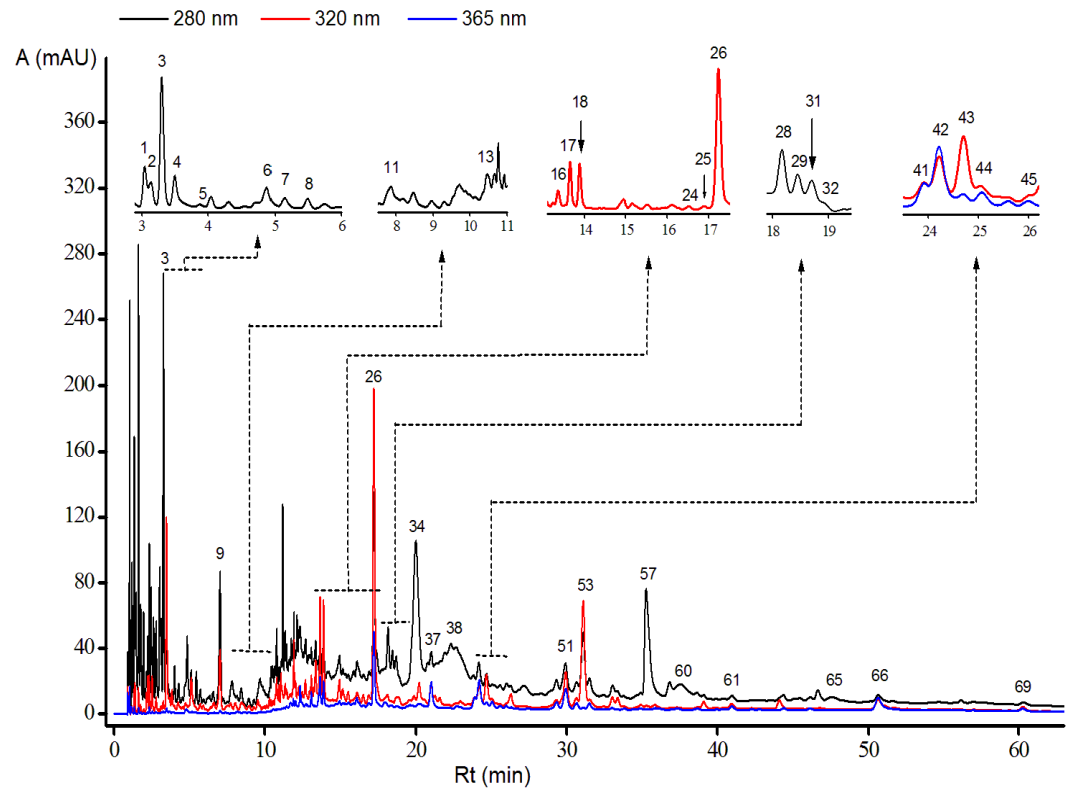


Figure 11 U(H)PLC-DAD phenolic profile of olive mill wastewater extract monitored at 280, 320 and 365 nm. Peak assignment refers to **Table 6** and **7**

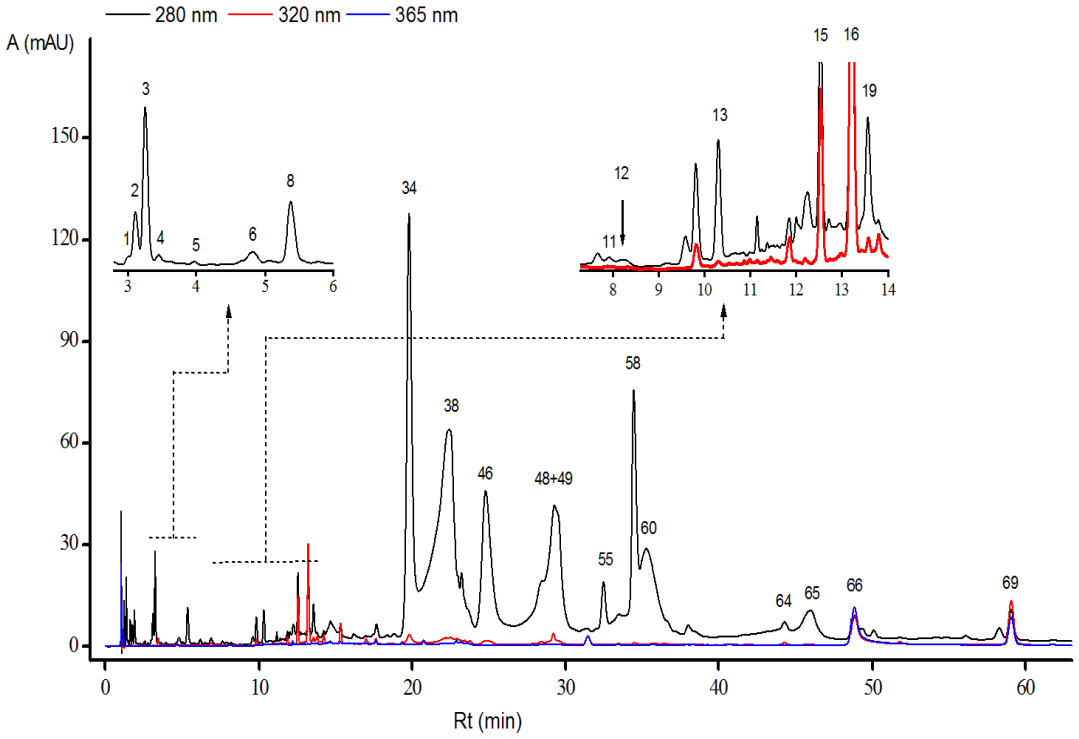


Figure 12 U(H)PLC-DAD phenolic profile of olive oil extract monitored at 280, 320 and 365 nm. Peak assignment refers to **Table 6** and **7**

Simple phenols. Among simple phenols, it was possible to confirm the presence of hydroxytyrosol, tyrosol and their glucosides, in addition to hydroxytyrosol acetate, which eluted as final among this class representatives. All of them were equally well detected by both detectors with an exception of tyrosol, which failed to ionise as already reported before (Obied et al., 2007a). Even so, its identity was easily confirmed by the standard co-elution and UV-Vis spectra comparison. While the DAD alone did not permit an unequivocal identification of hydroxytyrosol acetate in paste, pomace and wastewater due to co-elution problems, both detectors unambiguously confirmed the identity of two hydroxytyrosol glucosides (HTyr glu). Their presence has been rarely reported in *O. europaea* L. matrices, which could be attributed to analytical issues as demonstrated by Romero et al. (2002b) or their micro levels (Bianco et al., 1998). However, the EIC at *m/z* 315.1080 displayed the presence of two peaks (R_t 3.2 and 3.3 min) with the same fragmentation profile (*m/z* 153, 123) typical for this glucoside. There are three possible isomers differing in the glycosidation position (Obied et al., 2007a; Bianco et al., 1998), but only the UV-Vis spectra comparison allowed to distinct between both of them, further supported by their different yield behaviour during olive oil processing (Section 5.2.1). The first eluting isomer had λ_{max} of *B*-band at 276 suggesting that glycosidation occurred at one of the two OH groups at ring (HTyr glu 2 or HTyr 3, **Figure 13**), while an isomer 2 had its *B*-band shifted to a higher λ_{max} (~279 nm) indicating to an attached glucose moiety at OH group outside the phenol ring (HTyr glu 1), allowing us to tentatively identified it as hydroxytyrosol-1-*β*-glucoside.

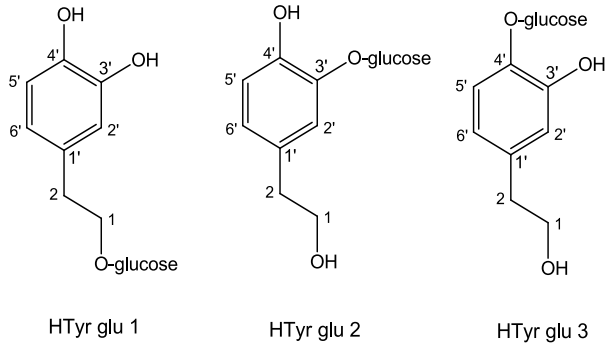


Figure 13 Chemical structures of hydroxytyrosol glucoside isomers (Adopted by Obied et al., 2007a)

Benzoic acids. Two representatives of benzoic acids were identified, namely vanillin and vanillic acid, commonly found in olive matrices. However, only the former was well detected by both detectors, while MS alone has failed to confirm the identity of vanillic acid. Its MS ions were clearly supressed under the analysis conditions applied, increasing error outside the tolerable range of 10 ppm. Even so, the presence of both phenols was unambiguously confirmed by the use of authentic standards. What infers next is the fact that vanillic acid was absent in fruits, but yet appeared in all of their process-derived matrices, unlike vanillin whose presence was confirmed in the stone from where it was apparently transferred to the oil.

Cinnamic acids. Four known cinnamic acids were found in olive extracts, among which three were esters (verbascoside and its two derivatives) and one appeared in the form of free acid, namely *p*-coumaric acid. Their presence and/or absence in extracts was easily confirmed by both, the DAD and MS, with an exception of *p*-coumaric acid in de-stoned fruit, whose UV-Vis spectra verification was hindered by the co-elution problems. However, none of the verbascosides were transferred to the olive oil, but instead were all lost with wastes. Moreover, the unexpected rise of two β-OH-verbascoside diastereoisomers was observed by DAD after crushing and malaxation, which could point to their technologically-induced formation and/or release. Indeed, their abundant presence has already been reported once in the Italian olive mill wastes (Mulinacci et al., 2005).

Lignans. One of the most interesting groups and of great analytical challenge was the class of lignans. Two representatives out of five previously reported (**Table 5**) were identified in olive extracts, however only in the oil, while in other matrices their detection could not be achieved in spite of several efforts made. Similar difficulties were described by López et al. (2008). However, in our study, the low ionisation of pinoresinol has restricted its accurate mass based identification, instead confirmed by the UV-Vis spectra and R_t match with standard. Interestingly, the same conditions were adequate for the identity confirmation of acetoxypinoresinol determined with uncertainty of 4.3 ppm. However, it must be emphasized that a high background signal of nearest eluting compounds (oleuropein and ligstroside aglycones) has almost miss-assigned their presence, similarly as DAD alone owing to a great UV-Vis spectra similarity with simple phenols (*e.g.* hydroxytyrosol).

Flavonoids. MS and DAD had comparable efficiency in the analysis of extracts' flavonoidal profile, where among thirteen phenols found, seven could be identified using standards, while others were tentatively assigned (if possible) as follows.

Six peaks were detected when scanning TIC traces at *m/z* 447.0927 characteristic for luteolin glucosides, which eluted at R_t 4.6, 15.9, 24.1, 26.3, 36.6 and 41.7 min. The accurate mass of the first eluting peak was far above the tolerable level (> 15 ppm) and the absence of fragment at *m/z* 285 has omitted the presence of luteolin glucoside. The identity of second peak (R_t 15.9 min) was tentatively assigned as luteolin diglucoside due to its relative short R_t, high mass accuracy (< 6 ppm) and characteristic MS ions at *m/z* 609, 447 and 285. Its UV-Vis spectra displayed two absorption maxima at 268 and 338 nm, which is identical to that of luteolin-4'-*O*-glucoside (**Annex A**). According to the literature data (Da Graça Campos et al., 2007) three sites of glycosidation are possible for luteolin diglucoside – at 3',4'-*O*, 3',7-*O* and 4',7-*O* positions. The λ_{max} at 338 nm pointed to a 4'-*O* substitution, while at 268 nm to additional 7-*O* glucosidation. The same rationale could also be established from its MS spectra interpret, where low intensity of aglycone ion at *m/z* 285 pointed to a loss of glucose from 7-*O* rather than from 3'-*O* position (Cuyckens et al., 2004). All together indicated to a presence of luteolin-4',7-*O*-diglucoside, which best to authors' knowledge has never been found in olives before, but instead once in the leaves (Meirinhos et al., 2005). The later eluting peaks were easily identified based on standards co-elution as luteolin-7-*O*-glucoside (R_t 24.1 min), quercitrin (R_t 26.3 min) and luteolin-4'-*O*-glucoside (R_t 36.6 min), whereas the identity of last peak was tentatively assigned as luteolin-3'-*O*-glucoside. As evident from the molecular structure of luteolin glucoside (**Table 4**), the sugar residue may be either *C*-linked (at 6 and 8 positions, *e.g.* orientin and homoorientin) or *O*-linked at 5, 3', 4' and 7 positions. This compound had long R_t (41.7 min), which immediately excluded the presence of orientin, homoorientin and 5-*O* glucoside, similarly as 4'-*O* and 7-*O* due to standards availability. This left us only to evidence the existence of luteolin-3'-*O*-glucoside, once indeed proved to elute after 4'-*O*-glucoside under RP-LC conditions (Ko et al., 2008). Its presence has already been confirmed before in the leaf, sharing identical UV-Vis absorption maxima (λ_{max} 268, 340) (Mylonaki et al., 2008). This, along with a high abundance of *m/z* 285 in its MS

spectrum strongly supported our decisive proof. Again, both detectors had indispensable role in the identity assignment with a higher degree of confidence.

Scanning for *m/z* 593.1506 corresponding to luteolin rutinoside revealed the presence of three peaks (R_t 24.3, 25.5 and 26.6 min) sharing identical fragmentation ions and their relative abundance (*m/z* 593, 477 and 285). Up to now, only two isomers have been confirmed in *O. europaea* L. matrices, namely luteolin 7-*O*-rutinoside and luteolin 4'-*O*-rutinoside with former having higher λ_{max} and a shorter R_t (Obied et al., 2007a; Cardoso et al., 2005). In our study, all three isomers eluted after luteolin-7-*O*-glucoside and prior to luteolin-4'-*O*- glucoside, suggesting that the nature of sugar had no influence on elution order in RP-LC. Moreover, the first two isomers presented similar UV-Vis spectra with λ_{max} around 243 nm, indicating that glycosidation most likely occurred away from the chromophore, whereas UV-Vis spectra verification of a third isomer was not feasible due to its trace amounts present. Such data compilation suggests that these isomers could be either 7-*O*, 5-*O*, 6-*C* or 8-*C* linked, however, only the use of other analyses (*e.g.* NMR) could provide their further structural assignment, hence the peaks were only tentatively assigned as luteolin rutinoside isomers.

Apart from apigenin-7-*O*-glucoside (R_t 30.3 min), another peak with the same accurate mass, *i.e. m/z* 431.0978 was detected in olive fruit, paste and pomace. This compound eluting at 35.1 min showed no affinity for liquid matrices, however, its trace levels were beyond the DAD detection and hence were not quantified in any of the fractions found.

Secoiridoids. The combined use of DAD and ESI-QTOF-MS analysis permitted a facile identification of common secoiridoids such as oleuropein, ligstroside, demethyloleuropein, 3,4-DHPEA-EDA, *p*-HPEA-EDA, comselogoside and caffeoyl-6'-secologanoside. However, it also allowed the detection of others structurally correlated to oleuropein and/or other secoiridoid representatives. The discussion below has only focused on those detected in several isomeric forms, while others were discussed among the "unknowns" (pp. 80).

Oleoside. The EIC at 389.1084 obtained by ESI-QTOF-MS displayed four peaks (R_t 2.7, 3.0, 3.5 and 8.9 min) with almost the same fragmentation profile characteristic for oleoside. The UV-Vis spectra of the first two peaks exhibited two absorption maxima, which is not typical for oleoside and suggest that oleoside is only in their structures. However, their identity has not been further investigated due to trace concentrations. By contrast, the third and fourth peak (R_t 3.5 and 8.9 min) had one characteristic λ_{max} at 236 nm, though the UV-Vis of former showed some deviation owing to co-elution with hydroxytyrosol. This peak, however, was tentatively identified as oleoside based on a high mass accuracy (< 6 ppm) and a typical fragmentation profile (*m/z* 345, 227, 209, 183), while the second as secologanoside owing to the absence of fragment at *m/z* 227 and a strong signal at *m/z* 345 characteristic for this phenol (Fu et al., 2010). Unlike before, both isomers in addition displayed the ion species at *m/z* 779 corresponding to a presence of dimers. What is of interest to add is the fact that their signals have sometimes over-dominated the molecular ions, which could easily lead to their identity miss-assignment.

Elenolic acid glucosides. Five peaks (R_t 7.5, 8.6, 9.2 10.2 and 11.9 min) in EIC at *m/z* 403.1240 were detected in olive fruits with the same fragmentation pattern (*m/z* 371, 223 and 179) typical for elenolic acid glucoside described in the literature also as 11-methyl oleoside. The fragment at *m/z* 371 corresponds to a neutral loss of methyl group, while the fragment at *m/z* 223 to the elimination of hexose, giving rise to *m/z* 179 by the neutral loss of CO_2. The presence of dimers $[2M-H]^-$ were also detected at *m/z* 807.2559. However, among these peaks only four (Peaks 2−5) presented the UV-Vis spectra with one absorption maximum, which varied depending on the matrix and isomer investigated (λ_{max} 236−245 nm), while the first eluting peak displayed two and presented a major signal at *m/z* 891. This could infer that 11-methy oleoside is in its structure, but its further identity assignment was not feasible (discussed elsewhere, **Table 7**, Unknown B). Olive stone was absent of this compound, but in addition displayed another isomer eluting at R_t 7.8 min. However, several isomers of elenolic acid glucoside have already been found in different olive tissues and process-derived matrices such as fruits, leaves, oil and wastes (**Table 5**), but their trail during oil processing has never been followed before. As evident from

our results, only one, *i.e.* an isomer 2, has been thoroughly sustained through all operative steps, while others showed no or lower resistance; in fact the majority has diminished already after crushing.

Oleuropein. The EIC at *m/z* 539.1765 in olive extracts showed the presence of three major peaks eluting at R_t 22.9, 27.0 and 29.0 min. The first two peaks displayed the fragments at *m/z* 377, 307, 275, 223, 149 and 139, and presented similar UV-Vis spectra characteristics (λ_{max} ~240 and 280 nm). The first eluting peak was identified as oleuropein using standard addition method, while the second as oleuroside known to elute afterwards under RP-LC conditions (Savarese et al., 2007). Interestingly, the third peak (R_t 29.0 min) also displayed the predominant ion at *m/z* 539.1765, but only in extract of de-stoned fruit, while in stone the predominant ion was at *m/z* 789.2466 (**Annex A**). No fragment characteristic for oleuropein aglycone (*m/z* 377) was observed in its MS spectrum, which along with distinct UV-Vis (λ_{max} 250, 280sh nm) excluded the presence of a third isomer (discussed elsewhere, **Table 7**, Unknown C). However, as evident from results, none of the two recognized isomers were transferred to oil, though constituting a major phenolic fraction in fruits. This, on one hand points to their rather low lipophilic character as already assumed before (Rodis et al., 2002), while on the other to a fast and facile transformation during processing. Their imperative role as precursors for the newly formed phenols is discussed later on.

Oleuropein diglucoside. Several peaks were detected in olive fruits at *m/z* 701.2293, however, only four (R_t 18.5, 19.5 19.8 and 20.8 min) exhibited the MS profile characteristic for oleuropein diglucoside (*m/z* 701, 539, 377, 307, 275, 223). As previously demonstrated by Fu et al. (2010) there are five possible isomers of this diglucoside, but the absence of fragment at *m/z* 341 suggested that none of them has the *O*-dihexosyl structure. Likewise before, all isomers eluted between demethyoleuropein and oleuropein, but unfortunately no UV-Vis spectra were provided for a further comparison. However, again, none of them were partitioned to the oily phase during processing, but yet two of them appeared in the wastes.

Oleuropein aglycone. Three major peaks with the same accurate mass (*m/z* 377.1236) and yielding fragments (*m/z* 345, 327, 307, 275, 149 and 139) were detected in extracts previously identified as oleuropein aglycone isomers in various tautomeric forms (Fu et al., 2009b). The peak of first eluting isomer was broad (elution 21.5–22.8 min) and in addition contained the signals of residual oleuropein (*m/z* 539) and its diglucoside (*m/z* 701), while in the second peak (R_t 37.5 min) the presence of dimers (*m/z* 755) could be observed. However, all three isomers appeared in all olive matrices, but the initial trace amounts in fruits prevented their DAD detection. A remarkable fact is that crushing implied their rise to a level easily quantifiable by DAD in all process-derived matrices, where in fact, they constituted a major phenolic fraction. However, the TIC traces in oily phase also revealed the presence of other peaks with the same MS and similar UV-Vis spectral characteristics; yet two of them were abundant and co-eluted with ligstroside aglycones (Peaks 48 and 49, **Figure 12**), whereas the identity of others could not be confirmed due to ultra-trace concentrations.

Ligstroside aglycone. Though an analogous behaviour was expected for ligstroside aglycones, these phenols displayed a distinct quantitative yield behaviour than oleuropein aglycones. None of the detectors could assign their presence in fruits, while in their process-derived matrices, only one, *i.e.* an isomer 2, and yet only in the oil, was above the detection limit of DAD. Such tiny amounts suggests to its facile degradation to *p*-HPEA-EDA whose appearance in olive oil should be marked at the end of processing. However, the identity of all was unambiguously confirmed based on their high mass accuracies of $[M-H]^-$ (≤ 6.1 ppm) and a well fitted fragmentation profile with previous report (Fu et al., 2009a).

3,4-DHPEA-EDA. The presence of two 3,4-DHPEA-EDA isomers (R_t 16.0 and 19.9 min) was confirmed based on accurate mass (*m/z* 319.1182) and characteristic fragment ions at *m/z* 301, 195, 183, 165 and 139, though a major signal in their MS spectra corresponded to a dimer (*m/z* 639.2442). However, none of them were detected in fruits, whereas both of them appeared in their solid process-derived matrices, and one (isomer 2) also in the liquid ones, where in fact, it presented one of the major phenol constituents. Their abundant evolution after brief crushing points to a fast and efficient interconversion of native fruit phenols, most likely of oleuropein

and/or ligstroside according to the mechanism proposed by Servili et al. (2004), while their apparent amphiphilic character could be of interest for forward technological partition regulation between the oil and water phases.

Nűzhenide. Four isomers of nüzhenide were identified in stone based on mass accuracy and fragmentation profile (*m/z* 523, 453, 421, 299, 223) match with previous reports (Silva et al., 2010; Obied et al., 2007a). Interestingly, three of them were also confined in the peel and/or pulp, disaffirming the notion of their seed restricted distribution (Servili et al., 1999a). However, only three (isomers 2, 3 and 4) were present in the levels quantifiable by DAD, of which two have further diminished after crushing. In fact, only one, *i.e.* nüzhenide isomer 2 was sustained through all operative steps, but has shown no affinity for the oil matrix. However, in addition to nüzhenide isomers, the presence of their esters, namely nüzhenide 11-methyl oleoside and nüzhenide di(11-methyl oleoside) could also be confirmed in the stone, but none in the other fruit compartments. While the former eluted as four peaks according to a previous report (Silva et al., 2010), the second eluted as three; at R_t 51.5, 52.0 and 58.7 min. Again, none of them were transferred to the oily phase, though some have remained occluded in the wastes.

Unknowns. Apart from known phenols described above, the presence of other compounds could also be detected in extracts from olive oil processing trial. However, only those whose spectroscopic evidence indicated to their potent phenolic structure by either sharing strong UV-Vis or MS spectral similarities with an already known phenols, were included in **Table 7** and hence quantified with the rest of phenolic fraction. Three of them have remained with a non-defined MW (Unknown A, B and C), while for four new molecular formulas were assigned, among which three were also tentatively identified.

Table 7 Unknown compounds of potent phenolic structure detected in olive extracts by UPLC-DAD-ESI-QTOF-HRMS analysis

N°	Compound	R_t (min)	λ_{max}	Major ESI^- peaks	Major ESI^+ peaks[B]	Olive matrix / Δm[A,B] (ppm)	Proposed formula / name / class	Co[B]
4[F]	Unknown A	3.7	236 313	409, 389, 355, 241, 209, 191	li	fruit: *pr*[B]; stone: *pr*; paste: *pr*; pomace: *pr*; wastewater: *pr*; oil: *pr*	–/–/secoiridoids	+
5	Unknown 408 MW compound 1	4.0	237 312	815[C], 407, 389, 377, 375, 357, 345, 313, 161, 151, 101	431[D], 415, 391, 247, 229, 211, 197, 169, 155	fruit: *0.5*; stone: *-3.4*; paste: *-0.7*; pomace: *3.7*; wastewater: *3.2;* oil: *-2.0*	$C_{17}H_{28}O_{11}$/ –/secoiridoids	+++
7	Unknown 408 MW compound 2	5.5	236 314	815[C], 407, 389, 375, 355, 191, 85	431[D], 415, 251, 211, 173, 132, 98	fruit: *-3.2*; stone: *-1.5*; paste: *-3.4*; pomace: *-3.2*; wastewater: *-3.7;* oil: *nd*	$C_{17}H_{28}O_{11}$/ –/secoiridoids	+++
9	Unknown B	7.5	242 283	891, 673, 651, 403, 325, 163	*	fruit: *pr*; stone: *nd*; paste: *pr*; pomace: *pr*; wastewater: *pr*; oil: *nd*	–/–/secoiridoids	+++
24	Unknown 654 MW isomer 1[E]	16.2	249 284sh 331	653, 621, 459, 179, 161	li	fruit: *-4.3*; stone: *-4.6*; paste: *-2.6*; pomace: *-2.6*; wastewater: *-0.9*; oil: *nd*	$C_{30}H_{38}O_{16}$/ β-Methyl-OH verbascoside isomer 1/cinnamic acids	+++
25	Unknown 654 MW isomer 2[E]	16.6	247 285sh 331	653, 621, 459, 179, 161	li	fruit: *0.3*; stone: *4.7*; paste: *-4.0*; pomace: *-4.1*; wastewater: *-2.1*; oil: *nd*	$C_{30}H_{38}O_{16}$/ β-Methyl-OH verbascoside isomer 2/cinnamic acids	+++
IV	Unknown 716 MW isomer 1[E]	17.1	–	1431[C], 715, 553, 483, 451, 329	739[D]	fruit: *nd*; stone: *4.3*; paste: *-2.8*; pomace: *-1.3*; wastewater:*nd*; oil: *nd*	$C_{32}H_{44}O_{18}$/ Methoxynüzhenide isomer 1/ secoiridoids	++
28	Unknown 484 MW isomer 1	17.8	242 280	967[C], 483, 347, 181, 139	507[D], 485, 429, 371, 250, 137	fruit: *nd*; stone: *nd*; paste: *nd*; pomace: *nd*; wastewater: *pr*; oil: *nd*	–/–/secoiridoids	+++
29	Unknown 484 MW isomer 2	18.1	246 278	967[C], 483, 347, 181, 139	507[D], 485, 429, 371, 250, 137	fruit: *nd*; stone: *nd*; paste: *nd*; pomace: *nd*; wastewater: *pr*; oil: *nd*	–/–/secoiridoids	+++
31	Unknown 484 MW isomer 3	18.5	245 281	967[C], 483, 347, 181, 139	507[D], 485, 429, 371, 236 137	fruit: *nd*; stone: *nd*; paste: *nd*; pomace: *nd*; wastewater: *pr*; oil: *nd*	–/–/secoiridoids	+++
36	Unknown 716 MW isomer 2[E]	20.9	250 277	1431[C], 715, 553, 483, 451, 329	739[D]	fruit: *nd*; stone: *-3.8*; paste: *2.8*; pomace: *nd*; wastewater:*nd*; oil: *nd*	$C_{32}H_{44}O_{18}$/ Methoxynüzhenide isomer 2/ secoiridoids	+++
50	Unknown C	29.0	250 280sh	789, 539, 403, 275, 223	li	fruit: *pr*; stone: *pr*; paste: *pr*; pomace: *pr*; wastewater: *pr*; oil: *nd*	–/–/ secoiridoids	+++
63	Uknown 1102 MW[E]	43.2	255 276	1101, 715, 553, 329	1125[D]	fruit: *nd*; stone: *5.0*; paste: *2.0*; pomace: *3.4*; wastewater: *-1.0*; oil: *nd*	$C_{49}H_{66}O_{28}$/ Methoxynüzhenide 11-methly oleoside/ secoiridoids	++

[A] *Δm*; calculated mass deviation; (*m/z* exp – *m/z* theor*) / m/z* theor × 10^6. [B]Compound is described as: (pr) present, (tr) present in traces, (nd) not detected, (*) the evidence for its presence is inconclusive, (+) major peak in DAD chromatogram, (++) major peak in TIC chromatogram, (+++) equally well detected by DAD and MS, (li) low ionisation. [C]$[2M–H]^-$. [D]$[M+Na]^+$. [E]Tentatively identified. [F]Arabic numbers refers to DAD detected/quantified phenols, while romans to only MS detected phenols.

Unknown A. The compound eluting at R_t 3.7 min was present in all olive matrices, where it presented the same UV-Vis spectra as comselogoside (**Figure 14a**). Its MS spectrum in NIM mode showed the fragments at *m/z* 409, 389, 355, 241, 209 and 191, while in fruit extract the presence of additional ion signal was detected at *m/z* 421 (**Figure 14b**). The latter was in fact the predominant ion as already observed by Cardoso et al. (2005) reporting its presence in fruit and pomace extracts, however, due to its total absence in other matrices, the MW of this compound was rather marked as undefined (Unknown A). Such decision was further supported by the lack of sufficient evidence for its molecular ion in PIM mode. However, it is possible that a signal of the precursor ion was week or absent due to its fast dissociation, but the presence of two common fragments at *m/z* 389 and 241 along with similar elution order indicated that this compound is at least similar to that previously reported by Cardoso et al. (2005). Unfortunately, no UV-Vis spectrum characteristics were provided in earlier study and hence no further comparison possible. Even so, both of these fragments are typical for secoiridoid phenols, which confidentially allowed us to classify it among secoiridoids. Moreover, this compound practically showed the identical UV-Vis spectrum with a well-known secoiridoid comselogoside eluting later at R_t 31.2 min. Undoubtedly, a further research is needed to assign its identity and structure, interesting also because of its ubiquitous presence in all matrices.

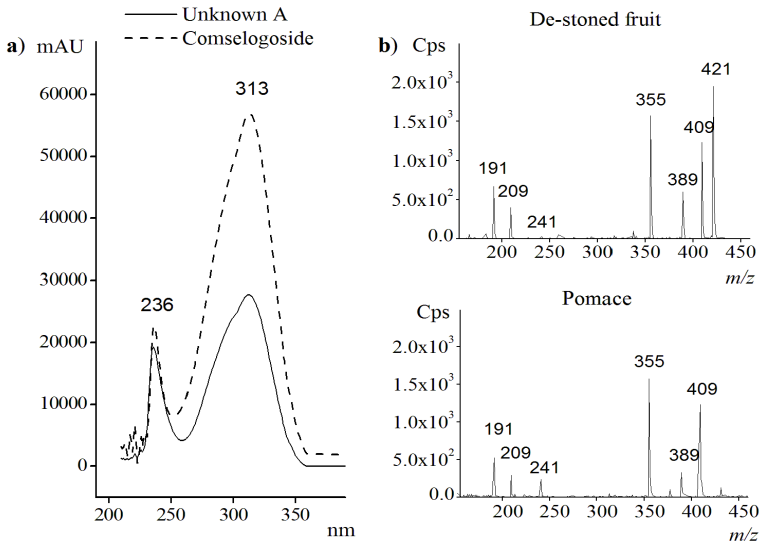


Figure 14 UV-Vis and MS spectra comparison; unknown A *vs.* comselogoside (a), and de-stoned fruit *vs.* pomace extracts (b)

Unknown 408 MW. Two peaks (R_t 4.0 and 5.5 min) with the same exact mass (*m/z* 407.1553) and molecular formula $C_{17}H_{28}O_{11}$ generated by QTOF were detected in olive extracts, sharing similar UV-Vis, but different MS fragmentation profile. The first eluting compound (Peak N° 5) was more polar and had its *B*-band shifted to a lower λ_{max} (312 nm, **Figure 15a**). Moreover, it presented a similar MS spectrum as previously reported by the two authors, *i.e.* Obied et al. (2007a) and Cardoso et al. (2005). The second eluting peak (Peak N° 7) was less polar and had a slightly higher λ_{max} of *B*-band at 314 nm (**Figure 15b**), but displayed much less MS fragment ions of lower intensities. Interestingly, both precursor ions corresponded to the same elemental formula ($C_{17}H_{28}O_{11}$) determined with a high mass accuracy (≤ 3.7 ppm), which could indicate to their equal chemical composition, but of likely different structure. As already demonstrated for secoiridoidal derivatives before, there are several models possible differing in the structure of elenolic ring; being in open or closed, aldehydic or non-aldehydic forms (De La Torre-Carbot et al., 2005). However, beside the molecular formula proposed, no further structural assignment could be established, which entails a further investigation using LC-MS^n, NMR *etc*. However, it is worth mentioning that both of them again shared a strong UV-Vis similarity with comselogoside (**Figure 15c**), and were hence classified among secoiridods. The presence of secoiridoidal diagnostic fragments at *m/z* 389, 377, 375 and 345 strongly supports such a decisive proof.

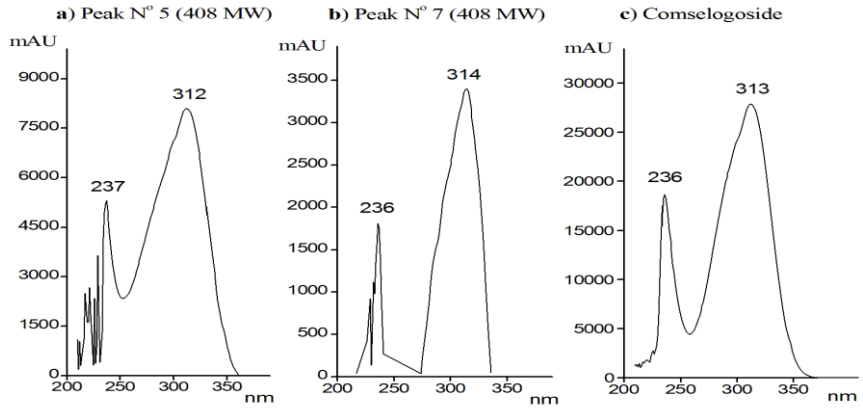


Figure 15 UV-Vis spectra comparison of two unknown 408 MW compounds (1 and 2) and comselogoside. Peak assignment refers to **Table 7**

Unknown B. Another compound with a non-defined MW eluted at R_t 7.5 min. Likewise to unknown A, no base peak could be evidenced in PIM mode, whereas in NIM the ion at *m/z* 891 could correspond to $[M-H]^-$, but owing to a highly variable intensity of signal among different matrices, we could not confidentially support such notion. However, in NIM mode, the additional MS ions were observed at *m/z* 673, 651, 403, 325 and 163, though the former was not detected in the extract of de-stoned fruit. Best to our knowledge such MS profile has never been reported before, but based on elution time and UV-Vis spectra resemblance with oleuropein (**Annex A**) this compound was classified among secoiridoids when quantified further on.

Unknown 654 MW. Two new diastereoisomers (R_t 16.2 and 16.6 min) of verbascoside derivative were discovered in olive extracts, that best to authors' knowledge have never been found before. Their tentative identification was performed by calculating the possible molecular formula from experimental *m/z* and MS fragments data interpret, yielding $C_{30}H_{38}O_{16}$ with a high mass accuracy (< 5 ppm) for all matrices. **Figure 16a** presents the EIC at *m/z* 653.2082 and the corresponding MS spectra of both compounds (**Figure 16b**) displaying identical MS profile (*m/z* 621, 459, 179, 161) typical for verbascoside derivatives (Innocenti et al., 2005). The ion at *m/z* 621 is formed by the loss of methyl group and the formation of a double bond between α- and β- carbons, which subsequently forms the fragment at *m/z* 459 by the loss of caffeoyl group. The common ions at *m/z* 179 and 161 correspond to caffeic acid and its dehydrated ion. On the basis of this MS profile, we proposed the structure for these two diastereoisomers, which is along with fragmentation pattern presented in **Figure 16c**. However, as the latter was almost identical with an already known β-Ethyl-OH verbascoside diastereoisomers previously found in pomace (Innocenti et al., 2005), we analogously referred the newly discovered compounds as β-Methyl-OH verbascoside isomers. Their structural correlation with verbascoside and β-OH verbascoside is further apparent from the UV-Vis spectra comparison (**Figure 17**) and supports their classification among cinnamic acids. What is of interest to add is their significant rise observed after fruits crushing, which kept increasing also during malaxation (discussed elsewhere, **Table 10**).

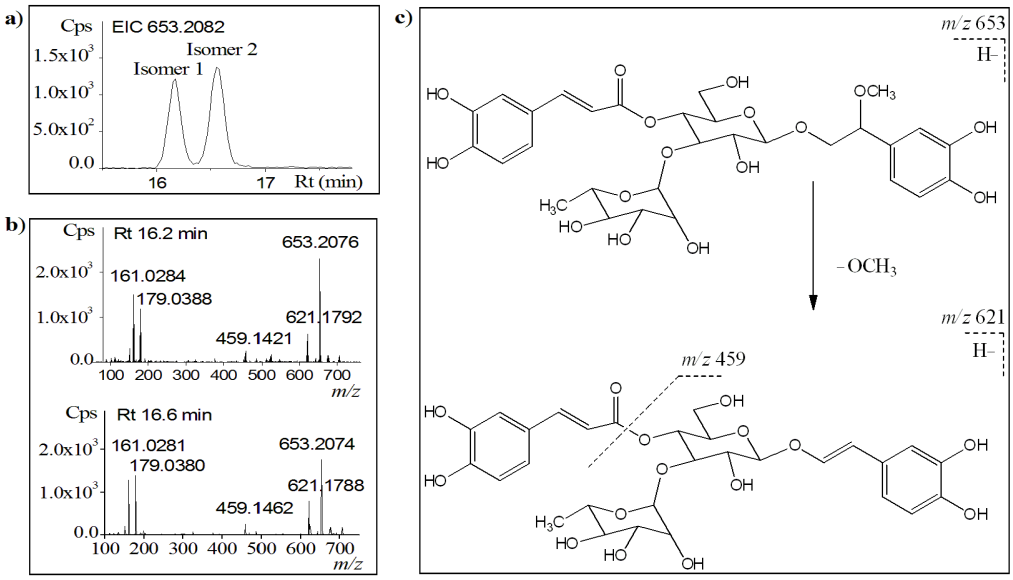


Figure 16 EIC of *m/z* 653.2082 (a), ESI-QTOF-MS spectra (b), the structure and fragmentation scheme proposed for β-Methyl-OH verbascoside isomers (c)

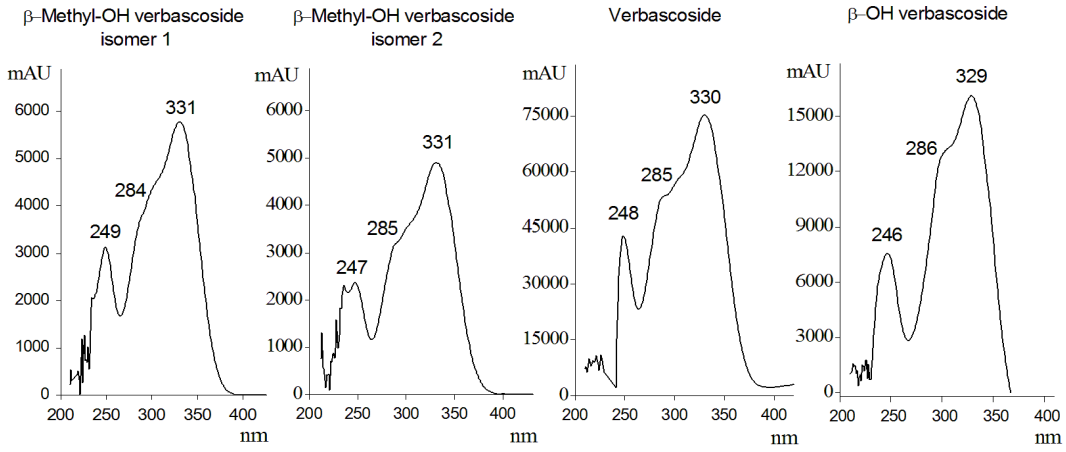


Figure 17 UV-Vis spectra comparison; β-Methyl-OH verbascoside isomers *vs.* verbascoside and β-OH verbascoside

Unknown 716 MW. This phenolic compound has already been detected before in olive stone, but not identified, though structurally correlated to nüzhenide having more 30 mass units (Silva et al., 2010). Likewise before, this compound eluted as two peaks (R_t 17.1 and 20.9 min) and presented the same fragmentation profile (*m/z* 715, 553, 483, 451, 329) suggesting to a presence of isomers. A HR-MS analysis of both peaks provided the exact masses of 715.2449 corresponding to $C_{32}H_{44}O_{18}$ determined with less than 5 ppm uncertainty. Their product ion spectra showed a series of ions typical for nüzhenide with *m/z* values increased by 30.0105 mass units

(**Figure 18a**), which is likely due to an attached methoxy group to tyrosol glucose moiety of nüzhenide. The structure proposed for unknown 716 MW is along with fragmentation scheme presented in **Figure 18c**, where the ion at *m/z* 553 is formed by a neutral loss of glucose, while the ion at *m/z* 329 by the consecutive loss of methoxytyrosol glucose. The common ions at *m/z* 483 and 451 originates from *m/z* 553 as previously demonstrated for nüzhenide (Silva et al., 2010). Nevertheless, its UV-Vis spectrum shares a strong similarity with nüzhenide **(Figure 18b)**, which reasonably suggests to a presence of methoxynüzhenide isomers. Such a decisive proof is further supported by a high mass accuracy of all characteristic fragment ions presented in **Table 8**. However, what infers next, is their unique presence in stone, which could point to a distinct biosynthetic metabolism of this fruit compartment, while the trace presence in process-derived matrices is only a result of their transference from stone.

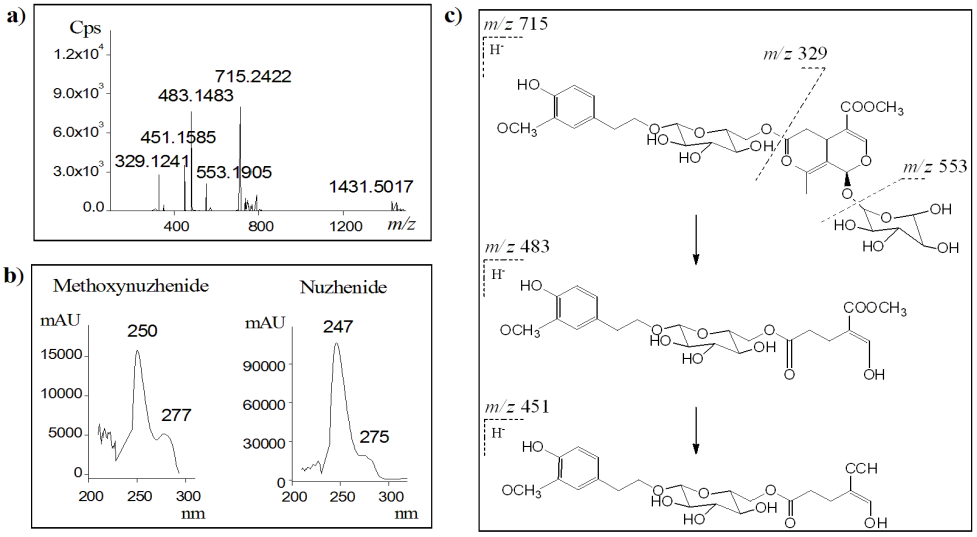


Figure 18 ESI-QTOF-MS (a), UV-Vis spectrum (b), the structure and fragmentation scheme proposed for Methoxynüzhenide (c)

Table 8 Accurate mass data of Methoxynüzhenide MS ions

Proposed fragment ions	m/z_{theor} [A]	m/z_{exp} [B]	Formula	Δm [C] (ppm)
$[2M–H]^-$	1431.4977	1431.5017	$C_{64}H_{88}O_{36}$	*2.8*
$[M–H]^-$	715.2449	715.2422	$C_{32}H_{44}O_{18}$	*-3.8*
$[M–H–C_6H_{10}O_5]^-$	553.1921	553.1905	$C_{26}H_{34}O_{13}$	*-2.9*
$[M–H–C_6H_{10}O_5–C_4H_4O]^-$	483.1503	483.1483	$C_{22}H_{30}O_{12}$	*-4.1*
$[M–H–C_6H_{12}O_6–C_4H_6O_3]^-$	451.1604	451.1585	$C_{22}H_{28}O_{10}$	*-4.2*
$[M–H–C_6H_{10}O_5–C_{11}H_{12}O_5]^-$	329.1236	329.1241	$C_{15}H_{22}O_8$	*1.5*

[A]Calculated monoisotopic mass $[M–H]^-$. [B]Detected mass. [C]Calculated mass deviation; (*m/z* exp - *m/z* theor) / *m/z* theor × 10^6).

Unknown 1102 MW. Another compound found in stone, but never characterised using HR-MS analysis was eluted at R_t 43.2 min. This compound is exactly 386.1213 mass units heavier than methoxynüzhenide and shares the same fragmentation pattern (*m/z* 715, 553, 329) and similar UV-Vis spectrum characteristics (λ_{max} 255 and 276) (**Figure 19a** and **b**). Silva et al. (2010) already assigned its presence in stone and hypothesised to its structure as 11-methyloleoside linked to a compound 716 MW. We in addition provide its molecular formula, *i.e.* $C_{49}H_{66}O_{28}$ determined with a high mass accuracy of 5.0 ppm, its structure and proposed fragmentation scheme (**Figure 19c)**. The accurate masses of detected fragment ions, their molecular formulas and the calculated errors between them are further provided in **Table 9**, indicating that 11-methyloleoside is indeed likely attached to a methoxynüzhenide (716 MW). All together allowed us to tentatively identify the unknown 1102 MW as methoxynüzhenide 11-methyl oleoside with a high degree of confidence. Again, its presence was restricted solely to the stone, from where it was apparently transferred to the paste, pomace and wastewater, however only in traces detectable by LC-MS.

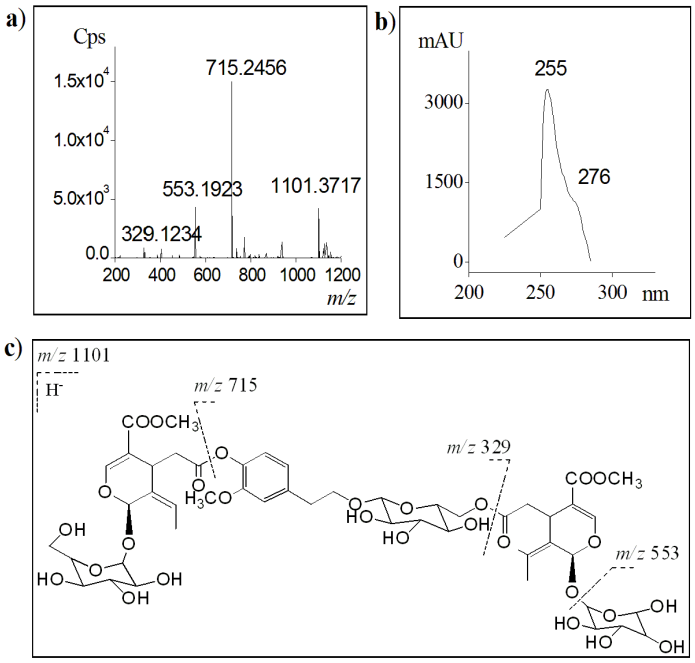


Figure 19 ESI-QTOF-MS (a), UV-Vis spectrum (b), the structure and fragmentation scheme proposed for Methoxynüzhenide 11-methyloleoside (c)

Table 9 Accurate mass data of Methoxynüzhenide 11-methyl oleoside MS ions

Proposed fragment ions	m/z_{theor} [A]	m/z_{exp} [B]	Formula	Δm [C] (ppm)
$[M–H]^-$	1101.3662	1101.3717	$C_{49}H_{66}O_{28}$	*5.0*
$[M–H–C_{17}H_{22}O_{10}]^-$	715.2449	715.2456	$C_{32}H_{44}O_{18}$	*1.0*
$[M–H–C_{17}H_{22}O_{10}–C_6H_{10}O_5]^-$	553.1921	553.1923	$C_{26}H_{34}O_{13}$	*0.4*
$[M–H–C_{17}H_{22}O_{10}–C_{11}H_{12}O_5]^-$	329.1236	329.1234	$C_{15}H_{22}O_8$	*-0.6*

[A]Calculated monoisotopic mass $[M–H]^-$. [B]Detected mass. [C]Calculated mass deviation; (*m/z* exp - *m/z* theor*) / m/z* theor × 10^6).

In total, eighty different phenolic compounds were found in six olive extracts using combined UPLC-DAD-ESI-QTOF-HRMS analysis, which is the first report with such a wide range of detection. However, only sixty-six were equally well detected by both detectors; while DAD alone allowed the detection of sixty-nine phenols, the ESI-QTOF-MS confirmed the presence of seventy-seven owing to trace detections beyond the DAD limits. As expected, MS was more selective than DAD and permitted insight into olive phenols structure, but only when using strong ionisation conditions. It also provided an elemental composition of known and novel constituents with a high mass accuracy below 10 ppm. For most phenols, NIM mode was much better than PIM and dimers were frequently observed in their MS spectra. By contrast, DAD allowed the identification of phenols which failed to ionise or whose ions were hindered by the matrix signal suppression effect, increasing error outside the tolerable range of 10 ppm. Moreover, it also provided a valuable and complementary UV-Vis spectra information that sometimes helped to distinct between the phenols isomeric forms and allowed to specify the site of glycosidation. But yet, it entailed a good chromatographic separation and was much more prone to the matrix interferences as evident from several absorption maxima deviations of phenols in different olive matrices. All together, both detectors were needed to assign the presence and identity of olive phenols in complex extracts with a high degree of confidence, demonstrating the power of such screening approach.

Matrix specifity. Only a portion of phenols was strictly related to a particular matrix, whereas others were widespread and present in all matrices. Secoiridoids, simple phenols, cinnamic acids and flavonoids were the most ubiquitous classes with at least one representative found in each, while lignans were only detected in oil, and benzoic acids were absent in the peel and/or pulp. While the profiles of paste, pomace and wastewater were rather similar, they on contrary differed from that of

oil, owing to its lipophilic character. Likewise, the phenol profiles of stone and the rest of fruit compartment displayed a rather distinct phenolic composition. However, among all, paste displayed the most diverse range of phenols (forty-one quantifiable out of fifty detected) due to evolution of new respective derivatives formed after fruits crushing. The pulp and peel together along with pomace had the second richest profile, followed by wastewater, stone and oil. Stone was unique in the absence of quantifiable flavonoids and the presence of nüzhenide-type phenols with an exception of nüzhenide isomer 2, also found in the peel and/or pulp. While the distinct profiles of different fruit compartments points to a different metabolism of olive tissues or to an easy transfer of their phenolic precursors (Ryan et al., 2003), the appearance of non-native fruit phenols in the process-derived matrices reasonably infers to their technologically-induced formation and/or release. Such is the case of vanillic acid, apigenin, 3,4-DHPEA-EDA, *p*-HPEA-EDA and ligstroside aglycones found in paste after fruits crushing. Likewise, the unique presence of pinoresinol, acetoxypinoresinol in olive oil, analogously as acetal of 3,4-DHPEA-EDA and unknown isomers of 484 MW in wastewater could point to their artefactual nature.

5.1.2 Quantitative analysis

In line with majority of existing reports, the quantitative phenol analysis was carried out by DAD at four chosen wavelengths, presenting a compromise for individual class detection. Simple phenols and secoiridoids were quantified at 280 nm with an exception of unknown A, uknowns 408 MW, caffeoyl-6'-secologanoside and comselogoside quantified at 320 nm along with the group of cinnamic acids and flavonoids. Flavonols, on the other side, were all quantified at 365 nm. A high background of mobile phase has restricted the quantification of nüzhenides at 240 nm, though displaying a greater absorption than at 280 nm. Co-elution was also sometimes observed that occasionally disturbed the quantification. All together, sixty-nine phenols was quantified in a single run, which is far more than ever reported before for such matrices.

5.1.3 Method validation

The suitability of U(H)PLC-DAD system was satisfactory, where the linearity of injector was always above 99% and the reproducibility of ten replicates has never exceeded 0.5% of RSD, respectively.

The linearity of method was excellent with high correlation coefficients (R^2) obtained for all standards over their broad concentration ranges (μg/mL) tested as follows; vanillin (R^2 = 0.9933; 0.33–64.83), vanillic acid (R^2 = 0.9957; 0.35–67.26), *p*-coumaric acid (R^2 = 0.9946; 0.33–64.21), caffeic acid (R^2 = 0.9982; 1.24–99.19), hydroxytyrosol (R^2 = 0.9970; 1.44–524.19), tyrosol (R^2 = 0.9937; 1.40–219.00), oleuropein (R^2 = 0.9963; 1.00–7953.12), verbascoside (R^2 = 0.9991; 0.39–1013.12), rutin (R^2 = 0.9952; 0.24–312.40), quercitrin (R^2 = 0.9925; 0.23–121.44), luteolin-4'-*O*-glucoside (R^2 = 0.9976; 0.25–896.56), apigenin (R^2 = 0.9988; 1.62–232.80), apigenin-7-*O*-glucoside (R^2 = 0.9999; 0.48–274.56), luteolin (R^2 = 0.9928; 0.56–483.12), luteolin-7-*O*-glucoside (R^2 = 0.9962; 1.26–765.24), pinoresinol (R^2 = 0.9975; 0.79–285.76).

Extraction methods for phenols isolation from olive matrices entailed in olive oil processing were adopted from our earlier reports performed as a preliminary step toward a high-yielding TP analysis in fruits, wastewater and oil, of which results are separately presented in the form of published papers (**Annex B1–B3**). However, prior to application, their efficiencies had to be re-checked owing to introduction of new matrices of richer quali- and quantitative phenolic profiles, and novel ones for which the recovery optimisations have not been yet performed (paste, pomace and stone). Even so, our results confirmed that both USLE and US-LLE extractions are efficient enough for the quantitative phenol analysis, where a three-step extraction provided recoveries superior to 98% on average for all six matrices (US-LLE for the oil and USLE for others), again testifying to a high powerful US extracting abilities.

The sensitivity of DAD detector was rather comparable to a previously employed DAD from Agilent (Jerman Klen et al., 2012b; Jerman et al., 2010), where the calculated LODs/LOQs for each standard expressed in μg/mL were as follows;

0.36/1.09 (vanillin), 0.23/0.69 (vanillic acid), 0.22/0.67 (*p*-coumaric acid), 2.46/7.44 (caffeic acid), 2.55/7.74 (hydroxytyrosol), 1.75/5.29 (tyrosol), 1.57/4.76 (oleuropein), 0.19/0.58 (verbascoside), 0.03/0.01 × e^{-1} (rutin), 0.01/0.04 (quercitrin), 0.12/0.36 (luteolin-4'-*O*-glucoside), 1.20/3.87 (apigenin), 0.17/0.52 (apigenin-7-*O*-glucoside), 0.53/1.60 (luteolin), 0.16/0.49 (luteolin-7-*O*-glucoside) and 0.04/0.11 (pinoresinol).

5.2 Olive oil processing trial

The second study of doctoral thesis was devoted to the olive fruit phenols fate assessment during olive oil processing in relation to different operative conditions by using a 3-phase extraction line with Abencor system. In order to quantify their transfer/partition rates from fruits to paste and its final products – oil and wastes (pomace and wastewater), all their yields were expressed per fruits initial phenolic content considered as the available pool of phenols derived from either de-stoned fruit and/or stone marked as input (**Table 10)**. The final proportion of phenols resulting in each matrix was thus dependant on both – its relative amount and the phenols concentration.

However, before embarking to a detailed discussion as regards to olive phenols transfer, transformation and partition trail initiated by crushing, and continued by malaxation, some aspects of paste's structure must be better defined to understand the physico-chemical changes altered by individual technological parameter applied.

Crushing modifies the physical structure of olive fruit; it disrupts the equilibrium between its components, creates the oil/water emulsion and induces several enzymatic and/or chemical processes. It breaks up the cell walls and releases cell constituents, including phenols, which in addition to several transformations, are transferred/partitioned between the macroscopic parts of paste. The latter is a multi-phasic system in a dynamic state composed of one solid (pulp and fragments of stone) and the two immiscible liquids (vegetation water and oil) that after centrifugation yields the three final products, *i.e.* pomace, wastewater and oil. Phenols, once released or formed as a result of transformation, are distributed between the water and oil phase according to their affinities and concentrations,

while some of them remains entrapped in the solid fraction. The rate of such transfers depend on several factors, *i.e.* the physical structure and chemical composition of each phase, the temperature and time of contact, the presence of surfactants and co-adjuvants, and the characteristics of interfacial regions - extension, curvature and renovation (Herrera, 2007; Parenti et al., 2008; Rodis et al., 2002).

During malaxation, the interfacial films of olive paste are disrupted, reducing its emulsion state and promoting coalescence of small oil droplets to release the "free oil". In general, only 80% of the oil from vacuoles of pulp can be easily extracted, while the rest (up to 20%) remains inside the unsheltered cells, is dispersed in the cytoplasm as micro-gels and/or is bound in an emulsion with vegetable water. This fraction is known as "bonded/difficult/emulsified" and is hardly extractable, therefore it is often lost with wastes mainly due to lipoprotein membranes that surround the oil droplets and tends to keep them in emulsion/dispersed form. Moreover, these membranes also hamper different kind of transfers including those of minor components. When these phenomena are more pronounced such paste is known as difficult and sometimes needs the use of co-adjuvants to break-down such systems. In general, the pastes containing moisture above 50% are classified as difficult (Clodoveo, 2012; Aguilera et al., 2010; Herrera, 2007).

Table 10 presents a detailed insight into olive fruit phenols transfer, transformation and partition trail during olive oil processing at 30 min/25 °C malaxation conditions (control), while the discussion is divided based on its two main operative steps, *i.e.* crushing and malaxation.

Table 10 The fate of olive fruit phenols during olive oil processing at 30 min/25 °C malaxation conditions (control trial)

Class/ Phenolic compound	Olive matrix										
	Input			Intermediate	*Crushing*[D]		Output			*Malaxation*[F]	
	De-stoned fruit[A]	**Stone**[A]	Σ[B]	**Paste**[A, C] (t =0)	*SignF*	*Δc (%)*	**Pomace**[A]	**Wastewater**[A]	**Oil**[E]	*SignF*	*Δc (%)*
Simple phenols											
Hydroxytyrosol glucoside	51.8 ± 1.0	4.8 ± 0.3	56.6 ± 1.0	40.1 ± 1.3	***	*-29*	34.9 ± 3.8	7.1 ± 0.4	1.4 ± 0.1	ns	–
Hydroxytyrosol-1-β-glucoside	18.7 ± 1.3	9.9 ± 0.5	28.6 ± 1.6	48.5 ± 1.0	***	*+70*	54.5 ± 0.9	5.5 ± 0.6	7.5 ± 0.6	***	*+24*
Hydroxytyrosol	26.0 ± 1.0	6.3 ± 0.3	32.3 ± 1.2	87 ± 3.3	***	*+170*	86.7 ± 3.0	21.6 ± 3.0	27.0 ± 5.6	***	*+25*
Tyrosol glucoside	10.2 ± 1.1	6.1 ± 0.3	16.3 ± 1.3	37.8 ± 1.7	***	*+134*	38.3 ± 1.8	10.9 ± 1.4	6.7 ± 0.4	***	*+30*
Tyrosol	8.1 ± 0.3	2.3 ± 0.1	10.4 ± 0.4	21.4 ± 1.1	***	*+107*	22.7 ± 1.5	5.2 ± 0.5	24.7 ± 3.9	**	*+32*
Hydroxytyrosol acetate	26.6 ± 0.5	nd[G]	26.6 ± 0.5	nq (co)	–[H]	–	nq (co)	nq (co)	17.9 ± 1.3	–	–
Total	*141.3 ± 4.9*	*29.4 ± 1.6*	*170.7 ± 5.7*	*235.0 ± 7.3*	***	*+38*	*237.1 ± 10.1*	*50.3 ± 6.0*	*85.2 ± 11.7*	**	*+23*
Benzoic acids											
Vanillic acid	nd	nd	–	10.0 ± 0.3	***	*+100*	7.9 ± 0.5	1.5 ± 0.1	6.4 ± 0.5	ns	–
Vanillin	nd	1.5 ± 0.1	1.5 ± 0.1	nd	***	*-100*	nd	nd	4.7 ± 0.3	***	*+100*
Total	–	*1.5 ± 0.1*	*1.5 ± 0.1*	*10.0 ± 0.3*	***	*+567*	*7.9 ± 0.5*	*1.5 ± 0.1*	*11.1 ± 0.8*	***	*-6*
Cinnamic acids											
p-Coumaric acid	nq (co)	nd	–	8.3 ± 1.0	–	–	6.4 ± 0.4	0.8 ± 0.1	5.5 ± 0.4	ns	–
β-OH verbascoside isomer 1	6.0 ± 0.3	0.5 ± 0.0	6.6 ± 0.2	16.9 ± 1.2	***	*+157*	21.6 ± 1.3	4.7 ± 0.4	nd	***	*+56*
β-OH verbascoside isomer 2	3.2 ± 0.2	0.4 ± 0.0	3.7 ± 0.2	15.4 ± 1.3	***	*+320*	20.3 ± 1.4	4.6 ± 0.4	nd	***	*+64*
β-OH-methyl verbascoside isomer 1	8.3 ± 0.9	nq (tr)	8.3 ± 0.9	24.1 ± 2.2	***	*+194*	26.7 ± 0.5	1.8 ± 0.1	nd	***	*+19*
β-OH-methyl verbascoside isomer 2	5.8 ± 0.7	nq (tr)	5.8 ± 0.7	26.1 ± 2.5	***	*+357*	30.2 ± 0.5	2.0 ± 0.1	nd	***	*+24*
Verbascoside	358.5 ± 10.8	13.3 ± 0.7	371.7 ± 11.0	258.0 ± 19.6	***	*-31*	202.4 ± 22.1	20.3 ± 0.6	nd	*	*-13*
Total	*381.8 ± 12.1*	*14.2 ± 0.7*	*396.0 ± 12.2*	*348.7 ± 27.4*	**	*-12*	*307.6 ± 26*	*34.3 ± 1.5*	*5.5 ± 0.4*	ns	–
Flavonoids											
Luteolin-4',7-*O*-diglucoside	19.5 ± 0.1	nd	19.5 ± 0.1	nq (ins)	–	–	nd	nd	nd	–	–
Rutin	290.1 ± 6.8	nq (tr)	290.1 ± 6.8	181.9 ± 11.9	***	*-37*	119.1 ± 3.1	3.5 ± 0.3	nq (tr)	***	*-32*
Luteolin-7-*O*-glucoside	315.5 ± 9.9	nd	315.5 ± 9.9	41.8 ± 9.5	***	*-87*	32.5 ± 3.0	1.7 ± 0.1	nd	ns	–
Luteolin rutinoside isomer 1	42.7 ± 1.3	nd	42.7 ± 1.3	29.5 ± 0.7	***	*-31*	30.2 ± 2.3	4.3 ± 0.3	nd	*	*+17*
Luteolin rutinoside isomer 2	15.0 ± 0.6	nd	15.0 ± 0.6	12.2 ± 0.3	***	*-19*	11.5 ± 0.9	1.4 ± 0.0	nd	ns	–
Quercitrin	50.1 ± 0.9	nd	50.1 ± 0.9	35.0 ± 2.1	***	*-30*	27.4 ± 0.7	1.2 ± 0.1	nd	***	*-18*
Apigenin-*7*-*O*-glucoside	79.9 ± 2.9	nd	79.9 ± 2.9	39.3 ± 2	***	*-51*	34.3 ± 2.8	4.1 ± 0.3	nd	ns	–
Luteolin-*4'*-*O*-glucoside	93.8 ± 2.3	nd	93.8 ± 2.3	29.7 ± 3.4	***	*-68*	24.7 ± 1.7	nq (ins)	nd	–	–

Table 10 *(Cont)*

Class/ Phenolic compound	Olive matrix										
	Input			Intermediate	*Crushing*		Output			*Malaxation*	
	De-stoned fruit	**Stone**	∑	**Paste** (t =0)	*SignF*	*Δc (%)*	**Pomace**	**Wastewater**	**Oil**	*SignF*	*Δc (%)*
Luteolin-3'-*O*-glucoside	29.4 ± 0.7	nd	29.4 ± 0.7	30.1 ± 0.7	ns	–	27.7 ± 2	2.3 ± 0.1	nd	ns	–
Luteolin	nd	nd	–	123.5 ± 10.3	***	*+100*	133.5 ± 16.2	5.1 ± 0.4	37.1 ± 2.8 d	ns	–
Apigenin	nd	nd	–	28.0 ± 1.9	***	*+100*	28.0 ± 3.6	1.8 ± 0.1	31.6 ± 2.3 cd	ns	–
Total	*936.1 ± 24.7*	–	*936.1 ± 24.7*	*551.1 ± 26.8*	***	*-41*	*469.0 ± 35.7*	*25.2 ± 1.6*	*68.7 ± 5.0*	*	*-10*
Lignans											
Pinoresinol	nd	nd	–	nd	–	–	nd	nd	69.4 ± 4.8	***	*+100*
Acetoxypinoresinol	nd	nd	–	nd	–	–	nd	nd	266.6 ± 18.3	***	*+100*
Total	–	–	–	–	–	–	–	–	*336.0 ± 23.0*	***	*+100*
Secoiridoids											
Unknown A	12.0 ± 0.4	nq (tr)	12.0 ± 0.4	10.6 ± 0.3	***	*-12*	8.9 ± 0.8	2.1 ± 0.1	0.5 ± 0.0	ns	–
Unknown 408 MW compound 1	2.0 ± 0.1	nq (ins)	2.0 ± 0.1	2.0 ± 0.1	–	–	1.7 ± 0.2	0.3 ± 0.0	0.1 ± 0 .0	ns	–
Unknown 408 MW compound 2	3.1 ± 0.2	nq (ins)	3.1 ± 0.2	3.5 ± 0.1	–	–	2.9 ± 0.3	0.6 ± 0.0	nd	ns	–
Unknown B	309.8 ± 10.8	nd	309.8 ± 10.8	284.9 ± 5	**	*-8*	261.9 ± 22.8	54.3 ± 3.5	nd	ns	–
Elenolic acid glucoside isomer 1	35.2 ± 1.3	5.4 ± 0.3	40.6 ± 1.4	50.1 ± 1.3	***	*+24*	nd	nd	nd	***	*-100*
Secologanoside	54.1 ± 2.6	nq (tr)	54.1 ± 2.6	67.0 ± 1.2	***	*+24*	56.1 ± 4.0	12.1 ± 2.5	9.4 ± 1.5	ns	–
Elenolic acid glucoside isomer 2	64.0 ± 4.1	5.2 ± 0.3	69.2 ± 4.3	nq (co)	–	–	nq (co)	nq (co)	11.4 ± 1.0	–	–
Elenolic acid glucoside isomer 3	151.4 ± 5.7	12.7 ± 0.6	164.1 ± 5.9	nd	***	*-100*	nd	nd	nd	–	–
Demethyloleuropein	3470.1 ± 99.2	nq (ins)	3470.1 ± 99.2	nq (tr)	–	–	nq (tr)	nq (tr)	nd	–	–
Dihydro-oleuropein isomer 1	118.5 ± 3.3	nd	118.5 ± 3.3	194.2 ± 6.8	***	*+64*	189.9 ± 15.4	21.1 ± 1.2	nd	ns	–
3,4-DHPEA-EDA isomer 1	nd	nd	–	168.6 ± 6.7	***	*+100*	118.9 ± 19.4	nd	nd	**	*-29*
Dihydro-oleuropein isomer 2	348.0 ± 10.9	34.4 ± 1.9	382.4 ± 11.9	275.1 ± 7.2	***	*-28*	105.3 ± 8.5	nq (tr)	nd	***	*-62*
Unknown 484 MW isomer 1	nd	nd	–	nd	–	–	nd	51.3 ± 2.6	nd	***	*+100*
Unknown 484 MW isomer 2	nd	nd	–	nd	–	–	nd	38.9 ± 4.1	nd	***	*+100*
Oleuropein diglucoside isomer 1	110.8 ± 8	30.0 ± 1.8	140.8 ± 8.9	230.8 ± 22.7	***	*+64*	nq (ins)	nq (ins)	nd	–	–
Unknown 484 MW isomer 3	nd	nd	–	nd	–	–	nd	37.1 ± 2.9	nd	***	*+100*
Nüzhenide isomer 2	57.0 ± 1.8	92.3 ± 4.9	149.3 ± 5.0	180.3 ± 2.7	***	*+21*	143.8 ± 8.3	22.2 ± 1.6	nd	*	*-8*
Oleuropein diglucoside isomer 2	143.1 ± 4.7	nd	143.1 ± 4.7	nd	***	*-100*	nd	nd	nd	–	–
3,4-DHPEA-EDA isomer 2	nd	nd	–	1828.6 ± 188.9	***	*+100*	3039.4 ± 382.9	241.6 ± 6.7	1925.8 ± 126.8	***	*+81*
Nüzhenide isomer 3	nd	17.0 ± 1.2	17.0 ± 1.2	nd	***	*-100*	nd	nd	nd	–	–

Table 10 *(Cont)*

Class/ Phenolic compound	Olive matrix										
	Input			Intermediate	*Crushing*		Output			*Malaxation*	
	De-stoned fruit	**Stone**	**∑**	**Paste** (t =0)	*SignF*	*Δc (%)*	**Pomace**	**Wastewater**	**Oil**	*SignF*	*Δc (%)*
Methoxynüzhenide	nd	34.0 ± 2.1	34.0 ± 2.1	nd	***	*-100*	nd	nd	nd	–	–
Oleuropein aglycone isomer 1	nd	nd	–	4729.6 ± 167.5	***	*+100*	3406.7 ± 198.3	331.4 ± 12.9[I]	3321.6 ± 238.6	***	*-20*
Nüzhenide isomer 4	nd	12.7 ± 0.8	12.7 ± 0.8	nd	***	*-100*	nd	nd	nd	–	–
Oleuropein	12222.0 ± 360.6	427.9 ± 25.0	12649.8 ± 369.0	698.5 ± 156.5	***	*-94*	377.6 ± 15.9	nd	nd	***	*-44*
Caffeoyl-6'-secologanoside	14.7 ± 0.6	nd	14.7 ± 0.6	14.7 ± 0.3	ns	–	13.2 ± 1.1	1.8 ± 0.1	nd	ns	–
p-HPEA-EDA	nd	nd	–	nd	***	–	nd	nd	1303.6 ± 90.0	***	*+100*
Oleuroside	152.7 ± 5.0	16.6 ± 1.5	169.2 ± 5.0	nd	***	*-100*	nd	nd	nd	–	–
Ligstroside + Oleuropein aglycone sum	nd	nd	–	nd	–	–	nd	nd	1879.4 ± 109.0	***	*+100*
Unknown C	227.5 ± 4.2	16.5 ± 1.3	243.9 ± 3.3	nq (co)	–	–	nq (co)	nq (co)	nd	–	–
Ligstroside	575.2 ± 17.2	52.0 ± 3.8	627.2 ± 18.1	nd	***	*-100*	nd	nd	nd	–	–
Comselogoside	33.7 ± 1	nd	33.7 ± 1.0	34.7 ± 2.0	ns		30.7 ± 2.5	3.9 ± 0.2	nd	ns	–
Nüzhenide 11-methyl oleoside isomer 1	nd	18.2 ± 1.7	18.2 ± 1.7	nd	***	*-100*	nd	nd	nd	–	–
Nüzhenide 11-methyl oleoside isomer 2	nd	18.3 ± 2.6	18.3 ± 2.6	nd	***	*-100*	nd	nd	nd	–	–
Acetal of 3,4-DHPEA-EDA	nd	nd	–	nd	–	–	nd	142.6 ± 9.5	nd	***	*+100*
Oleuropein aglycone isomer 2	nd	nd	–	693.6 ± 17.4	***	*+100*	661.7 ± 44.1	89.9 ± 3.8	1194.6 ± 78.8	*	*+10*
Nüzhenide 11-methyl oleoside isomer 4	nd	31.9 ± 3.6	31.9 ± 3.6	112.4 ± 2.5	***	*+258*	88.7 ± 4.0	nd	nd	***	*-21*
Methoxynüzhenide 11-methyl oleoside	nd	10.3 ± 2.3	10.3 ± 2.3	nd	***	*-100*	nd	nd	nd	–	–
Ligstroside aglycone isomer 2	nd	nd	–	nd	–	–	nd	nd	167.3 ± 4.4	***	*+100*
Oleuropein aglycone isomer 3	nd	nd	–	347.0 ± 3.5	***	*+100*	306.3 ± 9.2	46.2 ± 2.1	515.7 ± 40.4	ns	–
Nüzhenide di(11-methyl oleoside) isomer 1	nd	8.6 ± 2.5	8.6 ± 2.5	nd	***	*-100*	nd	nd	nd	–	–
Nüzhenide di(11-methyl oleoside) isomer 2	nd	15.0 ± 3.6	15 ± 3.6	nd	***	*-100*	nd	nd	nd	–	–
Total	*18104.9 ± 536.6*	*859.1 ± 58.3*	*18964 ± 547.9*	*9926.1 ± 321.5*	***	*-48*	*8813.7 ± 711.3*	*1097.4 ± 34.4*	*10329.3 ± 686.0*	ns	–
Σ TP[J]	19564.1 ± 577.7	904.2 ± 60.6	20468.3 ± 589.5	11070.9± 346.1	***	*-46*	9835.3 ± 783.3	1208.8 ± 42.1	10835.8 ± 725.5	ns	–

[A]Expressed as mean ± SD (mg/kg fruits FW). [B]Total initial fruit content (destoned fruit + stone). [C]t = 0, paste obtained immediately after fruits crushing. [D]Impact of crushing; SignF, means separated by *t*-test (***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; ns, not significant $P \geq 0.05$); Δc, calculated rate of increase/decrease with respect to fruits (Eqs 2, pp. 61). [E]Expressed as mean ± SD (mg/kg fruits FW × e^{-2}). [F]Impact of malaxation; SignF, means separated by *t*-test (***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; ns, not significant $P \geq 0.05$); Δc, calculated rate of increase/decrease with respect to paste (Eqs 3, pp. 61). [G]Compound is described as: (nd) not detected, (nq) not quantified due to trace amount (tr), inconsistent UV-Vis spectra (ins) or co-elution/separation problems (co). [H](–) Value could not be calculated. [I]Value includes oleuropein residual. [J]Total phenols, sum of HPLC-DAD quantified phenols.

5.2.1 Impact of crushing

The relative contribution of crushing step to a phenols reduction during olive oil processing has not been yet quantified in the existing literature. Similarly, a little scientific information is available on its role in their transformation process.

A direct comparison of olive fruits initial phenolic composition (de-stoned fruit + stone) with the corresponding paste obtained directly after crushing (t = 0) is the first approach to a such estimation. Looking quantitatively, a significant TP drop was observed immediately after crushing (46%) confirming the previous postulation of being the most critical step in the overall process (Servili et al., 2004). While the content of simple phenols and benzoic acids has increased, the yields of other classes declined or were not involved/affected. Although brief, this technological operation has also induced several transformation changes arriving from the mechanical mixing action, chemical and/or biochemical reactions (enzymatic and non-enzymatic hydrolyses and oxidations) as summarised by Parenti et al. (2008). As evident from results, out of fifty-one initially quantified fruit phenols, thirteen have irrevocably disappeared (mainly those from stone), but eight newly appeared in the paste immediately after milling. This suggests that its profile is a result of all, the transfer, liberation and transformation phenomena with none, partial or total hydrolysis/degradation of fruits native phenols leading to a formation of new respective derivatives. A visual comparison of de-stoned fruits', stone's and paste's phenolic profiles (**Figures 7–9**) reflects some of the main interconversion changes discussed below.

The highest decrease was observed for the main fruit secoiridoid glucosides, *i.e.* oleuropein, demethyloleuropein and ligstroside. While the content of oleuropein decreased for 94%, its demethylated form was almost completely degraded (traces of demethyloleuropein), whereas ligstroside could not be detected by DAD anymore. According to the mechanism of Servili et al. (2004) these fruit glucosides can be transformed into their respective aglycones; primarily to oleuropein or ligstroside aglycones and further to their decarboxymethylated forms, *i.e.* 3,4-DHPEA-EDA or *p*-HPEA-EDA. Indeed, both transformants of oleuropein and demethyloleuropein were found in the paste after crushing; their first interconversion product eluted as

three (oleuropein aglycone isomers), while the second as two peaks (3,4-DHPEA-EDA isomers) together accounting 35 and 12% of fruits initial oleuropein + demethyloleuropein content together. This could indicate that crushing accelerated an enzymatic degradation of both glucosides, which already began in the fruit and would likely to progress during maturation. Such an assumption is supported by the MS trace detection of oleuropein aglycones in de-stoned fruit (**Table 6**) and the fact that 3,4-DHPEA-EDA has already been found in the fruits before (Section 5.3; Ryan et al., 2003). Similarly, these transformants could also arrive from oleuropein diglucoside isomer 2, which completely diminished after milling, while the vague rise of its isomer 1 (+64%) so far constitutes unexplained and warrants a further investigation. Nevertheless, the degradation of these native secoiridoid glucosides could also yield some other oleuropein structurally correlated phenols whose yields have increased after crushing such as dihydro-oleuropein isomer 1 or others of lower MW phenols. In fact, there are many cleavage products possible upon the degradation of oleuropein, demethyloleuropein and ligstroside, of which some are illustrated in **Figure 20**.

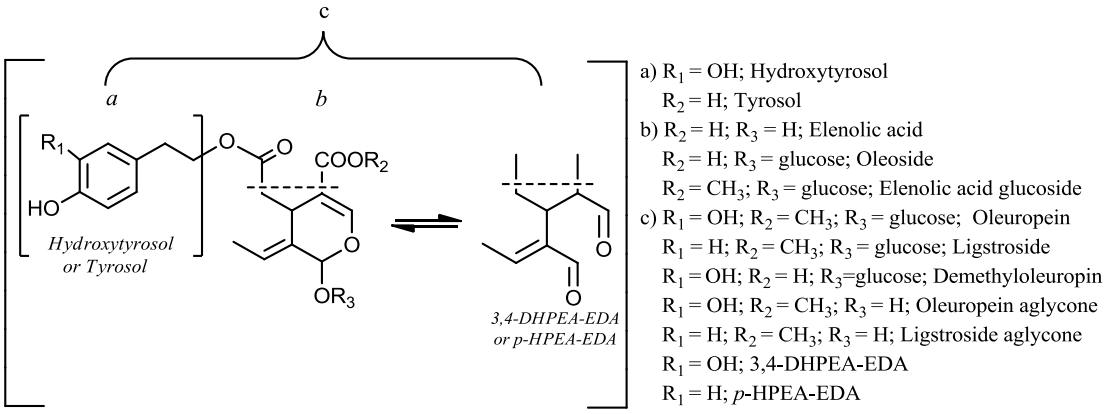


Figure 20 Possible interconversions of selected secoiridoids containing tyrosol and hydroxytyrosol in their structures

By contrast, the degradative mechanism of ligstroside was more ambiguous as none of its two potent transformants (ligstroside aglycones and *p*-HPEA-EDA) could be detected in paste by DAD, but instead could easily be assigned and quantified in the oil after malaxation/centrifugation operations were over. The relationship that quantitatively explains such vague interconversion remains unclear and may indicate that; i) ligstroside was completely transformed into both, but due to complexity of

paste's structure none of them could be detected in paste; indeed its composition immediately after fruits crushing strongly differs from that after malaxation (Herrera, 2007), or these two transformants could have formed the complexes with polysaccharides released only after malaxation (Vierhuis et al., 2001), or ii) ligstroside was completely oxidised and/or transformed into other products, while its respected aglycones found in oil are the hydrolysis products of others, but structurally related compounds.

However, beyond the knowledge of fruits's prime secoiridoid glucosides' behaviour, the degradative mechanism of stone's main secoiridoid glucosides *i.e.* nüzheonide and its esterified forms with methoxy group and 11-methyl oleoside has not been yet established. Interestingly, only two out of ten quantified representatives were increased upon olives crushing (nüzhenide isomer 2 for 21% and nüzhenide 11-methyl oleoside isomer 4 for 258%) most likely due to improved release and/or mill-prompted hydrolysis of higher MW phenols such as nüzhenide (di)esters giving rise to their formation. All the other nüzhenide representatives have totally diminished throughout the milling operation due to either low oxidation resistance and/or crushing-induced degradation. Nevertheless, based on their chemical structures they could all yield a tyrosol glucoside, whose significant rise in paste (134%) should be marked after crushing.

Crushing also implied a rise of other simple phenols; hydroxytyrosol increased for 170% owing to degradation of hydroxytyrosol-containing compounds, while tyrosol for 107% due to the hydrolysis of tyrosol-containing phenols. This could have occurred *via* cleavage of ester bond by the action of endogenous esterases splitting secoiridoid glucosides or their aglycones (**Figure 20c**) giving rise to elenolic acid glucoside, oleoside or elenolic acid (**Figure 20b**). Until now, their release from aglycones were only proved to ensue during oil storage (Boselli et al., 2009) and never during course of its production. Alternatively, hydroxytyrosol could also be released from the fruits native hydroxytyrosol glucoside (-29%) and verbascoside (-31%) by the activity of glucosidases, analogously as tyrosol from nüzhenide and its esters. Owing to such complexity, it was not possible to assign their exact origin as no obvious increase of the corresponding cleavage compounds could be observed in the paste. Another remarkable fact is also a distinct behaviour of the two

hydroxytyrosol glucosides displaying rise (70%, hydroxytyrosol glucoside-1-β-glucoside) and a drop (29%, hydroxytyrosol glucoside) after crushing. Such discrepancy clearly indicates to their different chemical structures and for the first time experimentally supports (during course of olive oil production) what has only been postulated before (Obied et al., 2007a); hydroxytyrosol-1-β-glucoside is a hydrolysis product of verbascoside enhanced by both, the crushing and malaxation, while the formation of hydroxytyrosol glucoside appears not be technologically induced; instead it rather contributes to its degradation.

The appearance of vanillic acid in olive paste after crushing raises an issue of its origin. The fact that it was absent in both input matrices, but present in all process-derived matrices suggests that is a formation product of technological process, *i.e.* the crushing step. Possible explanations include a crushing-induced hydrolysis of lignin from olive stone once already proved to yield vanillic acid under acid steam explosion (Fernández-Bolaños et al., 1998), although there is always a possibility that a lab-milling of stone in a sample preparation was not efficient as the mill crushers of Abencor system, hampering its expected detection in this fruit compartment. Interestingly, its quantitative yield was further unaffected by the malaxing conditions, which seems to only facilitated its full distribution among the final products. By contrast, vanillin was detected in olive stone, but not in the corresponding olive paste, which could be attributed to a brief duration of crushing step, suffering less tissue damage and hence a limited release immediately after it. The subsequent malaxation apparently induced its extractability, which allowed its detection in olive oil, though in a much lower amount with respect to its original content in stone (3%).

Among cinnamic acids, only verbascoside has diminished after fruits crushing (-31%), whereas all of its derivatives have considerably increased (> 150%). It is of interest to add that their accumulation has further proceeded also during malaxation, thus it is very likely that verbascoside derivatives had originated from any of the fruits' native unknown compound(s) hydrolysed upon crushing and malaxation. Moreover, a high level of these phenols found further in other Istrska belica *cv.* fruits (Section 5.3) suggests that the technology has only hasten the biosynthetic

interconversion, which would likely occur also at the fruit level, however, such a hypothesis undoubtedly warrants a further investigation, and is beyond the scope of present thesis.

Cruhing also contributed to a loss of glycosylated flavonoids with an exception of luteolin 3'-*O*-glucoside, which seemed to be resistant to any or all of enzymatic and/or non-enzymatic degradations, transformations or oxidations. The appearance of two new flavonoidal aglycones in olive paste, *i.e.* luteolin and apigenin, indicates that some of them were hydrolysed by the endogenous glucosidases, released and/or activated upon crushing, but a firm relationship could only be established for apigenin whose formation in olive paste was collaborated with a drop of apigenin-7-*O*-glucoside. Luteolin, on the other hand, may have arisen from any or all of the six luteolin glycosides, rutin and/or quercitrin.

In spite of several quali- and quantitative induced changes by crushing, a small portion of phenols remained unaffected, including luteolin-3'-*O*-glucoside comselogoside and caffeoyl-6'-secologanoside. A remarkable fact is that all of their yields have further remained constant also during malaxation, indicating to their high technological resistance, which could have a wide potential in a forward design of TP enriched food products.

5.2.2 Impact of malaxation

Although phenols yield behaviour during course of malaxation has not been separately monitored, their levels in the output matrices yet permitted a reliable insight into their further transformation/partition trail from paste to the final products, especially if considered that the time of paste's span in the centrifuge is too short to allow important modifications happen.

In general, the behaviour of phenols continued the trend initiated by crushing, though quantitatively in a lower extend; a positive yield rises kept an increasing trend due to improved releases and/or transformative reactions, while the yield losses were followed by a further drops owing to enzymatic and/or non-enzymatic hydrolyses and oxidations. However, there were also few exceptions; for example luteolin rutinoside isomer 1 showed a rise after initial drop, while some of the potent

transformants have decreased, *i.e.* elenolic acid glucoside isomer 1, 3,4-DHPEA-EDA isomer 1, nüzhenide isomer 2, oleuropein aglycone isomer 1 and nüzhenide 11-methyl oleoside isomer 4. This could indicate that the hydrolysis of their parent compounds has finished and their oxidative degradation prevailed due to operative conditions and/or enzymatic activities. By contrast, some other potent transformants (dihydro-oleuropein isomer 1 and oleuropein aglycone isomer 3) remained rather unaffected, similarly as a great fraction of flavonoidal representatives. Moreover, the evolution of some new phenols could be evidenced in the final products; both representatives of lignans and some of secoiridoids (all unknown 484 MW isomers, *p*-HPEA-EDA, ligstroside aglycones and acetal of 3,4-DHPEA-EDA) have appeared in the oil or in the wastewater, while not in the pomace, though some traces could still be detected by MS as well (**Table 6**). It is thus possible to deduce that only malaxation conditions were efficient enough to induce their formation and/or release due to prolonged mixing and/or enzymatic actions. However, there is always a possibility that the extraction method for these compounds was not equally efficient for all matrices owing to structural specifies, requiring other approaches as previously demonstrated for lignans in fruits (López et al., 2008).

Looking from a TP yield perspective, no losses could be observed during malaxation at 30 min/25 °C conditions (control), indicating to a balanced degradation/formation equilibrium of phenolic compounds quantified. In other words, the available pool of phenols detected in olive paste after crushing could also be quantified at the end of process *via* TP sum of output products (~54%). However, as evident further from our results, only 0.53% of the available fruit phenols have ended-up in olive oil and nearly 6% in the wastewater, while others have remained entrapped in the solid (48.12%, **Figure 21**). Such partition rate distribution is rather different from that of industrial-scale results (0.3–1.2% oil; 38.2–46.2% wastewater; 4.5–47.4% pomace; Jerman Klen et al., 2012a), but not surprising as the latter is govern by the quantity of final products formed. A high retention of phenols in pomace further suggests that the process conditions applied (30 min/25 °C) has not fully induced their transfer to liquid phases, yet constituting a challenge for some potent improvements. Our results also confirmed that a major proportion of fruit phenols is indeed lost with wastes, but some also owing to their technological

destruction. This, however, is not in full agreement with the only available results found in the literature, cited by Rodis et al. (2002) and several others (Takac et al., 2009; Obied et al., 2005 *etc.*), showing no quantitative TP losses during entire olive oil processing; but rather their unsuited yield distribution among the final products, *i.e.* 1–2% oil, 53% wastewater and 45% pomace. However, it is rather uncommon that the technology would quantitatively yield all the phenols confined in the initial fruit material (100% TP pool), especially not due to a well-known phenomenon of their oxidative catabolism (Servili et al., 2012; Servili et al., 2004).

As further evident from results, the majority of phenols originated from the pulp and/or peel, together accounting 95% of fruits initial TP content, while stone confined only a minor fraction (~5%). Secoiridoids were the predominant class in both input matrices and maintained its prominent role also in the process-derived matrices, though represented by different individuals. With an exception of olive stone, the class distribution in solid matrices decreased in the order; secoiridoids > flavonoids > cinnamic acids > simple phenols. By contrast, in the wastewater, simple phenols constituted the second largest class evidencing their hydrophilic nature, analogously as lignans in olive oil, showing high tendency for this oily matrix. Interestingly, none of the fruit flavonoid glycosides were transferred to the oil above their trace amounts, but instead were largely occluded in both of waste matrices. Similarly, all cinnamic acids of fruits were lost with the by-products, though one has scarcely been transferred to the oil as well, *i.e. p*-coumaric acid. The partition behaviour of secoiridoids was rather similar to flavonoids, where apart from the two known glucosides (secologanoside and elenolic acid glucoside isomer 2), only aglycones were partitioned to the oil, while the other glucosides have ended-up in the wastes (*e.g.* comselogoside and caffeoyl-6'-secologanoside). Such partitioning model is quite similar to a previously reported at industrial-scale level using different starting fruit material (Jerman Klen et al., 2012a), which indicates its rather common pattern.

5.2.3 Comparison of olive oil processing trials

In the next step of doctoral study, the potential of different technological variables was explored to improve the low TP transfer rates from fruits to oil observed in the

control trial, and to reduce their lost with wastes. Thirteen olive oil processing trials (control and twelve others), differing only in the malaxing operative conditions, were hence compared in terms of the final products' characteristics, *i.e.* TP concentration, quantitative extraction yield and phenols partition yields. Each trial had at least two inputs (paste and malaxing water) and three outputs (oil, pomace and wastewater), of which all were carefully measured to obtain a mass and water balance approach (**Table 11**). The malaxing water added to each trial accounted around 14% of paste's FW and was needed to improve its rheology and enhance the phases separation.

Figure 21 summarises the results of all thirteen trials grouped according to the impact investigated; malaxation time/temperature, malaxation time/lukewarm water addition and co-adjuvants addition, however, only in terms of TP yields, while the partition behaviour of individual classes and of their phenol representatives is presented in **Table 12** and **Figure 22**.

Qualitatively, no major differences in the phenolic profiles of individual matrices could be evidenced between different trials, signifying that none of the technological variables has altered their transformative trail. Again, all of them shared a common pattern as already described above, which seems to be regulated by the fruit enzymes, while the magnitude of transformations by their level and operative conditions applied.

As seen from results (**Table 11**, **Figure 21**), the solid olive residue (pomace) was the major by-product produced in all the trials, accounting 69.5–88.1% of the total input material (trials N° 7 and 2). By contrast, the yields of liquid products were much lower (oil; 6.8–11.3% (N° 2 and 4), wastewater; 4.7–22.8% (N° 5 and 7)), but yet more highly affected by the technological variables studied. Moreover, their yielding index attained was much lower *vs.* that previously obtained at industrial-scale level (~20% for oil and ~57% for wastewater; Jerman Klen et al., 2012a) most likely due to a less efficient oil/wastewater separation phase in the Abencor system. This also explains a lower TP partition rates obtained for the oil when compared to industrial ones; for example 0.53% in control *vs.* 0.9% in a rather comparable traditional press, as well as for the wastewater, but a higher for pomace. A remarkable fact is also that

the final wastewater yields were almost always lower *vs*. initial water adds (malaxing or lukewarm) as already observed before (Artajo et al., 2006c) and could evidence the presence of difficult paste. Interestingly, only in the trial with talc addition (trials N° 11 and 12), these were slightly higher, which is somewhat expected as this co-adjuvant acts *via* drainage effect as described further on (Servili et al., 2012).

5.2.3.1 Impact of malaxation time and temperature

First, the impact of two malaxing times combined with three temperatures (30 and 60 min/25, 35 and 45 °C) was investigated in the olive fruit phenols transfer, transformation and partition trail study during olive oil processing.

Products' TP concentration. As evident from results (**Figure 21a)**, the temperature rise had a biphasic effect on TP concentration of the final products; while the latter increased their yields in olive oil, it has on the other hand, decreased them in both of waste matrices (though not always statistically significant). In the literature contradictive results are reported as regard to malaxing temperature effect on TP content in olive oils, demonstrating a limited rise up to 27 and 30 °C (Parenti et al., 2008; Ranalli et al., 2001) or a non-limited one up to 40 and 42 °C (Stefanoudaki et al., 2011; Inarejos-García et al., 2009). By contrast, only one report (best to our knowledge) is available on its effect on TP yield in the waste matrices, *i.e.* wet pomace, showing decrease with temperature rise (15–30 °C) and no report on all the products from a single experiment. Our results, however, showed that the TP concentration of the final products has depended on their quantities produced and their abilities for TP perception. It appears that elevated temperatures improved the phenols transfer rate from paste's solid fraction to the liquid ones, which have due to increased solubility (Rodis et al., 2002) dissolved in a higher amounts in the oily phase rather than in aqueous. It could be assumed that a paralleled decrease of paste's hydrophilic phase with temperature rise (resulting in a decreased wastewater extraction yields) has limited their transfer to the water phase. Likewise, the previous report of Obied et al. (2008b) has ascribed the TP drop in pomace to a changed phenols partition pattern govern by the quantity of products' yields. Nevertheless, the TP decreases could always be associated with increased oxidoreductases' activities known to catalyse phenols degradation (Clodoveo, 2012; Servili et al., 2012; Parenti et al., 2008).

Table 11 Mass and water balance approach as affected by processing conditions

+	>101%	51–100%	26–50%	1–25%	0	1–25%	26–50%	51–100%	>101%	–

N° Trial		*Input*					*Output*									
		Paste		**Mlx water**[E]	**Extra add**[F]	*Total*	**Oil**			**Pomace**			**Wastewater**			*Total*
		mass (g)	Σ TP[A]	mass (g)	mass (g)	mass (g)	mass (g)	%[G]	Σ TP[B,G]	mass[C] (g)	%[G]	Σ TP[A,G]	mass (g)	%[G]	Σ TP[A,G]	%
Mass balance in process																
0	30 min/25 °C (Cnt)	700.7 ± 0.1	31.3	100.6 ± 0.3	0.0 ± 0.0	*801.3 ± 0.2*	71.3 ± 0.4	100.0 e	1064.6 f	659.7 ± 1.3	100.0 f	41.0 d	70.3 ± 1.1	100.0 g	52.8 e	*100.0*
1	30 min/35 °C	700.2 ± 0.0	31.3	101.4 ± 0.2	0.0 ± 0.0	*801.5 ± 0.2*	81.0 ± 1.3	13.6 d	1207.4 e	674.0 ± 0.6	2.2 e	40.6 de	46.6 ± 1.7	33.8 h	38.1 g	*100.0*
2	30 min/45 °C	700.4 ± 0.3	31.3	100.6 ± 0.0	0.0 ± 0.0	*801.0 ± 0.3*	54.5 ± 0.1	23.6 g	1361.4 d	705.6 ± 4.4	7.0 c	38.7 e	41.0 ± 4.8	41.5 h	31.0 h	*100.0*
3	60 min/25 °C	700.8 ± 0.0	31.3	100.9 ± 0.5	0.0 ± 0.0	*801.7 ± 0.5*	80.3 ± 0.2	12.6 d	1150.7 e	637.4 ± 3.0	3.4 g	46.7 b	84.0 ± 2.3	19.5 f	46.5 f	*100.0*
4	60 min/35 °C	702.3 ± 0.2	31.3	100.0 ± 0.0	0.0 ± 0.0	*802.3± 0.2*	90.7 ± 0.3	27.1 a	1150.0 e	628.2 ± 1.8	4.8 gh	41.8 d	83.5 ± 1.7	18.8 f	45.3 f	*100.0*
5	60 min/45 °C	700.6 ± 0.2	31.3	100.1 ± 0.0	0.0 ± 0.0	*800.7 ± 0.2*	60.3 ± 0.3	15.5 f	1446.5 c	702.5 ± 5.2	6.5 c	42.5 cd	38.0 ± 5.8	45.7 h	31.2 h	*100.0*
6	200 mL/30 min	701.0 ± 0.6	31.3	101.0 ± 0.6	200.4 ± 0.2	*1002.4 ± 1.4*	84.8 ± 0.1	18.9 c	859.7 hi	740.8 ± 4.9	12.3 b	44.1 c	176.9 ± 3.4	151.7 c	73.0 ab	*100.0*
7	300 mL/30 min	701.0 ± 0.7	31.3	101.3 ± 0.1	301.2 ± 0.5	*1103.5± 1.2*	85.1 ± 0.4	19.4 c	793.4 i	766.5 ± 0.0	16.2 a	47.0 b	251.9 ± 0.9	258.6 a	71.2 bc	*100.0*
8	200 mL/60 min	700.2 ± 0.0	31.3	101.1 ± 0.6	200.2 ± 0.1	*1001.4 ± 0.5*	92.2 ± 0.1	29.2 a	881.0 gh	745.4 ± 0.2	13.0 b	49.1 a	163.9 ± 0.4	133.4 d	74.6 a	*100.0*
9	300 mL/60 min	700.7 ± 0.4	31.3	100.5 ± 0.2	300.5 ± 0.2	*1101.6± 0.1*	88.8 ± 0.3	24.5 b	938.9 g	773.8 ± 1.7	17.3 a	46.5 b	239.1 ± 2.0	240.6 b	73.1 ab	*100.0*
10	NaCl	701.1 ± 0.0	31.3	100.8 ± 0.2	10.0 ± 0.0	*811.8 ± 0.2*	81.4 ± 0.3	14.2 d	1620.0 b	690.8 ± 0.3	4.7 d	40.9 d	39.7 ± 0.8	43.5 h	44.7 f	*100.0*
11	Talc	701.2 ± 0.3	31.3	100.4 ± 0.2	10.0 ± 0.0	*811.6 ± 0.5*	87.9 ± 0.3	23.2 b	1213.4 e	618.1 ± 0.8	6.3 hi	44.1 c	105.6 ± 1.6	50.3 e	69.1 c	*100.0*
12	NaCl + Talc	701.4 ± 0.3	31.3	101.2 ± 0.1	10.0 ± 0.0	*812.6 ± 0.4*	87.6 ± 0.1	22.8 b	1691.3 a	615.0 ± 0.6	6.8 i	47.5 ab	110.1 ± 1.0	56.8 e	55.7 d	*100.0*
SignF[D]			ns					***	***		***	***		***	***	
Water balance in process																
0	30 min/25 °C	452.7 ± 0.1		100.6 ± 0.3	0.0 ± 0.0	*553.3 ± 0.2*	0.0 ± 0.0			491.3 ± 1.9			54.2 ± 1.0			*98.6*
1	30 min/35 °C	452.4 ± 0.0		101.4 ± 0.2	0.0 ± 0.0	*553.7 ± 0.2*	0.0 ± 0.0			513.0 ± 0.6			35.7 ± 1.0			*99.1*
2	30 min/45 °C	452.5 ± 0.2		100.6 ± 0.0	0.0 ± 0.0	*553.1 ± 0.2*	0.0 ± 0.0			515.3 ± 3.3			30.4 ± 4.4			*98.6*
3	60 min/25 °C	452.8 ± 0.0		100.9 ± 0.5	0.0 ± 0.0	*553.6 ± 0.5*	0.0 ± 0.0			480.5 ± 0.8			65.1 ± 1.9			*98.5*
4	60 min/35 °C	453.7 ± 0.2		100.0 ± 0.0	0.0 ± 0.0	*553.7± 0.2*	0.0 ± 0.0			470.7 ± 0.9			69.6 ± 1.8			*97.6*
5	60 min/45 °C	452.7 ± 0.1		100.1 ± 0.0	0.0 ± 0.0	*552.7± 0.2*	0.0 ± 0.0			518.0 ± 2.7			27.4 ± 4.7			*98.7*
6	30 min/200 mL	452.9 ± 0.4		101.0 ± 0.6	200.4 ± 0.2	754.3 ± 1.2	0.0 ± 0.0			595.1 ± 1.4			149.4 ± 3.9			*98.7*
7	30 min/300 mL	452.9 ± 0.4		101.3 ± 0.1	301.2 ± 0.5	855.4 ± 1.0	0.0 ± 0.0			633.1 ± 1.1			214.0 ± 1.3			*99.0*
8	60 min/200 mL	452.4 ± 0.0		101.1 ± 0.6	200.2 ± 0.1	753.6 ± 0.5	0.0 ± 0.0			604.0 ± 0.8			137.2 ± 0.2			*98.6*
9	60 min/300 mL	452.7 ± 0.3		100.5 ± 0.2	300.5 ± 0.2	853.6 ± 0.2	0.0 ± 0.0			633.6 ± 5.0			201.8 ± 2.4			*97.9*
10	NaCl	452.9 ± 0.0		100.8 ± 0.2	0.0 ± 0.0	553.7 ± 0.2	0.0 ± 0.0			518.7 ± 1.7			28.5 ± 0.6			*98.8*
11	Talc	453.0 ± 0.2		100.4 ± 0.2	0.0 ± 0.0	553.4 ± 0.4	0.0 ± 0.0			455.8 ± 0.3			88.0 ± 1.2			*98.2*
12	Talc + NaCl	453.2 ± 0.2		101.2 ± 0.1	0.0 ± 0.0	554.4 ± 0.3	0.0 ± 0.0			458.6 ± 2.3			90.0 ± 1.1			*98.9*
SignF																ns

[A]Total phenols, sum of HPLC-DAD quantified phenols (mg/g DW). [B]Total phenols, sum of HPLC-DAD quantifed phenols (µg/g oil). [C]Calculated mass; ($m_{total\ input}$ - m_{oil} - $m_{wastewater}$). [D]Means separated by Duncan test; ns, non significant ($P \geq 0.05$); *** ($P \leq 0.001$). [E]Malaxation water add. [F]Extra lukewarm water or coadjuvant add. [G]Scale of relative increase/decrease in respect to control (Cnt).

Figure 21 Products' TP concentrations, extraction yields and TP partition rates as affected by processing conditions. Values marked with the same letter are not significantly different; small letters refer to a matrix comparison, while the capital to a products' sum comparison

Table 12 Phenol class distribution in pomace, wastewater and oil as affected by processing conditions

N° Trial		Phenol Class						*Total*
		Simple phenols	**Benzoic acids**	**Cinnamic acids**	**Flavonoids**	**Lignans**	**Secoiridoids**	
Pomace[A]								
0	30 min/25 °C (Cnt)	237.1 c	7.9 cd	307.6 cd	469.0 b	nd[D]	8813.7 de	9835.3 def
1	30 min/35 °C	220.6 e	7.3 de	246.7 g	413.8 ef	nd	8457.8 ef	9346.2 fgh
2	30 min/45 °C	218.8 e	7.9 cd	268.0 f	430.7 def	nd	9602.1 b	10527.6 bc
3	60 min/25 °C	271.8 a	9.5 a	289.3 e	537.2 a	nd	9340.5 bc	10448.3 bc
4	60 min/35 °C	240.2 c	6.9 e	246.1 g	422.5 ef	nd	8463.8 ef	9379.5 fgh
5	60 min/45 °C	258.0 b	7.9 cd	293.2 de	450.2 bcd	nd	10187.1 a	11196.2 a
6	30 min/200 mL	223.3 de	7.3 de	266.1 f	473.7 b	nd	8205.3 f	9175.7 gh
7	30 min/300 mL	197.6 g	6.9 e	256.5 fg	425.6 def	nd	8057.9 f	8944.5 h
8	60 min/200 mL	205.4 fg	8.1 bc	269.3 f	438.6 cde	nd	8717.3 de	9638.7 efg
9	60 min/300 mL	201.7 g	7.3 de	259.5 fg	421.6 ef	nd	8367.0 ef	9257.1 gh
10	NaCl	215.9 ef	8.6 b	318.1 bc	408.4 f	nd	9102.8 cd	10053.8 cde
11	Talc	233.1 cd	8.7 b	329.7 ab	464.5 b	nd	9174.7 bcd	10210.7 bcd
12	Talc + NaCl	206.0 fg	8.1 bc	335.1 a	457.4 bc	nd	9595.1 b	10601.8 b
SignF[C]			***	***	***		***	***
Wastewater[A]								
0	30 min/25 °C	50.3 ef	1.5 f	34.3 f	25.2 f	nd	1097.4 f	1208.8 f
1	30 min/35 °C	18.7 i	0.6 gh	14.3 i	13.1 hi	nd	541.3 i	588.0 i
2	30 min/45 °C	17.4 ij	0.5 gh	11.5 j	12.1 i	nd	426.0 j	467.6 j
3	60 min/25 °C	48.4 f	1.5 f	33.0 f	27.9 e	nd	1145.3 f	1256.2 f
4	60 min/35 °C	31.5 g	0.7 g	22.8 g	21.2 g	nd	821.1 g	897.3 g
5	60 min/45 °C	15.2 j	0.4 h	11.5 j	12.3 i	nd	434.1 j	473.6 j
6	30 min/200 mL	86.7 d	5.9 a	76.9 d	57.9 c	nd	2635.3 c	2862.6 c
7	30 min/300 mL	110.6 b	3.1 c	101.1 b	80.4 a	nd	3556.0 a	3851.3 a
8	60 min/200 mL	93.9 c	3.0 c	81.4 c	63.3 b	nd	2841.4 b	3083 b
9	60 min/300 mL	122.1 a	4.2 b	106.2 2 a	79.5 a	nd	3584.9 a	3896.8 a
10	NaCl	26.3 h	0.7 g	20.5 h	14.7 h	nd	647.5 h	709.7 h
11	Talc	52.9 e	2.6 d	51.5 e	33.0 d	nd	1598.4 d	1738.4 d
12	Talc + NaCl	53.4 e	1.8 e	50.6 e	32.2 d	nd	1451.9 e	1590.0 e
SignF			***	***	***		***	***
Oil[B]								
0	30 min/25 °C	85.2 de	11.1 e	5.5 d	68.7 d	336.0 d	10329.3 g	10835.8 hi
1	30 min/35 °C	110.3 c	12.3 d	4.6 efg	31.9 f	389.4 c	13366.0 d	13914.3 d
2	30 min/45 °C	74.5 ef	9.5 f	3.1 j	12.0 h	284.2 fg	10202.7 g	10586.1 i
3	60 min/25 °C	82.2 de	12.5 d	4.8 ef	69.4 d	404.3 c	12609.3 de	13182.5 de
4	60 min/35 °C	93.6 d	13 1 d	4.8 e	84.6 b	434.9 b	14225.7 c	14856.6 c
5	60 min/45 °C	114.2 bc	14.1 c	3.5 i	40 e	330.7 de	11943.4 ef	12445.8 ef
6	30 min/200 mL	66.8 f	8.4 g	4.1 h	31.3 f	301.0 fg	9981.4 gh	10393.0 ij
7	30 min/300 mL	64.0 f	8.0 g	3.8 i	26.7 g	281.4 g	9254.2 h	9638.0 j
8	60 min/200 mL	73.1 ef	9.9 f	4.4 gh	29.2 fg	307.9 ef	11169.1 f	11593.6 gh
9	60 min/300 mL	75.9 ef	10.1 f	4.5 fg	39.9 e	327.5 de	11436.2 f	11894.2 fg
10	NaCl	125.6 b	17.7 b	7.6 b	76.3 c	444.2 b	18147.8 b	18819.2 b
11	Talc	85.7 de	12.2 d	6.3 c	108.5 a	404.9 c	14585.7 c	15203.5 c
12	Talc + NaCl	139.1 a	19.6 3 a	8.4 a	77.9 c	484.7 a	20396.2 a	21125.8 a
SignF			***	***	***	***	***	***

[A]Expressed in mg/kg fruits FW. [B]Expressed in mg/kg fruits FW × e^{-2}. [C]**M**eans separated by Duncan test;*** ($P \leq 0.001$), values marked with the same letter are not significantly different. [D]Not detected. [E]Scale of relative increase/decrease in respect to control (Cnt).

+	>101%	51–100%	26–50%	1–25%	0	1–25%	26–50%	51–100%	>101%	–

Phenolic compound / N° trial	a) Pomace (mg/kg fruits FW)												
	0	1	2	3	4	5	6	7	8	9	10	11	12
Hydroxytyrosol glucoside	34.9 b	b	b	b	b	a	c	c	c	c	b	b	b
Hydroxytyrosol-1-*β*-glucoside	54.5 c	cd	cd	a	b	b	b	d	e	cd	f	cd	g
Hydroxytyrosol	86.7 b	de	f	a	cd	bc	de	f	ef	f	cd	b	de
Tyrosol glucoside	38.3 d	cd	c	a	b	a	cd	e	e	e	e	d	e
Tyrosol	22.7 ab	de	d	a	d	ab	d	e	d	e	bc	c	c
Hydroxytyrosol acetate													
Vanillic acid	7.9 cd	de	cd	a	e	cd	de	e	bc	de	b	b	bc
Vanillin													
p-Coumaric acid	6.4 ab	ef	d	ab	f	c	def	ef	de	def	bc	a	b
β-OH verbascoside isomer 1	21.7 c	ef	d	b	c	a	de	ef	ef	ef	def	c	f
β-OH verbascoside isomer 2	20.3 c	d	c	b	b	a	de	e	de	de	d	c	de
β-OH-methyl verbascoside isomer 1	26.7 b	fg	gh	a	d	c	e	gh	gh	h	ef	b	ef
β-OH-methyl verbascoside isomer 2	30.2 b	g	fg	a	cd	c	de	g	g	g	ef	b	ef
Verbascoside	202.4 c	f	de	ef	g	de	de	de	d	de	b	b	a
Rutin	119.1 bc	de	f	a	f	g	ab	de	d	e	e	c	e
Luteolin-7-*O*-glucoside	28.5 h	gh	gh	g	f	f	e	d	cd	bcd	bc	b	a
Luteolin rutinoside isomer 1	30.2 cde	g	cde	b	cde	a	cde	fg	def	efg	cde	c	cd
Luteolin rutinoside isomer 2	11.5 ab	de	bc	a	cd	a	e	e	de	de	bc	a	a
Quercitrin	27.4 b	fg	fgh	a	gh	h	b	cd	c	de	ef	b	cd
Apigenin-7-*O*-glucoside	34.3 cde	def	b	ab	cd	a	cd	ef	cdef	ef	f	c	cd
Luteolin-4'-*O*-glucoside	24.7 fg	de	c	a	cd	cd	b	de	ef	ef	h	g	g
Luteolin-3'-*O*-glucoside	27.7 bc	def	b	a	cd	b	d	f	d	ef	de	b	b
Luteolin	133.5 b	e	de	a	cd	b	cd	cd	bc	cd	cd	b	a
Apigenin	28.0 b	d	bc	a	bc	a	bc	c	bc	cd	cd	b	a
Pinoresinol													
Acetoxypinoresinol													
Unknown A	8.9 bc	ef	bc	b	de	a	f	g	ef	g	b	bc	cd
Unknown 408 MW compound 1	1.7 de	f	bcd	b	ef	a	g	hi	gh	i	bc	de	cde
Unknown 408 MW compound 2	2.9 bcde	defg	b	bc	defg	a	g	h	fg	h	bcd	cdef	efg
Unknown B	261.9 cd	def	bc	b	de	a	ef	g	f	g	de	de	ef
Secologanoside	56.1 cd	bc	bc	a	b	d	bc	e	e	e	e	c	e
Elenolic acid glucoside isomer 2													
Dihydro-oleuropein isomer 1	189.9 b	e	c	b	cd	a	cd	de	cd	e	bc	b	c
3,4-DHPEA-EDA isomer 1	118.9 e	bcd	a	ab	ab	abc	de	de	cd	de	de	ab	e
Dihydro-oleuropein isomer 2	105.3 e	c	d	a	e	b	c	d	d	f	e	e	e
Unknown 484 MW isomer 1													
Unknown 484 MW isomer 2													
Unknown 484 MW isomer 3													
Nüzhenide isomer 2	143.8 ef	bc	b	a	b	b	b	de	c	f	d	d	ef
3,4-DHPEA-EDA isomer 2	3039.4 h	gh	b	fgh	c	a	i	gh	def	cde	fg	efg	cd
Oleuropein aglycone isomer 1	3406.7 b	de	f	b	g	h	c	e	cd	e	b	b	a
Oleuropein	377.6 ab	cd	a	de	fg	bc	abc	hi	gh	i	ef	efg	fgh
Caffeoyl-6'-secologanoside	13.3 cd	f	de	ab	f	a	ef	f	de	f	cd	abc	bcd
p-HPEA-EDA													
Ligstroside + Oleuropein aglycone sum													
Comselogoside	30.7 bc	g	d	c	fg	c	fg	g	de	ef	c	a	ab
Acetal of 3,4-DHPEA-EDA													
Oleuropein aglycone isomer 2	661.7 f	ef	b	c	de	a	gh	gh	fg	h	cd	cd	c
Nüzhenide 11-methyl oleoside isomer 4	88.7 g	b	a	a	c	b	d	f	e	h	g	de	de
Ligstroside aglycone isomer 2													
Oleuropein aglycone isomer 3	306.3 d	gh	ef	a	de	bc	fg	h	fg	h	de	cd	b

Phenolic compound / N° trial	b) Wastewater (mg/kg fruits FW)												
	0	1	2	3	4	5	6	7	8	9	10	11	12
Hydroxytyrosol glucoside	7.1 e	h	i	e	f	i	c	a	c	b	g	d	d
Hydroxytyrosol-1-*β*-glucoside	5.5 e	h	h	e	f	h	c	b	c	a	g	d	e
Hydroxytyrosol	21.6 f	h	hi	f	g	i	d	b	c	a	g	f	e
Tyrosol glucoside	10.9 e	j	j	fg	h	j	d	b	c	a	i	f	g
Tyrosol	5.2 e	h	h	f	g	h	d	b	c	a	g	e	e
Hydroxytyrosol acetate													
Vanillic acid	1.5 f	gh	gh	f	g	h	a	c	c	b	g	d	e
Vanillin													
p-Coumaric acid	0.8 e	h	h	f	g	h	c	a	b	a	g	d	d
β-OH verbascoside isomer 1	4.7 d	h	h	e	f	h	b	a	c	a	g	d	e
β-OH verbascoside isomer 2	4.6 d	h	h	e	f	h	b	a	c	a	g	d	e
β-OH-methyl verbascoside isomer 1	1.8 d	g	g	d	e	g	b	a	b	a	f	c	d
β-OH-methyl verbascoside isomer 2	2.0 d	i	i	f	g	i	c	b	c	a	h	d	e
Verbascoside	20.3 g	i	j	g	h	j	d	b	c	a	h	f	e
Rutin	3.5 f	g	g	e	f	g	d	b	c	a	g	e	e
Luteolin-7-*O*-glucoside	1.7 gh	g	g	e	e	h	c	a	b	a	i	f	d
Luteolin rutinoside isomer 1	4.3 f	j	j	g	h	j	c	a	b	a	i	d	e
Luteolin rutinoside isomer 2	1.4 h	j	k	g	i	k	d	b	c	a	j	e	f
Quercitrin	1.2 g	j	j	f	h	j	d	b	c	a	i	e	e
Apigenin-7-*O*-glucoside	4.1 g	j	j	g	h	j	d	a	c	b	i	e	f
Luteolin-4'-*O*-glucoside													
Luteolin-3'-*O*-glucoside	2.3 h	jk	k	g	i	k	d	a	c	b	j	e	f
Luteolin	5.1 e	h	h	e	f	h	c	a	b	a	g	d	d
Apigenin	1.8 e	g	g	de	f	g	c	a	c	b	g	e	d
Pinoresinol													
Acetoxypinoresinol													
Unknown A	2.1 e	h	h	e	f	h	c	a	b	a	g	d	d
Unknown 408 MW compound 1	0.3 f	i	j	f	g	j	c	a	c	b	h	d	e
Unknown 408 MW compound 2	0.6 f	i	i	f	g	i	c	a	c	b	h	d	e
Unknown B	54.3 f	i	i	f	g	i	c	a	b	a	h	d	e
Secologanoside	12.1 c	g	gh	e	f	h	c	a	b	b	g	d	d
Elenolic acid glucoside isomer 2													
Dihydro-oleuropein isomer 1	21.1 f	j	k	g	hi	k	c	a	d	b	i	e	h
3,4-DHPEA-EDA isomer 1													
Dihydro-oleuropein isomer 2													
Unknown 484 MW isomer 1	51.3 g	ij	i	f	h	j	e	c	b	a	h	f	d
Unknown 484 MW isomer 2	38.9 e	i	ij	g	g	i	c	a	d	b	i	f	h
Unknown 484 MW isomer 3	37.1 h	j	j	f	i	k	e	c	b	a	i	g	d
Nüzhenide isomer 2	22.2 e	i	i	f	g	i	c	a	c	b	h	d	e
3,4-DHPEA-EDA isomer 2	241.6 g	i	j	g	h	j	d	b	c	a	i	e	f
Oleuropein aglycone isomer 1	331.4 f	i	j	e	h	j	c	a	c	b	g	d	d
Oleuropein													
Caffeoyl-6'-secologanoside	1.8 f	i	i	f	g	i	c	a	b	a	h	d	e
p-HPEA-EDA													
Ligstroside + Oleuropein aglycone sum													
Comselogoside	3.9 g	j	j	h	i	j	d	b	c	a	i	e	f
Acetal of 3,4-DHPEA-EDA	142.6 f	h	hi	e	ef	hi	d	a	c	b	i	ef	g
Oleuropein aglycone isomer 2	89.9 e	h	i	f	g	h	c	a	b	a	g	d	d
Nüzhenide 11-methyl oleoside isomer 4													
Ligstroside aglycone isomer 2													
Oleuropein aglycone isomer 3	46.2 g	j	j	g	h	j	d	a	c	b	i	e	f

Phenolic compound / N° trial	c) Oil (mg/kg fruits FW × 10^{-2})											
	0	1	2	3	4	5	6	7	8	9	10	11	12
Hydroxytyrosol glucoside	1.4 e	c	ef	hi	fg	a	gh	i	fg	gh	d	gh	b
Hydroxytyrosol-1-*β*-glucoside	7.5 c	b	d	d	d	a	e	e	e	e	f	f	f
Hydroxytyrosol	27.0 c	b	d	c	bc	cd	c	cd	c	bc	a	bc	a
Tyrosol glucoside	6.7 c	b	de	d	d	a	fgh	ef	h	gh	gh	gh	efg
Tyrosol	24.7 b	b	d	b	b	bc	d	d	cd	cd	a	b	a
Hydroxytyrosol acetate	17. 9 h	d	c	f	d	a	i	i	gh	fg	b	e	a
Vanillic acid	6.4 c	d	g	c	c	e	e	f	de	de	b	c	a
Vanillin	4.7 g	de	f	ef	d	c	h	h	g	g	b	f	a
p-Coumaric acid	5.5 d	efg	j	ef	e	i	h	i	gh	fg	b	c	a
β-OH verbascoside isomer 1													
β-OH verbascoside isomer 2													
β-OH-methyl verbascoside isomer 1													
β-OH-methyl verbascoside isomer 2													
Verbascoside													
Rutin													
Luteolin-7-*O*-glucoside													
Luteolin rutinoside isomer 1													
Luteolin rutinoside isomer 2													
Quercitrin													
Apigenin-7-*O*-glucoside													
Luteolin-4'-*O*-glucoside													
Luteolin-3'-*O*-glucoside													
Luteolin	37.1 d	g	i	d	c	f	gh	h	g	e	b	a	b
Apigenin	31.6 cd	g	i	c	b	e	g	h	h	f	d	a	d
Pinoresinol	69.4 e	de	h	d	c	f	fg	gh	fg	f	b	c	a
Acetoxypinoresinol	266.6 de	c	fg	c	b	d	fg	g	ef	de	b	c	a
Unknown A	0.5 d	b	e	ef	fg	a	h	i	gh	gh	d	hi	c
Unknown 408 MW compound 1	0.1 c	b	def	def	ef	a	de	def	def	d	c	f	b
Unknown 408 MW compound 2													
Unknown B													
Secologanoside	9.4 c	a	def	defg	de	b	de	fgh	h	gh	def	efgh	d
Elenolic acid glucoside isomer 2	11.4 b	b	f	c	c	a	fg	h	h	gh	e	fg	d
Dihydro-oleuropein isomer 1													
3,4-DHPEA-EDA isomer 1													
Dihydro-oleuropein isomer 2													
Unknown 484 MW isomer 1													
Unknown 484 MW isomer 2													
Unknown 484 MW isomer 3													
Nüzhenide isomer 2													
3,4-DHPEA-EDA isomer 2	1925.8 h	fg	f	f	d	c	h	h	g	g	b	e	a
Oleuropein aglycone isomer 1	3321.6 gh	d	i	gh	ef	j	e	h	fg	gh	b	c	a
Oleuropein													
Caffeoyl-6'-secologanoside													
p-HPEA-EDA	1303.6 f	e	e	d	c	c	g	g	f	e	b	c	a
Ligstroside + Oleuropein aglycone sum	1879.4 ef	c	h	f	de	i	g	g	g	g	b	cd	a
Comselogoside													
Acetal of 3,4-DHPEA-EDA													
Oleuropein aglycone isomer 2	1194.6 g	g	g	f	ef	def	de	d	d	c	c	b	a
Nüzhenide 11-methyl oleoside isomer 4													
Ligstroside aglycone isomer 2													
Oleuropein aglycone isomer 3	167.3 d	d	fg	d	c	e	fg	g	d	ef	b	c	a
	515.7 de	c	ef	b	a	b	g	g	g	f	f	f	cd

Figure 22 Phenols individual yield distribution in the final products as affected by processing conditions. Values marked with the same letter are not significantly different. The number of trial and legend refer to the **Table 11/12**

As regards to malaxing time, a positive TP yield relationship could be evidenced for oil and pomace matrices, whereas its effect on wastewater was less consistent. It appears that prolonged malaxations have in addition released the entrapped phenols from olive paste, of which some have partitioned between the oil and water fractions, according to their hydro-soluble nature, affinity, temperature and the relative proportions of these phases. However, not all of them were successfully dissolved, but have also remained kept in the solid as evident from TP concentration rise in pomace. Similar results were obtained earlier by Obied et al. (2008b), demonstrating a significant TP rise in pomace at longer malaxations (60 *vs*. 30 min), while in a study of Di Giovacchino et al. (2002b), the TP yields of wastewater showed a limited rise up to 45 min (). Some authors obtained such a bell-shape distribution also for the oil matrix (Youssef et al., 2013; Di Giovacchino et al., 2002b), while others reported only a positive (Inarejos-García et al., 2009) or a negative TP yielding trend with malaxing time increase (Stefanoudaki et al., 2011; Ranalli et al., 2003; Angerosa et al., 2001).

Products' extraction yields. The curve of olive oil extraction yields as a function of temperature rise (25–45 °C) showed a biphasic behaviour according to a previous study of Kalua et al. (2006). Its extractability increased when the temperature rose from 25 to 35 °C (~13%) followed by a marked drop (up to 23%) at 45 °C. While the initial rise could be attributed to a reduced viscosity of the oily phase favouring coalescence and extraction from olive paste (Ranalli et al., 2001; Fang et al., 1989), the subsequent decline may be ascribed to a paste´s rheology change and increased interactions between the lipids, proteins and carbohydrates, which culminates in the entrapment of oil in olive paste (Kalua et al., 2006). In fact, increasing temperatures up to 40 °C has already been proved to promote oils' extraction yields on account of its reduced lost with wastes (mainly pomace and slightly wastewater), especially when dealing with difficult pastes (Aguilera et al., 2010; Di Giovacchino et al., 2002b). It appears that temperatures above 35 °C has re-established an emulsion state in olive paste, which additionally entrapped the portion of "free oil" as released olive oil yields at 45 °C were lower *vs*. control (up to 26%). On the other hand, the yields of wastewater were consistently decreased upon increasing temperature though not always in a significant manner. It seems that higher temperatures have raised the

water holding capacity of paste, decreasing availability of free water released after centrifugation.

A time rise (30–60 min), on the other hand, improved the extractability of olive oils at all temperatures investigated (10.6–12.6%) presumably due to advanced degradation of oil-containing cells derived from the activity of endogenous enzymes and/or mechanical mixing actions (Clodoveo, 2012; Inarejos-Garcia, 2009). Such favoured release was previously associated with the incidence of difficult paste (Aguilera et al., 2010; Di Giovacchino et al., 2002b) and could support its presence in our study too. This could furter explain an enhanced extractability of wastewater at longer malaxations (up to 79%) as the vegetative water has been parallelly released with the oil fraction from oil/water emulsion until re-emulsification occurred again at 45 °C, respectively.

TP partition rate. The curve of TP partition rate to olive oil as a function of temperature rise had a bell-shape distribution indicating that only malaxations at 35 °C improved their partition to olive oil (28%) owing to a simultaneous rise of TP and extraction yields. Though at 45 °C the TP concentration in olive oil still kept an increasing trend, the proportion of phenols resulting in oily phase has decreased due to a lower physical extractability of oil, reaching TP yields comparable to control (~0.53%). Conversely, the TP retention of pomace displayed an opposite behaviour, whereas the TP partition to wastewater has gradually decreased with each temperature rise. Interestingly, on a total quantitative level both temperatures of 25 and 45 °C presented a comparable TP pool gained at the end of process (~54 and ~58% at 30 and 60 min malaxations), while at 35 °C the latter was markedly decreased (~49 and 51% at 30 and 60 min malaxations). This abrupt could be explained with a phenols degradation/formation equilibrium altered by the temperatures of malaxation. As evident from pomace's phenolic composition (**Table 12**), all phenolic classes have declined with temperature rise presumably due to advanced oxidative degradations, except that of secoiridoids, which dropped in the range of 25–35 °C, but further increased reaching maximal levels at 45 °C. This could be ascribed to a progressive hydrolysis of major secoiridoids giving rise to a lower MW phenols considered as transformants (*e.g.* isomers of 3,4-DHPEA-EDA,

Figure 22a). It appears that malaxation at 45 °C increased these transformative reactions to a level that over-dominated an advanced oxidative degradations, while malaxation at 35 °C mainly affected those whose presence did not directly depend on enzymatic rate (*i.e.* non-transformants), displaying high degradation/formation equilibrium and hence a greater TP loss *vs.* control.

On the other side, a higher TP transfer rates from fruits to the final products were observed at longer malaxations as evident from a higher TP pool attained at the end of process *vs.* control (~58 *vs.* ~55% or 51 *vs.* ~49%). This suggests that Istrska belica *cv*. paste requires longer malaxations to facilitate additional TP release from its difficult fraction, obviously not detected in olive paste immediately after crushing. While the TP transfer rates to olive oil were always higher at longer malaxations, the TP yields in the corresponding waste matrices were not always statistically increased. Interestingly, none of the extra released TP pool has remained occluded in the solid when malaxation took place at 35 °C, but instead was fully distributed between the oil and water phase, while at 25 and 45 °C its major portion remained kept in the pomace, and only a minor was further partitioned to the oily phase.

Phenol classes and their representatives. Not all the classes responded equally to a malaxation time/temperature change (**Table 12**). Simple phenols were of main interest in our study due to their recognised antioxidant activities and hence of great urge to improve their partition to olive oil. As seen from results, their transference to olive oil was only slightly improved at 60 min/35 °C malaxation conditions (~10%), and greatly at 30 min/35 °C (~29%) and 60 min/45 °C (~34%). All other time/temperature combinations distinct from the control trial have decreased their loss with wastes, except longer malaxations raised their occlusion in the pomace. At the level of individual phenols (**Figure 22a–c**), the picture was more complicated, especially in olive oil matrix, where no unique response of their representatives could be observed; instead, their behaviour most likely depended on their individual characteristics such as solubility, thermal and oxidative stability. However, all phenolic classes displayed a lower partition to wastewater with the temperature rise, often paralleled with a lower occlusion in the pomace. This could point to their

rather low oxidative resistance and/or increased thermal degradation, giving rise to some of the transformants whose increased levels should be marked in the final products, *i.e.* in pomace (*e.g.* isomers of 3,4-DHPEA-EDA, nüzhenide 2, oleuropein aglycone isomer 2 and nüzhenide 11-methyl oleoside isomer 4), and in olive oil (*e.g.* 3,4-DHPEA-EDA isomer 2, *p*-HPEA-EDA and oleuropein aglycone isomer 2).

On the other hand, a time rise has favoured the partition to wastewater for all classes and most of their representatives, however, only at 35 °C, while at 25 °C in spite of increased physical extractability of wastewater; the latter remained occluded in the pomace. In fact, the majority of supposedly released phenols from difficult fraction as a result of prolonged malaxations have remained in the solid as evident by their increased levels in the pomace. Only few exceptions showed a decrease upon increasing malaxation time. For example, a drop of verbascoside could be ascribed to a prolonged enzymatic hydrolysis, yielding hydroxytyrosol-1-β -glucoside (already discussed in Section 5.2.1) whose yield was always higher at longer malaxation. Likewise, the lower levels of oleuropein at 60 min malaxations could be ascribed to a prolonged enzymatic actions, which could theoretically give rise to a number of compounds increased in any of the final products (*e.g. p*-HPEA-EDA in olive oil).

All together, these results confirmed that both parameters, *i.e.* malaxation time and temperature, have altered physical extractability of the final products as well as affected the phenols transformative and partition behaviour observable through all classes and most of their representatives. At this point, it may be concluded that 60 min/35 °C malaxation conditions offer the best compromise for Istrska belica *cv.* fruits olive oil processing in terms of extraction yields and TP quality. These conditions provided the maximal olive oil extraction yields with rather high TP concentration (1150.0 μg/g, **Table 11**) and the most efficient process in terms of TP transference from fruits to oil (0.73%). Moreover, it also offered the lowest wastes' production and related TP lost, but still of high TP concentration quality interesting for further utilisation (TP in pomace; 41.8 mg/g DW, TP in wastewater 45.3 mg/g DW, **Table 11**).

5.2.3.2 Impact of lukewarm water addition

Secondly, the impacts of two lukewarm water additions (200 and 300 mL) in a combination with two malaxing times (30 and 60 min) were assessed in the olive fruit phenols fate study during olive oil processing.

Products' TP concentration. All lukewarm water paste dilutions decreased the TP concentration in olive oils, where addition of 200 mL resulted in ~19% and ~23% TP drops in comparison to control malaxations at 30 and 60 min, while higher paste dilutions (300 mL) have not triggered any further reductions (**Figure 21b**). By contrast, the TP concentration in the corresponding wastes has markedly increased after lukewarm water additions; in wastewater (up to 60%) and in pomace (though not always) up to 14%. It appears that the addition of lukewarm water improved the extractability of phenols from olive paste (most likely those entrapped in the oil/water emulsion), of which the majority has further dissolved in a water phase owing to its enhanced extraction yield and hydrophilic character until reaching maximum, while some also remained kept in the solid. This caused a TP reduction in olive oil, amplified *via* dilution effect due to its parallelly raised extraction yield. A similar wash outs of olive oil phenols have already been observed before (Carrapiso et al., 2013; Stefanoudaki et al., 2011; Ben-Bavid et al., 2010; Issaoui et al., 2009; Salvador et al., 2004; Di Giovacchino et al., 2002; Gimeno et al., 2002; De Stefano et al., 1999; Angerosa et al., 1996).

Products' extraction yields. Both lukewarm water additions (200 and 300 mL) brought a significant improvement of olive oil extraction yields raised between 18.9 and 29.2%, which is a major incidence of difficult paste. This also indicates that more than one fifth of the available oil fraction in Istrska belica *cv*. paste is emulsified and needs the help to facilitate its extraction or will remain lost with the wastes produced. However, no substantial oil yield rises could be evidenced with a higher paste dilutions; instead, together with longer, *i.e.* 60 min malaxations, they caused a slight yield reduction (29.2 *vs.* 24.5%), suggesting that after achieving the maximum, the prolonged mixing actions favours re-establishment of paste's emulsion state as already observed before (Clodoveo, 2012). However, on the other side, none of the two lukewarm water adds seems to be feasible from the

environmental perspective as they have all triggered significant wastewater yield production, being raised toward higher paste dilutions (133–259%).

TP partition rate. A remarkable fact is that lukewarm water adds to olive paste have always allowed a higher TP pool quantification in the final products, which substantially increased toward higher paste dilutions, *i.e.* from ~55 to ~59 and ~63% (at 30 min malaxations) and from ~58 to ~63 and ~65% (at 60 min malaxations). This suggests that lukewarm water addition has favoured an extra TP release from olive paste, most likely those entrapped in its difficult fraction liberated only after breakdown of oil/water emulsion (vida supra). At the same time, it seems that these water adds have largely flushed away the available pool of phenols as evident from a significant TP yield rise in wastewater, collaborated with TP drops in the pomace and in oil. At maximal paste dilutions, the TP lost with wastewater was more than doubled *vs.* control (19% *vs.* 6%), but decreased with the pomace (5–6%) due its lowered relative amount produced (**Table 11**). Likewise, the final proportion of phenols resulting in the oily phase has decreased, though not thoroughly due to contradictive effects of lukewarm water addition on oil's TP concentration (yield decrease) and physical extractability (yield rise). In overall, the TP partitioning behaviour was similar to that previously observed at industrial-scale level, where three commercially available extraction systems differing in the requirement for water addition were compared (Jerman Klen et al., 2012a). Likewise here, the TP drops in olive oil has decreased toward higher paste dilutions (2-phase centrifuge > traditional press > 3-phase centrifuge) paralleled by the TP drops in pomace and TP rises in wastewater. Nevertheless, a positive TP yield-malaxation time relationship could be verified again (discussed previously in Section 5.2.3.1).

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Phenol classes and their representatives. All phenolic classes experienced a large drop in olive oil after lukewarm water addition, where flavonoids were the most (up to 61%) and secoiridoids the least sharply reduced (up to 11.4%). The content of simple phenols decreased up to 23% at 30 malaxations and slightly less at 60 min, *i.e.* up to 11% (**Table 12**). Among their representatives, tyrosol glucoside was the most significantly decreased and hydroxytyrosol the least by being rather unaffected. The same behaviour could also be evidenced for other classes and their

representatives, displaying more or less substantial drops or indifference to lukewarm water adds with an exception of one phenol, *i.e.* oleuropein aglycone 2, showing consistent rise towards higher paste dilutions. By contrast to previous reports (De Stefano et al., 1999; Angerosa et al., 1996), no obvious decrease could be confirmed for *o*-diphenols, including hydroxytyrosol as previously observed at industrial-scale level (Jerman Klen et al., 2012a), which could be ascribed to a different characteristics of industrial and lab-scale systems, in addition to a cultivar diversity as demonstrated before (Stefanoudaki et al., 2011).

On the other side, all phenols showed an important rise in the wastewater matrix, rarely accounting below 100% (**Table 12**, **Figure 22b**). The highest increase was observed for benzoic acids (up to 293%), followed by secoiridoids, cinnamic acids and flavonoids, displaying rather comparable yield upsurges (up to ~220%), and the least for simple phenols (152%). By contrast, their lost with pomace has markedly decreased, where simple phenols were the most (18% on average) and secoiridoids the least sharply reduced (8% on average). However, there were also few exceptions, which showed a rise after lukewarm water addition, but only luteolin-7-*O*-glucoside has consistently increased with higher paste dilutions most likely due to improved extractability from olive paste.

All together, no lukewarm water adds to olive paste are suggested when producing olive oils from Istrska belica *cv*. fruits due to apparent loss of process' functionally and sustainability. Though lukewarm water paste dilutions have largely improved the extractability of olive oil and phenols release from olive paste, this technological approach resulted in no appreciable changes in the final products' characteristics, instead, it increased the wastewater yield production, which largely flushed away the phenols and caused a TP drop in olive oil.

5.2.3.3 Impact of NaCl and talc addition

Finally, three different treatments involving addition of two co-adjuvants; NaCl, talc and their combination (NaCl + talc) were assessed in the last study of olive fruit phenols transfer, transformation and partition trail during olive oil processing.

Products' TP concentration. As seen from results (**Figure 21c**), all co-adjuvants increased the TP concentration in olive oil**,** where a combination of NaCl + talc was the most efficient (~58%), whereas adds of individual co-adjuvants resulted in a lower upsurges equal to 52% (NaCl) or less, 14% (talc). This is rather comparable to a previous report of Pérez et al. (2008) recording a 45% TP rise for NaCl + talc for a similar range addition, but rather lower when compared to Ben-David et al. (2010) displaying up to 1.5-fold increase for talc (though attained with a 4-times higher addition). The impact of NaCl alone on TP quality has best to our knowledge not been yet examined in any of matrices, largely ignored also in the wastes of talc addition studies. However, as evident from our results, all of them have also altered the TP concentration in olive wastes, though in a distinct manner. While NaCl alone reduced the TP concentration in the wastewater for 15% without affecting that in pomace, talc alone or combined with NaCl raised them in both of wastes, *i.e.* in wastewater for 5% (talc) or 131% (talc + NaCl) and in pomace for 7% (talc) or 16% (talc + NaCl). Such data compilation points to a different mechanism of two co-adjuvants acts. The addition of NaCl has most likely altered phenols partition equilibrium between the oily and aqueous phase as the TP rise in olive oil was in accord with the TP reduction in wastewater. This has already been hypothesized before by Pérez et al. (2008), but experimentally not proved due to restricted analysis of oil sample alone. As explained, the presence of NaCl in olive paste increases the density and ionic strength of an aqueous phase, which further reduces the solubility of phenols in water and hence increases their partition to oil. On the other hand, talc is known as emulsifier breaker acting *via* drainage effect (Servili et al., 2012; Artajo et al., 2006b), whose addition apparently improved the release of entrapped phenols from difficult fraction, of which the majority has further partitioned to the liquids, while some also remained entrapped in the solid, adding on to a TP rise in pomace.

Products' extraction yields. As expected, the addition of co-adjuvants altered the extraction yield behaviour of final products. The physical extractability of olive oil was always significantly higher *vs.* control; talc was more efficient than NaCl (23% *vs.* 14%), which is in line with earlier report of Cruz et al. (2007), but no synergism between both of them could be confirmed by contrast to Pérez et al. (2008). Salts are known to act as demulsifiers when in the ionized form, making extraction easier and efficacious. While the mechanism of NaCl emulsion breakage is based on a repulsion

between the oily and hydrophilic phase (Chumsantea et al., 2012), the mechanism of talc is based on a drainage effect (Servili et al., 2012). Such difference also explains a distinct yield behaviour of the corresponding waste matrices after talc or NaCl additions. Talc apparently absorbed the part of aqueous phase in olive paste, which helped to break-down the oil/water emulsion and released the entrapped fractions of both – the oil and wastewater after centrifugation. On the other hand, the addition of NaCl to olive paste has seemingly increased its water-holding capacity that consequently discharged a nearly half-lower wastewater yields in comparison to control. Such liquid waste reduction could be of great commercial interest, especially as NaCl is much cheaper than talc, is recognized as GRAS and yet considerably improves the extractability of olive oil from olive paste.

TP partition rate. The highest TP transfer rate from fruits to oil was achieved by NaCl + talc addition (1.03%), followed by NaCl (0.92%) and talc alone (0.74%), corresponding to 94, 74 and 40% increases of process' efficiency *vs.* control. However, only NaCl has not additionally raised the TP yields in waste matrices; instead, it reduced their partition to wastewater for nearly 41% supposedly mainly on account of increased transference to olive oil as the TP pool quantified at the end of process and occlusion in pomace was comparable to the control (~55% and ~49%). By contrast, it may be reasonable suggested that addition of talc has favoured an additional release of phenols from olive paste, most likely those entrapped in the difficult fraction as already hypothesized before in the cases of lukewarm water adds and longer malaxations. This, again, is supported by the fact that the TP pool quantified at the end of process was always higher *vs.* non-talc additions, *i.e.* ~59% *vs.* 55% (in case of talc) and ~61% *vs.* 55% (in case of NaCl + talc).

Phenol classes and their representatives. A remarkable fact is that addition of NaCl improved the partition of simple phenols to olive oil for 47%, while talc alone did not affect their transference, but it synergistically helped the action of NaCl (~63%). Interestingly, this rise was in accord with the drop in pomace, suggesting that NaCl favoured their transference from solid to oily phase during paste malaxation (**Table 12**). However, not all of their representatives responded equally; talc slightly raised the content of hydroxytyrosol and its acetate form, while NaCl in

addition of hydroxytyrosol glucoside and tyrosol, but always in a much marked manner. Their synergistic action could also be evidenced for the two phenols, *i.e.* hydroxytyrosol glucoside and hydroxytyrosol acetate (**Figure 22c**). Other classes and their individuals showed a rather similar behaviour, where partition efficiencies to olive oil decreased in the order; NaCl + talc > NaCl > talc, with an exception of flavonoids (luteolin and apigenin) and one secoiridoid representative (oleuropein aglycone isomer 2) being raised by talc more than by NaCl. In fact, this common salt improved the partition to olive oil of almost all phenols with few minor exceptions, whose levels have significantly decreased after its addition; hydroxytyrsol-1-*β*-glucoside, tyrosol glucoside, apigenin, secologanoside, elenolic acid glucoside 2 and oleuropein aglycone isomer 3. In the case of talc, additional drop of hydroxytyrosol glucoside and of unknown A could be observed in olive oil. However, for most of phenols the addition of NaCl + talc was the most efficient, but especially significant for *o*-diphenols, *i.e.* hydroxytyrosol and its derivatives, according to a previous report (Pérez et al., 2008).

On the other hand, NaCl decreased the phenols partition rates to wastewater, often paralleled with a decrease in pomace, though some yield rises could still be evidenced, including that of 3,4-DHPEA-EDA. Its simultaneous rise in pomace and in oil could point to an increased activity of β-glucosidase after NaCl addition. By contrast, talc increased the loss of phenols with wastewater, which is in line with previous report of Artajo et al. (2006b), but in particular of vanillic acid, 3,4-DHPEA-EDA, rutin, luteolin rutinoside isomer 2 and oleuropein aglycone isomer 1 (**Figure 22b**). However, it also implied a slight rise of phenols in pomace, but statistically significant only for benzoic and cinnamic acids, suggesting that majority of released phenols have distributed between the liquid phases, mainly to the water and less to the olive oil. Interestingly, the partition behaviour of phenols after NaCl + talc addition seems to be a result of both – the talc and NaCl individual actions described above.

Among all co-adjuvant combinations, addition of NaCl alone seems to be the best compromise for Istrska belica *cv*. olive oil processing in terms of nutritional, economical and environmental benefits. This co-adjuvant improved the physical

extractability of olive oil for 14% and raised its TP concentration for 52%, which together allowed the second highest TP partition rate assessment to olive oil among all trials (0.92%). Though the latter could still be slightly improved with the help of talc (NaCl + talc, 1.03%), its high price and a parallely increased wastewater yield production is not a reasonable counterbalance, and could perhaps simply be compensated with longer paste malaxations using NaCl alone.

In summary, many indices suggested that Istrska belica *cv*. olive paste used in our lab-scale processing trial could be classified as difficult, which largely contributed to the results obtained above. A minute crushing of olive fruits did not allow a total phenolic quantification in this paste, but apparently, only those not entrapped in its oil/water emulsion known as difficult. The quantified TP drop before and after fruits crushing (46%) could hence not be related to a destructive loss of crushing step only, which, however, is still significant taking into account the TP pool quantified in the final products (49–65%). Such behaviour is rather different to that of industrial-scale trial (Jerman Klen et al., 2012a), where the TP transfer rates from fruits to paste were similar (50–60%), but did not showed any further increases during malaxation. This abrupt could be ascribed to different characteristics of olive paste and/or to a lower efficiency of hammer crushers in the Abencor system, suffering less tissue damage and hence a limited TP release immediately after milling. In addition, crushing was also associated with the highest transformative changes, facing new phenolic evolutions, which kept ensued also during malaxation.

The final amount of phenols in the output products has mainly depended on the malaxing conditions, regulating phenols degradation/formation equilibrium and hence the TP pool quantified at the end of process. This appeared to be balanced when using 30 min malaxations at 25 or 45 °C or with NaCl addition, allowing the same TP yield quantification as in olive paste immediately after milling. Others were either lower, *i.e.* malaxations at 35 °C or higher owing to postulated extra releases of entrapped phenols from difficult fraction when using lukewarm water and talc additions or longer (60 min) malaxations. Why in their absence this TP fraction was not detected in pomace remains unknown, but is not surprising as the same inconsistency could be observed elsewhere. For example in the study of Artajo et al.

(2006b), the TP pool quantified in the final products after talc addition was more than doubled in comparison to that found in olive paste, but in control trial without talc, this fraction was similarly not confined in the pomace. This could have been associated with paste's structural change altered by all – the talc and lukewarm water adds or longer mixing actions. The entrapped phenols could have been linked with other compounds *e.g.* polysaccharides, released only after physical disruption of olive paste, allowing higher TP detection in the final products, including that in pomace. A similar extra TP releases have been observed after enzymes addition and ascribed to a reducing complexation of phenols with polysaccharides (Vierhuis et al., 2001).

However, different TP distribution rates among the final products further suggest that all examined parameters have also altered the phenols partition trail, which next to the concentration and affinity of phenols for particular matrix, mainly depended on the quantity of final products formed along with their ability for TP perception. Longer malaxations and temperatures at 35 °C, with NaCl and talc additions were the only parameters that improved the phenols partition rates to olive oil, but NaCl offered the most value-added olive oil processing from all – the nutritional, economical and environmental perspectives. Its addition to olive paste improved the TP concentration in olive oil (52%) along with simple phenols yield (47%), increased its physical extractability (14%) and reduced the wastewater yields for nearly half *vs*. control without NaCl addition. Nevertheless, this co-adjuvant is relatively cheap, recognized as safe and thus holds the best potential for forward large-scale investigations using other cultivars and multiple range of other parameters combinations.

5.3 Istrska belica *cv.* fruits seasonal and geographical phenol profile variation

The third part of doctoral thesis was dedicated to the investigation of Istrska belica *cv*. fruits phenolic profile variation in relation to the growing season and olive orchard location. All drupes were harvested at optimal ripening stage before being processed to olive oil from four randomly chosen olive groves with a view to determine the quality and quantity of phenolic antioxidants available in the main Slovenian olive cultivar for their transference to olive oil. Moreover, the study also

aimed to improve the local and international knowledge about this variety and its behaviour in response to different environmental stimuli.

Table 13 presents the phenolic composition of Istrska belica *cv*. fruits collected from three Slovenian cultivation regions, *i.e.* Vipava Valley, Goriška Brda and Slovenian Istria over two crop seasons 2009–2010. In addition, the available geo-climatic data from orchards' nearest weather stations are collectively presented in **Table 14** to establish (if possible) any correlation with the fruits phenol yield variation.

Many factors affect the phenolic profile of olive fruits, including the cultivar and its genetic make up (Morelló et al., 2004). Several studies have been carried out describing the differences between various profiles of olive cultivars from different countries such as Spain (Gómez-Rico et al., 2008), Italy (Sivakumar et al., 2005), Portugal (Vinha et al., 2005), Australia (Ryan et al., 1999) and Tunisia (Bouaziz et al., 2010), but never from the Slovenia yet.

The use of improved chromatographic analysis allowed us to identify and quantify a great phenolic fraction in Istrska belica *cv.* extracts comprised of forty individual representatives (**Table 13**). This is superior to existing literature data, demonstrating poorer qualitative profiles, including with our previous reports, *i.e.* nine (Jerman Klen et al., 2012a; Jerman et al., 2010) as well as others, *i.e.* ten (Gómez-Rico et al., 2008), eleven (Vinha et al., 2005), thirteen (Boskou et al., 2006), nineteen (Morelló et al., 2004), twenty-one (Bouaziz et al., 2010), twenty-six (Savarese et al., 2007) and twenty-seven phenols (Ryan et al., 1999). However, what infers next is the fact that some of these phenols have never been found before in the drupes of *O. europaea* L. such as luteolin-4'-7-*O*-diglucoside and luteolin-3'-*O*-glucoside. This could reflect any or all of varietal, agronomic or analytical differences, similarly as the absence of some commonly present fruit phenols (*e.g.* benzoic acids) not detected in Istrska belica *cv.* extracts.

Table 13 Phenol composition of Istrska belica *cv*. fruit extracts with regard to olive harvest season and orchard location

Class/ Phenolic compound	Concentration (mg/g fruits DW)								
	2009				**2010**				*SignF*[D]
	Martinjak[A]	*Gradno*[A]	*Šalara*[B]	*Šmihel*[C]	*Martinjak*	*Gradno*	*Šalara*	*Šmihel*	
Simple phenols									
Hydroxytyrosol glucoside	0.13 b	0.13 c	0.08 e	0.07 g	0.17 a	0.16 a	0.12 d	0.08 f	***
Hydroxytyrosol-1-β-glucoside	0.05 g	0.09 d	0.04 h	0.09 e	0.1 c	0.15 a	0.13 b	0.08 f	***
Hydroxytyrosol	0.09 h	0.15 g	0.18 e	0.2 d	0.17 f	0.25 c	0.36 a	0.32 b	***
Tyrosol glucoside	0.03 d	0.07 a	0.03 d	0.04 c	0.05 b	nq	0.07 a	0.05 bc	***
Tyrosol	0.03 g	0.04 0 e	0.05 h	0.05 g	0.06 c	0.08 b	0.12 d	0.06 a	***
Hydroxytyrosol acetate	0.12 f	0.13 bc	0.07 e	0.08 a	0.26 e	0.28 d	0.22 f	0.29 c	***
Total	*0.45 g*	*0.61 e*	*0.45 g*	*0.52 f*	*0.82 d*	*0.92 b*	*1.02 a*	*0.87 c*	***
Cinnamic acids									
β-OH verbascoside isomer 1	0.05 c	0.06 b	0.03 e	0.07 a	0.04 c	0.05 c	0.03 d	0.06 b	***
β-OH verbascoside isomer 2	0.05 b	0.05 bc	0.03 e	0.06 a	0.03 e	0.04 d	0.02 f	0.04 c	***
β-OH-methyl verbascoside isomer 1	nd	0.1 b	0.04 e	0.11 a	0.05 d	0.04 f	0.08 c	0.03 g	***
β-OH-methyl verbascoside isomer 2	nd	0.11 b	0.04 e	0.11 a	0.05 d	0.03 f	0.09 c	nd	***
Verbascoside	2.28 b	1.55 e	3.6 a	1.74 d	1.58 e	1.06 f	1.79 c	0.71 g	***
Total	*2.37 b*	*1.87 e*	*3.75 a*	*2.09 c*	*1.75 f*	*1.22 g*	*2.02 d*	*0.85 h*	***
Flavonoids									
Luteolin-4',7-*O*-diglucoside	0.07 c	0.06 d	0.1 b	0.1 a	0.04 f	0.03 g	0.04 f	0.06 e	***
Rutin	1.15 c	1.05 d	1.38 b	1.42 a	0.56 f	0.35 h	0.53 g	0.69 e	***
Luteolin-7-*O*-glucoside	1.27 c	1.13 e	1.77 a	1.61 b	0.7 f	0.42 h	0.57 g	1.21 d	***
Luteolin rutinoside isomer 1	0.15 d	0.16 c	0.2 b	0.23 a	0.07 f	0.05 h	0.06 g	0.09 e	***
Luteolin rutinoside isomer 2	0.06 c	0.04 f	0.05 de	0.05 d	0.06 c	0.05 e	0.07 b	0.1 a	***
Quercitrin	0.22 c	0.21 d	0.24 b	0.26 a	0.13 f	0.1 h	0.12 g	0.17 e	***
Apigenin-7-*O*-glucoside	0.26 d	0.23 0 e	0.29 c	0.32 b	0.16 f	0.11 h	0.15 g	0.33 a	***
Luteolin-4'-*O*-glucoside	0.39 b	0.35 c	0.57 a	0.57 a	0.18 e	0.11 g	0.14 f	0.29 d	***
Luteolin-3'-*O*-glucoside	0.1 c	0.12 b	0.17 a	0.18 a	0.06 d	0.05 e	0.06 d	0.1 c	***
Total	*3.68 b*	*3.35 e*	*4.78 a*	*4.74 c*	*1.96 f*	*1.26 g*	*1.73 d*	*3.03 h*	***

Table 13 *(Cont)*

Class/ Phenolic compound	Concentration (mg/g fruits DW)								*SignF*
	2009				**2010**				
	Martinjak	*Gradno*	*Šalara*	*Šmihel*	*Martinjak*	*Gradno*	*Šalara*	*Šmihel*	
Secoiridoids									
Unknown A	0.05 c	0.04 e	0.03 g	0.03 h	0.06 a	0.05 b	0.05 d	0.04 f	***
Unknown 408 MW	0.01 a	0.01 b	0.01 d	0.01 e	0.01 a	0.01 a	0.01 c	0.01 de	***
Unknown 408 MW	0.01 a	0.01 a	0.01 de	0.01 c	0.01 d	0.01 e	0.01 b	0.01 d	***
Unknown B	1.23 c	1.71 a	1.2 d	1.33 b	0.69 e	0.64 f	0.62 f	0.59 g	***
Elenolic acid glucoside isomer 1	0.22 d	0.29 ab	0.21 d	0.3 a	0.26 c	0.28 b	0.3 a	0.26 c	***
Elenolic acid glucoside isomer 2	0.18 e	0.3 b	0.08 g	0.33 a	0.21 c	0.2 d	0.18 e	0.16 f	***
Elenolic acid glucoside isomer 3	0.48 d	0.63 a	0.22 g	0.55 b	0.43 e	0.5 c	0.19 h	0.28 f	***
Demethyloleuropein	7.01 e	3.54 f	0.48 g	0.57 g	19.66 b	18.85 c	9.74 d	20.15 a	***
Dihydro-oleuropein isomer 1	0.35 c	0.33 c	0.24 d	0.38 b	0.33 c	0.35 c	0.37 b	0.4 a	***
Dihydro-oleuropein isomer 2	1.02 a	1.01 a	0.98 a	0.96 a	0.69 a	0.87 a	0.93 a	0.97 a	***
Oleuropein diglucoside isomer 1	0.34 c	nd	0.52 b	0.57 a	nd	nd	nd	nd	***
Nüzhenide isomer 2	0.2 b	nd	nd	0.41 a	nd	nd	nd	nd	***
Oleuropein diglucoside isomer 2	0.47 b	0.61 a	0.61 a	nd	nd	nd	nd	nd	***
3,4-DHPEA-EDA isomer 2	nd	nd	nd	1.05 e	4.63 d	6.46 b	11.66 a	5.72 c	***
Oleuropein	57.11 a	51.44 b	52.21 b	43.24 c	16.4 d	12.35 e	8.5 f	16.52 d	***
Caffeoyl-6'-secologanoside	0.08 f	0.11 c	0.08 f	0.1 e	0.1 e	0.1 d	0.11 b	0.14 a	***
Oleuroside	0.51 h	1.03 d	0.73 f	0.61 g	1.08 c	1.16 b	1.89 a	0.89 e	***
Unknown C	0.89 c	1.02 a	1.0 b	1.0 ab	0.72 d	0.72 d	nd	0.7 e	***
Ligstroside	5.43 c	4.89 d	7.73 a	6.01 b	2.82 f	2.36 g	2.4 g	3.88 e	***
Comselogoside	0.26 g	0.31 d	0.34 b	0.36 a	0.3 e	0.24 h	0.32 c	0.29 f	***
Total	*75.84 a*	*67.28 b*	*66.67 b*	*57.81 c*	*48.39 e*	*45.15 f*	*37.27 g*	*50.99 d*	***
Σ TP[E]	82.35 a	73.11 c	75.65 b	65.16 d	52.92 f	48.55 g	42.04 h	55.74 e	***

[A]Goriška Brda, Slovenia. [B]Slovenian Istria, Slovenia. [C]Vipava Valley, Slovenia. [D]**M**eans separated by Duncan test ; ***, $P \leq 0.001$, values marked with the same letter are not significantly different. [E]Total phenols, sum of HPLC-DAD quantified phenols.

Table 14 Distribution of monthly rainfall (Rain), average temperature (T_{avr}) and the difference between the maximal and minimal temperatures (T_{max}-T_{min}) during olive fruit growing seasons 2009–2010 at four orchard locations

Orchard location	**Martinjak**[A]						**Gradno**[B]						**Šalara**[C]						**Šmihel**[D]					
Altitude	164 nm						198 m						105 m						185 m					
Year	**2009**			**2010**			**2009**			**2010**			**2009**			**2010**			**2009**			**2010**		
Month	*Rain (mm)*	*T_{avr} (℃)*	*$T_{max-min}$ (℃)*	*Rain (mm)*	*T_{avr} (℃)*	*$T_{max-min}$ (℃)*	*Rain (mm)*	*T_{avr} (℃)*	*$T_{max-min}$ (℃)*	*Rain (mm)*	*T_{avr} (℃)*	*$T_{max-min}$ (℃)*	*Rain (mm)*	*T_{avr} (℃)*	*$T_{max-min}$ (℃)*	*Rain (mm)*	*T_{avr} (℃)*	*$T_{max-min}$ (℃)*	*Rain (mm)*	*T_{avr} (℃)*	*$T_{max-min}$ (℃)*	*Rain (mm)*	*T_{avr} (℃)*	*$T_{max-min}$ (℃)*
January	84	6.1	5.4	74	2.6	5.0	113	4.5	5.6	66	2.2	5.2	66	4.5	8.3	95	3.5	6.7	87	3.6	8.6	78	2.0	7.3
February	132	5.3	7.8	246	5.2	6.1	127	4.2	8.5	242	4.6	6.8	65	5.2	8.6	118	5.6	7.8	100	3.8	10.3	163	4.5	8.1
March	259	9.0	8.8	40	8.4	7.4	255	8.2	9.7	44	7.8	8.5	101	8.6	9.8	33	7.7	8.6	249	7.8	11.7	48	7.1	9.8
April	89	15.3	9.3	59	13.8	9.4	91	14.8	9.7	67	13.0	10.8	59	14.1	9.9	43	12.7	11	77	14.0	11.9	47	12.4	12.9
May	41	19.9	11.1	224	16.5	7.7	51	19.2	11.4	200	16.0	8.3	26	18.7	12.2	140	16.8	8.8	26	18.7	13.9	258	16.0	9.6
June	162	20.2	9.6	4	21.4	10.5	133	20.1	10.0	91	20.8	10.5	92	21.0	10.4	83	20.7	10.7	80	20.5	11.3	108	20.7	12.1
July	108	23.3	10.4	458	21.9	7.6	99	23.1	10.5	157	24.2	11.3	21	23.3	12.3	164	23.9	12.1	123	22.7	12.6	200	23.5	12.9
August	27	25.6	12.7	105	22.6	10.2	86	24.3	11.0	148	22.0	9.8	43	24.0	12.8	65	21.6	10.9	82	23.8	13.6	75	21.1	12
September	76	21.0	9.3	354	17.8	8.8	129	19.5	9.4	282	17.4	8.6	55	20.0	11.8	249	17.5	10.4	65	19.5	12.5	367	16.7	11.1
October	113	14.3	8.1	97	13.3	7.7	149	11.7	8.5	161	12.9	7.8	62	13.2	10.4	57	12.8	10	117	12.5	11.3	63	11.9	10.9
November	134	10.0	3.9	389	10.2	5.9	157	9.2	5.1	494	9.9	4.8	140	10.8	5.7	199	10.6	6.6	113	9.6	6	342	9.5	6.8
December	256	5.4	5.2	0	2.0	5.9	184	3.9	5.0	269	3.4	6.2	203	5.7	7.8	147	4.4	7.7	291	4.4	7.9	260	2.5	7.7
Calc./year[E]	***1481***	***14.6***	***8.5***	***2050***	***13.0***	***7.7***	***1574***	***13.6***	***8.7***	***2221***	***12.9***	***8.2***	***933***	***14.1***	***10.0***	***1393***	***13.2***	***9.3***	***1410***	***13.4***	***11.0***	***2009***	***12.3***	***10.1***

[A]Weather station of Biljana, Goriška Brda, Slovenia. [B]Weather station of Prepotto, Collio, Italy. [C]Weather station of Portorož, Slovenian Istria, Slovenia. [D]Weather station of Bilje, Vipava Valley, Slovenia. [E]Calculated amount per year: sum of rain, average of temperatures, the difference between the maximal and minimal temperatures.

However, no major qualitative differences could be observed between the extracts of Istrska belica *cv*. fruits from different growing conditions, but a major quantitative could be established on the other side. Looking from a quantitative TP yield perspective, the drupes of Istrska belica *cv.* could be considered as a rich source of phenolic compounds reaching yields up to 82.3 mg/g DW. This is slightly higher *vs.* other reports using the same strategy of HPLC-DAD quantification in freeze-dried fruits, where the maximal levels reported accounted up to 31.0 mg/g DW (Sivakumar et al., 2005), 51.0 mg/g DW (Vinha et al., 2005) and 64.8 mg/g DW (Vinha et al., 2002). By contrast, using Folin-Ciocalteu TP based analysis, the reported levels were typically higher reaching up to 150 mg/g DW (McDonald et al., 2001).

Secoiridoids were the predominant class in all Istrska belica *cv.* extracts, while the content of other classes showed a higher inter-seasonal and inter-geographical variation. At the level of individual phenols, hydroxytyrosol and its acetate form were the most abundant among simple phenols, analogously as verbascoside among cinnamic acids. Luteolin-7-*O*-glucoside and rutin were the prominent among flavonoids, while secoiridoidal profile was dominated by oleuropein or demethyloleuropein, depending on a growing season.

From the growing season perspective, a significant TP drop in olive drupes could be observed in y. 2010 at all orchard locations with Vipava Valley facing the lowest (~14% in Šmihel) and Slovenian Istria the highest TP yield reduction (~44% in Šalara), while the decrease in two other groves from Goriška Brda was rather comparable (~34% in Gradno *vs*. ~36% in Martinjak). Taking into account the geo-climatic conditions (**Table 14**), the season 2010 had a considerably higher cumulative rainfall in comparison to y. 2009, paralleled with a lower average temperatures of air and their differences between the maximal and minimal levels. This could have explain a higher TP yields obtained in the fruits from season 2009 as the water stress has already been associated with higher biosynthesis of phenols in olive drupes *via* increased activity of specific enzymes (Romero et al., 2003; Patumi et al., 2002; Tovar et al., 2002). As evident from the monthly distribution of rainfall, the maximal accumulation of rain could be evidenced for May and

July–September, which corresponded to the fruits growing season (starting in April), while another rise in November, coinciding with the harvested period.

However, not all phenol classes and their representatives decreased in a wetter season 2010. Among individual groups, only simple phenols have increased, but yet considerably, *i.e.* 51–127%, where hydroxytyrosol acetate was the most and hydroxytyrosol-1-β-glucoside the least affected. Some exceptional yield rises could also be evidenced among secoiridoid representatives, including demethyloleuropein and 3,4-DHPEA-EDA. Based on these results, it is possible to deduce that different levels of water availability have not only altered the biosynthesis of phenols, but also their metabolic relationships. Patumi et al. (2002) reported a similar increase of hydroxytyrosol in drupes of *O. europaea* L. under tree irrigation, accompanied with a drop of other phenols.

Another possible explanation for phenolic profile inter-seasonal variation of Istrska belica *cv*. fruits could be attributed to an alternate bearing as the tree of *O. europaea* L. is known to provide a high fruit production in one, and a low in the next year. The previous study of Ryan et al. (2003) observed that in a high-fruiting season the content of oleuropein increased during sampling season, inversely to 3,4-DHPEA-EDA, while in the next, *i.e.* low-fruiting season, both of them have declined in the Hardy's Mammoth fruits. The inverse relationship between 3,4-DHPEA-EDA and oleuropein could also be evidenced in our study, but the quantitative fruit production of olive trees has not been monitored to establish any firm relationship with the alternate bearing phenomenon.

As regard to olive orchard location, the picture was more complicated as no general trend on TP yield behaviour in olive drupes and geo-climatic data available nearby their groves could be evidenced. As seen from results, the TP yield in Istrska belica *cv*. fruits varied significantly among orchard locations (65.16–82.35 mg/g DW in y. 2009 and 42.04–55.74 mg/g DW in y. 2010), but their pattern change between the two seasons was poorly comparable. In season 2009, the fruits from grove of Martinjak in Goriška Brda had the richest TP yields, followed by Šalara in Slovenian Istria, Gradno (Goriška Brda) and Šmihel (Vipava Valley). Such pattern is not in line

with the regime of total rainfall, the difference between the maximal and minimal temperatures nor the altitude of olive groves. In fact, the only positive correlation could be obtained with the average temperature of air (R^2 = 0.8974), which showed a decreasing trend in fruits' TP yield toward lower growing temperatures. A higher TP accumulation with a higher air temperatures (max T_{avr} up to 25.6 °C) could be linked with the activity of enzymes responsible for phenols biosynthesis. A similar positive relationship with air temperatures up to 35 °C and olive explants' TP yields was observed by Roussos et al. (2007).

However, in a wetter season 2010, the regime of air temperatures was not consistent with the fruits TP yield behaviour, which decreased in the following order; Šmihel > Martinjak > Gradno > Šalara. In fact, no correlation could be found with any of the geo-climatic data available, which may be a result of their combination or some other factors not considered/monitored within our study. Even at the level of their class distribution, no general pattern between the orchard locations could be evidenced in season 2010, though in a previous season the same order was observed in the fruits from all olive groves; secoiridoids > flavonoids > cinnamic acids > simple phenols. What is of interests to add is the fact that fruits from Šalara grove, which is the most southern location studied, were always characterised by the highest content of cinnamic acids comprised of valuable phenols such as verbascoside and its derivatives. Similarly, a rather high content of some flavonoidic phenols in the grove of Šmihel should be marked.

5.4 Slovenian commercial olive mill wastes phenolic composition

In the last part of doctoral study, the Slovenian commercial olive mill wastes from two types of industrial-scale mill facilities were screened for the presence of phenolic compounds never investigated before. All the wastes were chosen arbitrarily from the unknown fruit material as the purpose of study was to assess the current state of by-products phenolic content in Slovenian olive oil production, important prior any valorisation and/or potent scale-up utilisations.

Qualitatively, a great similarity could be observed between the phenolic profiles of commercial and lab-scale olive waste extracts, where a major phenolic fraction was

composed of the same representatives in detailed described before (Section 5.1). On the other side, there was a marked quantitative dissimilarity evidenced for both, though a direct comparison is not the most relevant owing to their distinct initial fruit material. However, based on HPLC-DAD sum of individual phenols, the industrial-scale wastes contained much lower TP yields *vs.* lab-scale ones, which is in line previous observation for the oil using the same olive fruit cultivar (Stefanoudaki et al., 2011). The TP content in our commercial 2- and 3-phase pomaces varied between 3.8 and 24.6 mg/g DW (**Figure 23**), while in a lab-scale trial between 38.7 and 47.5 mg/g DW, depending on operative conditions applied (**Table 11**). Similarly, the TP yields in the wastewater were rather lower at industrial (22.0–44.8 mg/g DW) *vs.* lab-scale generation (31.0–74.6 mg/g DW).

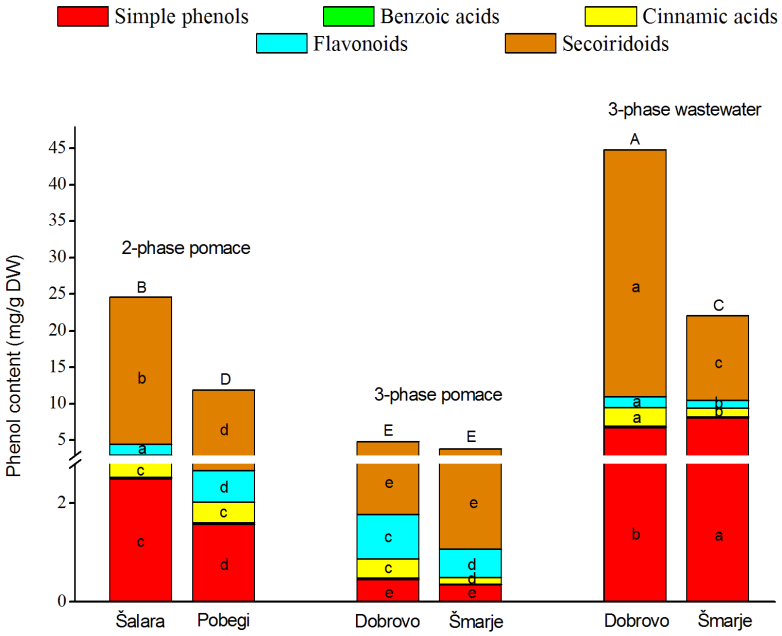

Figure 23 Phenol class distribution in the Slovenian commercial olive mill wastes. Values marked with the same letter are not significantly different; small letters indicate a comparison between different classes, while the capital compares their total sum

A further comparison with literature data revealed that the TP yields in Slovenian commercial olive mill by-products are rather comparable with other existing reports. The TP yields in 2-phase pomaces from the two Slovenian olive mills varied largely between 11.9 mg/g DW (Pobegi) and 24.6 mg/g DW (Šalara), which could be attributed to any or all of varietal, climatic and agronomic differences (**Figure 23**). These results are within the ranges reported for the olive wet-cakes from Croatia

(18.5 mg/g DW, Jerman Klen et al., 2012a), Australia (12.6–32.9 mg/g DW, Obied et al., 2008c), France (20.4.mg/g DW, Lesage-Meessen et al., 2001), Jordan (4.4 mg/g DW, Alu'datt et al., 2010) and Portugal (33.4–45.8 mg/g DW, Ramos et al., 2013). Similarly, the TP yields in Slovenian olive mill wastes from 3-phase decanters are rather comparable to those that already exploits them for commercial utilisation. As seen from results in y. 2010 (**Figure 23**), the TP content in our 3-phase wastes varied between 3.8 and 4.8 mg/g DW for pomace, and between 22.0 and 44.8 mg/g DW for wastewater, being always higher in Dobrovo than in Šmarje, most likely due to a richer initial fruit material. These ranges are in line with our already published data from the same olive mill of Dobrovo for y. 2009 (wastewater; 34.1 mg/g DW, **Annex B2**) and y. 2010 (wastewater; 57.3 mg/g DW and pomace; 1.9 mg/g DW, **Annex B4**), which all together may confidentially classified them as a rich source of valuable phenolic compounds. While the levels in Slovenian olive mill wastewaters are consistent with other valorisation reports, *i.e.* 24.0 mg/g DW (Lesage-Meessen et al., 2001), 414 mg/g DW (Suárez et al., 2010), 2.3 mg/g DW (Visioli et al., 1999), the 3-phase pomaces have scarcely been investigated and hence difficult to compare with the literature data. To our best knowledge, the only reported TP yields for 3-phase pomace expressed per DW are much higher, *i.e.* 22.9–32.9 mg/g DW (Obied et al., 2008b), which could be attributed to a richer starting fruit material or perhaps to their lab-scale generation.

The wastewater had 9.3- and 5.7-fold higher TP yields than its corresponding pomace in both 3-phase decanters of Dobrovo and Šmarje (**Figure 23**). This clearly indicates that a much higher portion of phenols is lost with wastewater than with pomace when processing olive oils with systems requiring higher paste dilutions. Moreover, there were also some unique patterns found for their class distribution observable in both of olive mills despite of different initial fruit materials processed; while secoiridoids were the predominant class in both of waste matrices, simple phenols constituted the second largest class in wastewaters and only the third in pomaces, which again signifies their high affinity for the water matrix. By contrast to both wastewater profiles, cinnamic acids in pomaces were over-dominated by flavonoids, whereas benzoic acids were the least confined in both, the liquid and solid ones. The wastewater was also greater in the abundance of individual phenols

(**Table 15**), with an exception of rutin in Dobrovo, and luteolin and comselogoside in Šmarje, being equally well represented in both wastes' types.

The 2-phase pomaces had always significantly higher phenol yields in comparison to 3-phase pomaces, which, however, is expected as the vegetation water from fruits containing phenols is confined in this wet by-product. On the other side, they contained much lower TP yields in comparison to 3-phase wastes together (pomace + wastewater), which may reflect varietal and/or processing differences. What is of interest next is the class distribution in the 2-phase pomaces, which seems to be the combination of that found for the wastewater and pomace from 3-phase decanters. From a qualitative viewpoint, all the phenols found in a 2-phase pomace had also been confined in at least one of the 3-phase waste matrices, and 3,4-DHPEA-EDA was clearly the predominant phenol, accounting more than 60% of all the phenols quantified.

Table 15 shows the content of selected phenolic compounds in Slovenian olive mill wastes, important from the commercial and/or scientific interests. As already noted earlier for the TP yields, the ranges of these phenols are likewise in line with above valorisation reports. A high content of hydroxytyrosol in wastewaters and 3,4-DHPEA-EDA in all the by-products, known for their high antioxidant activities could be a great commercial opportunity for the Slovenian olive oil sector trading them to pharmaceutical, food and/or cosmetic industries. Analogously, verbascoside and comselogoside are gaining significance in their value to mankind, presenting important functional constituents in the pharmacy and medicine. In this context, four verbascoside derivatives found in the Slovenian olive mill wastes could be of great economic and/or research interests. To our best knowledge, there are only two reports about the presence of β-OH verbascoside isomers in the Italian olive wastes together accounting up to 50.7 mg/g DW (Innocenti et al., 2006; Mulinacci et al., 2005). By contrast, the presence of two newly discovered β-Methyl-OH verbascoside diastereoisomers has not been yet reported, though ubiquitous in all of Slovenian wastes, showing higher yields in the liquid than in solid matrices. All together, the commercial by-products from Slovenian olive oil industry seems to be a promising source of different exploitable phenols, whose recovery would have a double benefit, *i.e.* environmental *via* reduced toxicity and economic *via* marketing trade.

Table 15 The content of selected phenolic compounds in Slovenian olive mill wastes

Phenolic compound	Phenol content (mg/g DW)						*SignF*[A]
	2-phase centrifuge		3-phase centrifuge				
	Pomace		Pomace		Wastewater		
	Šalara	Pobegi	Dobrovo	Šmarje	Dobrovo	Šmarje	
Hydroxytyrosol glucoside	1.19 b	0.17 d	0.06 e	0.03 e	1.24 a	0.59 c	***
Hydroxytyrosol-1-β-glucoside	0.35 c	0.10 d	0.13 d	0.13 d	0.70 b	1.21 a	***
Hydroxytyrosol	0.48 d	0.80 c	0.11 e	0.09 e	3.47 b	3.63 a	***
3,4-DHPEA-EDA	14.96 a	7.9 b	1.56 d	1.38 d	14.62 a	2.56 c	***
β-OH verbascoside isomer 1	0.04 c	0.06 b	0.06 b	0.01 d	0.56 a	nd[B]	***
β-OH verbascoside isomer 2	0.04 c	0.07 b	0.07 b	0.01 d	0.58 a	nd	***
β-Methyl-OH verbascoside isomer 1	0.05 d	0.05 d	0.07 c	0.03 e	0.14 b	0.26 a	***
β-Methyl-OH verbascoside isomer 2	0.05 e	0.06 d	0.08 c	0.03 f	0.13 b	0.32 a	***
Verbascoside	0.25 c	0.15 d	0.08 e	0.04 f	1.07 a	0.56 b	***
Rutin	0.26 a	0.07 c	0.2 b	0.06 c	0.19 b	0.22 b	***
Luteolin	0.23 b	0.12 d	0.23 b	0.15 c	0.35 a	0.15 c	***
Apigenin	0.04 e	0.04 e	0.06 c	0.05 d	0.13 a	0.12 b	***
Comselogoside	0.33 a	0.16 c	0.05 d	0.04 e	0.26 b	0.04 de	***

[A]Means separated by Duncan test; *** ($P \leq 0.001$), values marked with the same letter are not significantly different. [B]Not detected.

6 CONCLUSSIONS

6.1 Olive phenol analysis

As regard to olive phenol analysis, the most important conclusions could be summarised as follows.

- Ultrasound has provided the basis for new methods development in the phenol profiling study of complex olive matrices. The US-probe high power agitation abilities has been proved to efficiently assist the extraction of phenols and provide a rich quali- and quantitative profiles of all matrices entailed in olive oil processing, *i.e.* of fruits, stone, paste, pomace, wastewater and oil.

- A combined screening approach using UPLC-DAD-ESI-QTOF-HRMS analysis allowed the detection of eighty different phenols, of which sixty-nine could be quantified using DAD alone. It provided an elemental composition of seventy-seven phenols determined with a high mass accuracy that along with a complementary UV-Vis spectra information allowed their identification with a high degree of confidence. In addition, four new molecular formulas were proposed and three new tentative identities assigned to a newly discovered phenol constituents, *i.e.* β-methyl-OH verbascoside, methoxynüzhenide and methoxynüzhenide 11-methly oleoside.

- Secoiridoids were the most ubiquitous and abundant class among phenolic compounds, while the most diverse range of phenols was confined in olive paste, followed by extracts of de-stoned fruit and pomace, wastewater, stone and olive oil. Some of these matrices were unique in the presence of certain phenolic classes and their representatives, which could be a result of a distinct fruit tissue metabolism at plant level, while in the process-derived matrices of their tendency for partition to a particular matrix.

6.2 Olive oil processing trial

A simultaneous screening of all matrices entailed in olive oil processing provided us some basic insights into olive fruit phenols transfer, transformation and partition trail, while the comparison of different trials using the same biological fruit material into their further quali- and/or quantitative induced changes by individual technological variables. Some of the main concluding remarks from all could be drawn as follows.

- Crushing was associated with the highest transformations and degradations of initial fruit phenols, where out of fifty-one initially quantified phenols, thirteen have irrevocably disappeared (mainly those from stone) and eight newly appeared in the paste immediately after milling.

- A minute crushing did not allow a total phenolic quantification in olive paste, but only those not entrapped in its oil/water emulsion known as difficult fraction. The TP pool quantified in olive paste presented 54% of the initial fruit phenols, but due to postulated extra TP releases under specific malaxing conditions, the crushing step contribution to a total TP loss accounts of no more than 35% in respect to fruits initial phenolic content.

- In all trials, olive fruit phenols followed a simple partitioning model, where with few minor exceptions, only aglycones were transferred to the olive oil, while the fruit glycosides that yet reached the final products remained occluded in the wastes.

- Based on phenols yield behaviour during olive oil processing some new potent transformative relationships could be established. Secoiridoids; i) oleuropein/ligstroside/demethyloleuropein → oleuropein/ligstroside aglycone → 3,4-DHPEA-EDA/p-HPEA-EDA → hydroxytyrosol/tyrosol, ii) nüzhenide (di)esters → nüzhenide isomer 2 and nüzhenide 11-methyl oleoside isomer 4 → tyrosol glucoside → tyrosol. Flavonoids; i) vebascoside → hydroxytyrosol-1-*β*-glucoside, ii) apigenin7-*O*-glucoside → apigenin, iii) luteolin glycosides/rutin/quercitrin → luteolin.

- Operative conditions of malaxation step has regulated the phenols transfer, transformation and partition trail from olive paste to the final products *via* altering their physical extractability from paste, their degradation/formation equilibrium and sometimes their solubility properties. These parameters also affected the quantity of final products formed and their ability for TP perception, which along with the fruits' characteristics (level of enzymes, TP content) and phenols nature determined the quantity of phenols resulting in the final products.

- Longer paste malaxations and additions of talc and lukewarm water provided higher TP transfer rates from olive paste to the final products owing to postulated extra releases of entrapped phenols from paste's difficult fraction.

- In control trial, only 0.53% of the available fruit phenols have ended-up in olive oil, while the remaining and not technologically destructed fraction, were lost with the wastes; nearly 6% with wastewater and 48% with pomace.

- Among various malaxation time/temperature combinations, a 60 min/35 °C provided the highest TP yield partition to olive oil, accounting 0.73% of the available fruit initial phenolic content. Longer malaxations and higher temperatures improved the extractability of olive oil from olive paste until re-emulsification occurred at 45 °C.

- Lukewarm water additions increased the physical extractability of olive oil, but also triggered a significant wastewater yield production, which largely flushed away the phenols and reduced their yields in olive oil.

- NaCl and talc additions were the most efficient in TP enrichment of olive oil, improving their partition rates for 94% (NaCl + talc), 74% (NaCl) and 40% (talc), but only NaCl was also feasible from the economic and environmental perspectives as it reduced the wastewater yield production (44%) and improved the extractability of olive oil (14%).

6.3 Istrska belica *cv*. fruits seasonal and geographical phenol profile variation

The main results corresponding to the phenolic profile characterisation of the predominant Slovenian olive fruit cultivar Istrska belica *cv*. and its variability in response to different growing seasons and orchard locations could be summarised as follows.

- The extracts of Istrska belica *cv*. fruits displayed a rich qualitative and quantitative phenolic profile, unique in the presence of luteolin-4'-7-*O*-diglucoside and luteolin-3'-*O*-glucoside. While secoiridoids were the predominant class in all olive drupes, the yields of flavonoids, cinnamic acids and simple phenols varied with harvest season and orchard location.

- The impact of growing season and orchard location had no major qualitative effect on Istrska belica *cv*. phenolic composition, but a major quantitative impact was observed at both levels, *i.e.* the individual and total.

- All fruits of Istrska belica *cv*. experienced a sharp decrease from season 2009 to 2010, characterised by a higher cumulative rainfall, lower temperatures of air and their differences between the maximal and minimal levels. The TP drops in season 2010 varied among orchard locations (14–44%) and were consistent with a lower abundance of individual phenols with an exception of simple phenols and some of secoiridoid representatives, including demethyloleuropein and 3,4-DHPEA-EDA, whose levels were markedly increased in y. 2010.

- The impact of orchard location on Istrska belica *cv*. fruits' TP yields was significant, but their pattern change between the two seasons was poorly comparable and showed no or low consistency with the geo-climatic data from the nearest weather stations. However, some growing sites could also be characterised by a higher abundance of particular phenols.

6.4 Slovenian commercial olive mill wastes phenolic composition

The commercial olive mill wastes from Slovenian olive oil production were investigated as a potent source of priced phenolic compounds and some of the main findings could be delineated as follows.

- The quality and quantity of phenolic compounds confined in the Slovenian commercial olive mill wastes is at least equal to the other value-adding ranges from literature, holding a great potential for forward commercial utilisation.

- The qualitative phenol composition of commercial olive mill wastes was similar to a lab-scale generated wastes and shares a great analogy with the profiles of olive matrices from various extraction systems.

- The Slovenian olive mill wastewaters from 3-phase centrifuges contained much higher phenol yields than their corresponding pomaces (up to 9.3-times) and were particularly rich in the content of hydroxytyrosol and 3,4-DHPEA-EDA.

- A 2-phase pomace had the second richest quantitative phenolic profile and confined considerable amounts of 3,4-DHPEA-EDA, hydroxytyrosol glucoside, rutin and comselogoside.

- The ubiquitous presence of verbascoside derivatives in the Slovenian olive mill wastes constitutes a challenge from both, the commercial and scientific interests.

7 RESEARCH PERSPECTIVES

The research interests to redesign the current technological approaches to provide higher extractability of olive oils with richer phenol yields and of lower ecological footprints have been great in recent years and are still significant due to increasing demands for functionally and sustainability in olive oil industry. The results of present study have made some progress toward improved phenol analysis and profile characterisation of olive matrices as well as toward better understanding of phenols behaviour during technological process under different operative conditions, but is still far from being complete. There are many issues yet to be answered, clarified and/or proved, but were beyond the scope of present thesis whose results should rather be considered as a starting point material for forward research investigations. In a hope of motivating some further research interests, a few of most essential have been listed below.

Many promising results were obtained as regard to potent technological control of olive fruit phenols partition regulation during olive oil production, which deserve to be verified in a pilot or industrial scale-up investigations using different cultivars and/or their blends. Although lab-scale experiments are ideally suited as a first preliminary step, the results obtained are yet too scant to allow general rules on their behaviour in the large-scale systems with different characteristics. This, however, is necessary before any value-adding of individual technological variables or their combinations, which in addition to the starting fruit material indeed seems to be the key factors in the phenol profile shaping of final products. This would not only provide a new opportunity to re-assess their impacts, but would also allow to check for the potent drawbacks perhaps overlooked in the lab-scale trials.

The degradation mechanism of all phenol representatives confined in any of the fruit compartments (peel, pulp or stone) is another key factor that remains to be studied carefully using model or the real olive oil production systems. Similarly, the role of phenolic compounds oxidative catabolism during olive oil processing is far from being conclusively established.

The research attention should also be given to a study investigating the relationship between the plant and technologically induced phenols transformative pathway, which seems to share the same or at least similar pattern dictated by the activity of endogenous enzymes present in the fruits. Such knowledge could lead to the manipulation of their selective presence in both, *i.e.* in fruits and their process-derived matrices.

A high resistance to the technologically induced changes established for particular phenols is another important item worth of forward research investigations, especially in terms of finding the potent correlation with their chemical structures. This could have a wide scope in the food industry continuously engineering the TP enriched food products.

Nevertheless, the newly discovered phenols should be (re)characterised using other analyses apart from LC-MS and checked for their potent bioactivities. Such discoveries could also provide a new insight into secondary metabolism of *O. europaea* L. plants.

8 SUMMARY

The fate of olive fruit phenols during olive oil processing was investigated using a laboratory 3-phase extraction line in relation to different technological parameters; malaxation time/temperature, NaCl, talc and lukewarm water additions with aim to improve the phenols partition rates to olive oil and reduced their lost with wastes. The olive fruit phenols transfer, transformation and partition trail was followed by UPLC-DAD-ESI-QTOF-HRMS analysis through all operative steps from fruits (peel/pulp and stone) to paste and the final products, *i.e.* pomace, wastewater and oil, and the mass balance approach was parallelly assessed for each of the trial. In addition, the variability of phenols in Istrska belica *cv*. fruits under different growing conditions was evaluated and the Slovenian commercial olive mill wastes from various mill facilities screened for the presence of priced phenolic compounds.

Crushing was associated with the highest transformations and degradations of native fruit phenols, where out of fifty-one initially quantified phenols, thirteen have irrevocably disappeared (mainly those from stone) and eight newly appeared in the corresponding paste. The latter have further continued also during malaxation, where different operative conditions regulated their transfer, transformation and partition rates from paste to the final products, but all the changes were of quantitative and not of qualitative nature. In all the trials, olive fruit phenols followed a simple partitioning model, where with few minor exceptions, only aglycones were partitioned to the olive oil, while the fruit glycosides that yet reached the final products remained occluded in the wastes. In addition, all technological variables investigated have strongly influenced the extraction yields of final products, which along with the phenols nature and its related affinity for a particular matrix determined the quantity of phenols resulting in each of them.

In control trial, only 0.53% of the available fruit phenols have ended-up in olive oil, while the remaining not technologically destructed fraction, was lost with wastes, nearly 6% with wastewater and 48% with pomace. The results further showed that their quantitative yield distribution among the final products could be somewhat regulated and phenols partition to olive oil improved by using longer malaxations and temperatures at 35 °C, with additions of NaCl and talc, but no extra lukewarm

water adds. Among all parameters examined, NaCl and talc were the most efficient in phenols olive oil enrichment, increasing fruit–oil partition rates for 94% (NaCl + talc), 74% (NaCl) and 40% (talc), but only NaCl alone was also feasible from the economic and environmental perspectives as it reduced the wastewater yield production (44%) and improved the extractability of olive oil (14%).

The fruits of main Slovenian olive cultivar Istrska belica *cv*. could be considered as a rich source of various phenolic compounds whose yields varied significantly in response to different growing conditions, but presented a similar qualitative profiles unique in the presence and absence of particular phenols. Likewise, the Slovenian commercial olive mill wastes could be recognized as a promising source of valuable phenols, holding a great potential for their commercial utilisation.

Keywords: Olive oil processing, Phenols, Olive fruits, Olive stone, Olive mill wastes, UPLC-DAD-ESI-QTOF-HRMS analysis, Istrska belica *cv*., Malaxation time/temperature, Lukewarm water, NaCl, Talc.

9 POVZETEK

PORAZDELITEV IN ANTIOKSIDATIVNI POTENCIAL FENOLOV OLJK MED PROIZVODNJO OLJČNEGA OLJA

V doktorski disertaciji smo raziskovali vpliv tehnologije na kvantitativni prenos in transformacijo fenolnih spojin oljk med proizvodnjo oljčnega olja pri čemer smo za preučevanje uporabili najpogosteje zastopan 3-fazni kontinuirni sistem stiskanja na laboratorijski ravni ter UPLC-DAD-ESI-QTOF-HRMS detekcijo. Raziskovali smo učinkovitost prenosa fenolnih spojin iz oljk v pasto ter v olje in odpadne produkte, tj. pogačo in odpadno vodo v odvisnosti od procesnih spremenljivk kot so čas in temperatura malaksacije, dodatek tople vode, soli (NaCl) in talka ter tako skušali oceniti potencial za morebitno regulacijo fenolne particije z namenom izboljšave prehranske vrednosti oljčnega olja ter zmanjšanja fenolnih izgub z odpadnimi produkti. Raziskava je hkrati vključevala preučitev vpliva geografske in sezonske variabilnosti fenolnega profila oljk Istrske belice *cv*. ter različnih komercialnih odpadkov stiskanja oljčnega olja, saj nudi pomembno informacijo o kvaliteti in količini fenolov, ki jih lahko pričakujemo v olju ter odpadkih njegove proizvodnje.

Največji vpliv na izgubo in pretvorbo fenolov se je zgodil v začetni fazi proizvodnje, tj. v času mletja oljk, kjer smo od skupno enainpetdesetih fenolov oljk zabeležili izgubo trinajstih ter nastanek osmih novih fenolnih spojin. Transformacije in izgube fenolov so se nato nadaljevale tudi tekom malaksacije, kjer so procesne spremenljivke močno vplivale na stopnjo njihovih nadaljnih pretvorb, količinski prenos ter porazdelitev med tri končne produkte, tj. oljem, pogačo in odpadne vodo. Z manjšimi izjemami smo lahko v olju zasledili le aglikonske dele fenolov nastale pri razgradnji komplesnejših fenolov oljk s pretežno lipofilnim značajem, medtem ko smo glukozide in preostale lahko zasledili le v odpadkih. Končni produkti proizvodnje oljčnega olja različnih tehnologij se kvalitativno medsebojno niso razlikovali, medtem ko je bil vpliv preučevanih procesnih spremenljivk na njihov kvantitativni profil izjemen. Slednje pa so močno vplivale tudi na količinski izplen nastalih produktov, ki je poleg narave fenolov, pomembno vplival na končno vsebnost fenolov v oljčnem olju ter v odpadnih produktih.

Rezultati analiz kontrolnega poskusa so pokazali na slab količinski prenos fenolov oljka-olje, saj je med stiskanjem v olje prešlo le 0.53% razpoložljivih fenolnih spojin, medtem ko je večji del ostal v pogači (48%), preostali del, ki se ni izgubil med stiskanjem pa je bil odplaknjen z odpadno vodo (6%). Nadalje so rezultati pokazali, da je večji fenolni prenos v olje moč doseči pri daljših časih malaksacije in temperaturah 35 °C, brez dodatka tehnološke vode ter ob prisotnosti NaCl in talka. Predvsem dodatek slednjih je izrazito povečal prenos fenolov v olje, ki je bil v primeru NaCl + talk večji za 94%, pri njihovih samostojnih dodatkih pa za 74% (NaCl) in 40% (talk). Upoštevajoč ekonomski in okoljski vidik različnih tehnologij, smo največji potencial opazili pri tehnologiji z dodatkom NaCl, ki je poleg večjega fenolnega prenosa oljka–olje, povečala tudi količinski izplen oljčnega olja za 14% ter zmanjšala količino nastale odpadne vode za 44%.

Dvoletno spremljanje količine in vrste fenolov oljk Istrska belica *cv*. je potrdilo visoko vsebnost fenolov v plodovih oljk ter velik geografski in sezonski vpliv na njihovo količinsko variacijo, medtem ko pri profilu nismo opazili večjih kvalitativnih razlik. Analiza fenolov komercialnih odpadkov slovenske oljčne proizvodnje je pokazala na velik ekonomski potencial za izolacijo različnih tržno zanimivih fenolnih spojin.

Ključne besede: Proizvodnja oljčnega olja, Fenoli, Oljka, Koščica, Odpadni produkti proizvodnje oljčnega olja, UPLC-DAD-ESI-QTOF-HRMS analiza, Istrska belica *cv*., Čas in temperatura malaksacije, Topla voda, NaCl, Talk.

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ACKNOWLEDGMENTS

I would like to thank …

For the financial support:

- Slovenian Research Agency, ARRS (Ljubljana, Slovenia)
- World Federation of Scientists, WFS (Geneva, Switzerland)
- L'OREAL-UNESCO Women in Science (Ljubljana, Slovenia)

For the technical, scientific and moral support:

- People from Centre for Validation Technologies and Analytics (*National Institute of Chemistry Slovenia*)
- People from Experimental Olive Centre (*Agricultural Forestry Institution of Nova Gorica*)
- People from Kobis (*Company for Service and Validation Facilities*)
- People from Laboratory for Environmental Research (*University of Nova Gorica*)
- People from Metabolomics Technological Platform (*Research and Innovation Centre – Fondazione Edmund Mach, Italy*)
- People from Wine Research Centre (*University of Nova Gorica*)

For the kind donations of olive samples:

- People from Society of Olive Growers "Brda"
- People from Society of Olive Growers "Goriško"
- People from different olive mill facilities and independent olive growers

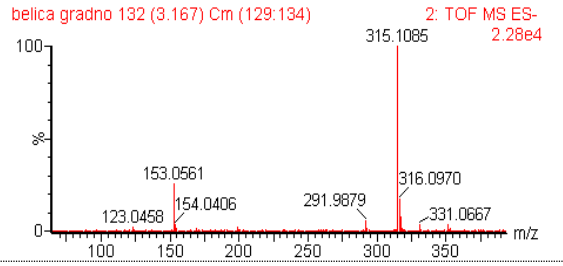
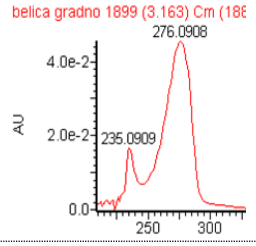
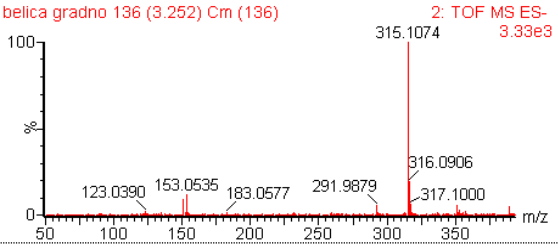
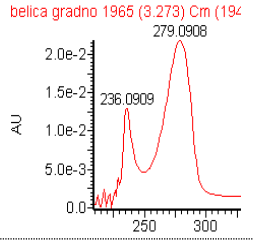
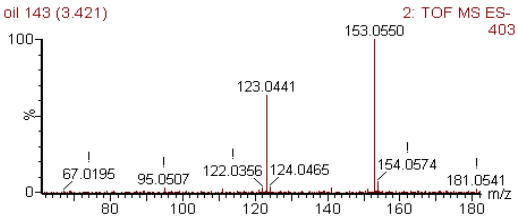
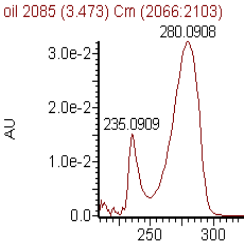
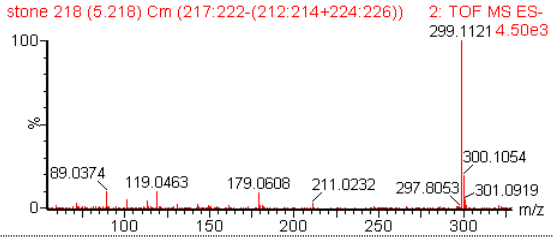
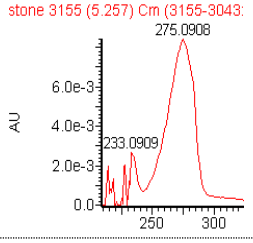
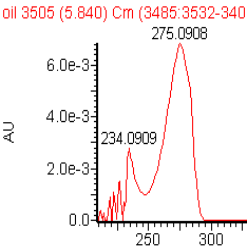
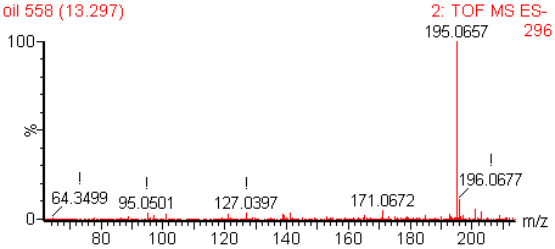
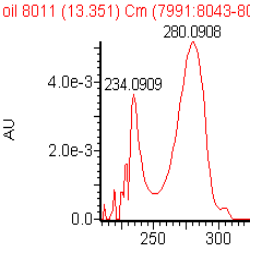
Now at the end, I can easily say that it is just rewarding to see how many great people I have met on this trip, and from whom I learned so much, not only about the work and science, but life itself in general. I will specifically not name all of them, though each deserves to be known, and each I will carry with me for a while, and some for a lifetime.

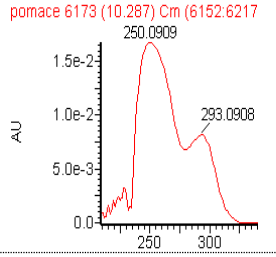
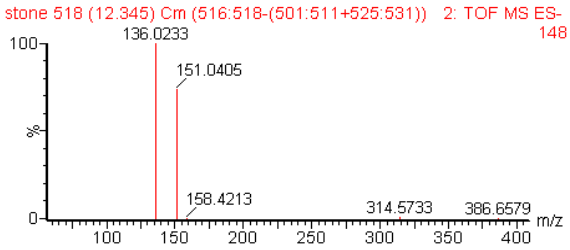
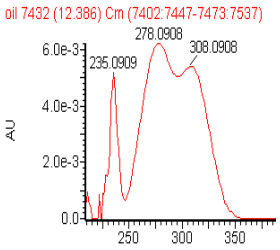
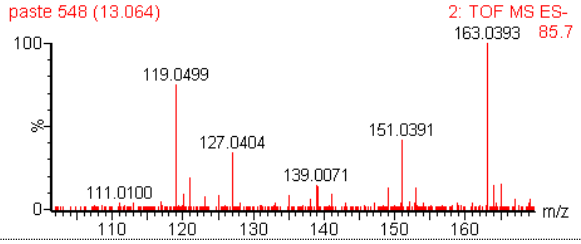
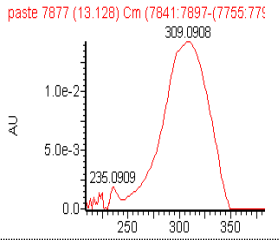
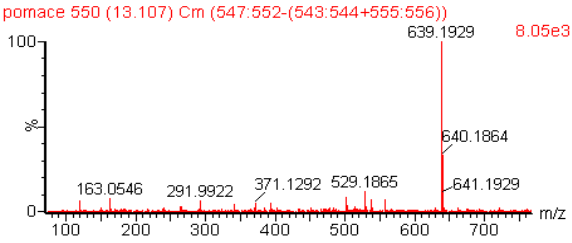
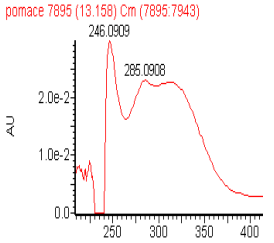
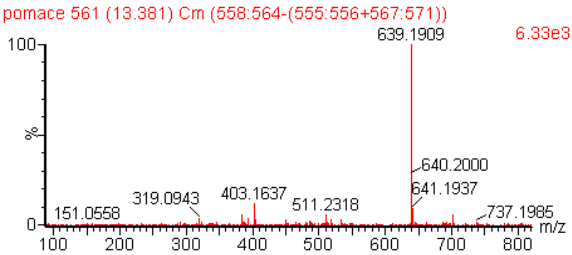
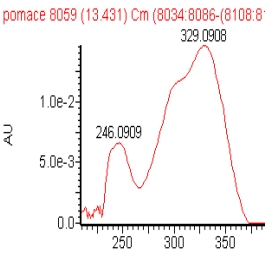
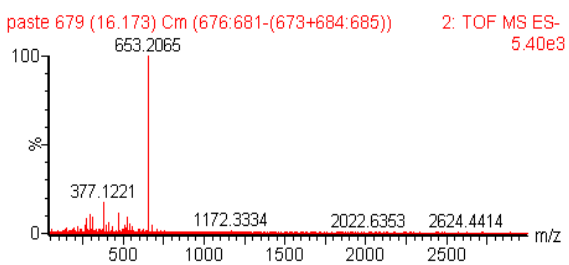
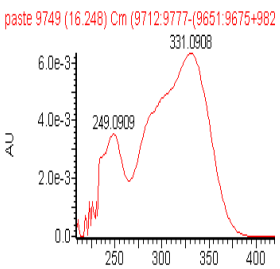
But yet, two groups of people I have to expose by expressing them my deepest gratitude ever – my dearest family and friends at home, and my ex co-workers for simply always being by my side. And Branka to you, for believing in me!

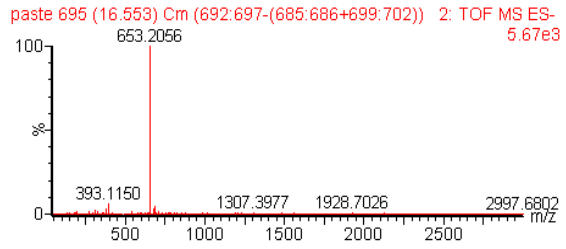
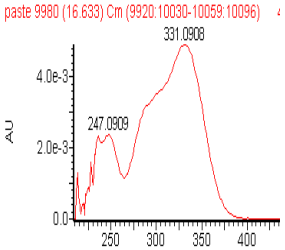
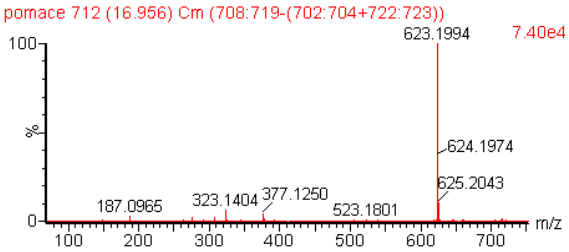
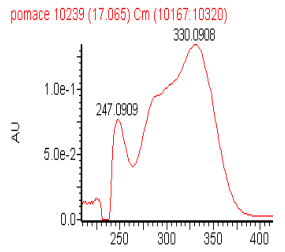
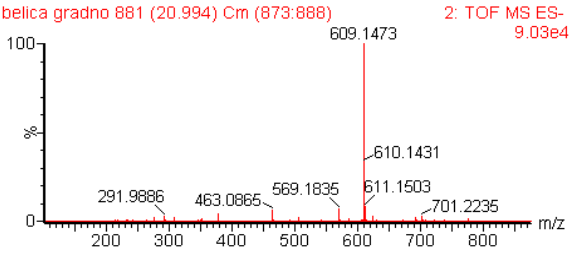
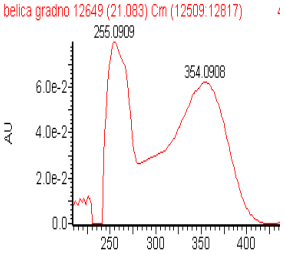
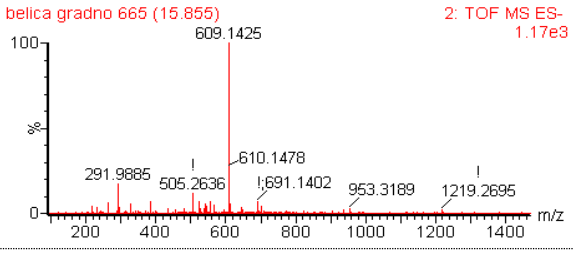
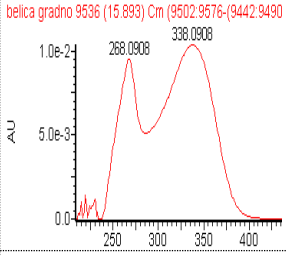
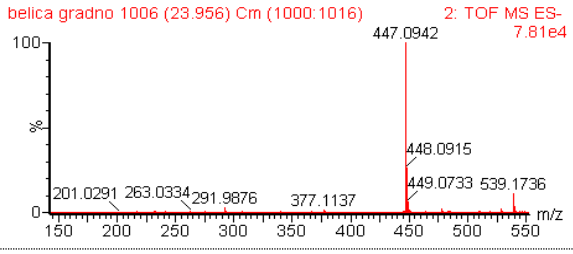
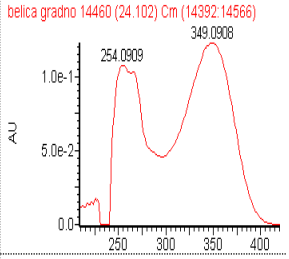
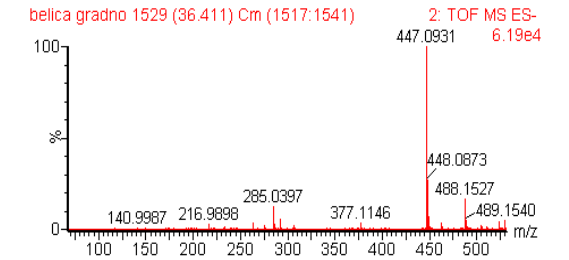
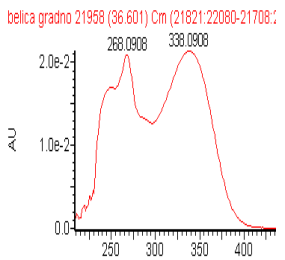
In overall, it has been an amazing journey in all the aspects, and again I would follow it mainly because of you – the people!

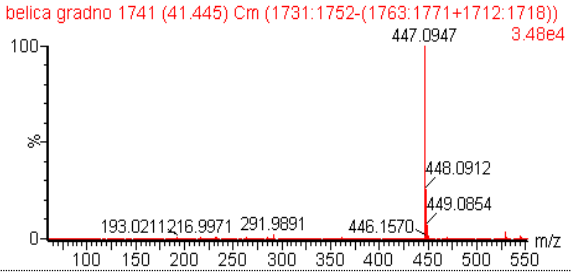
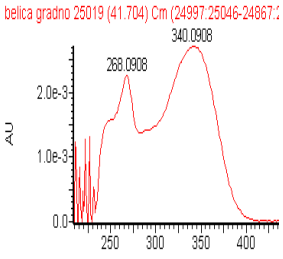
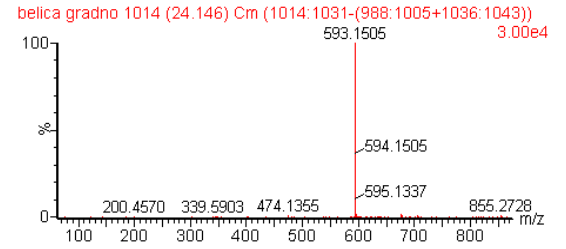
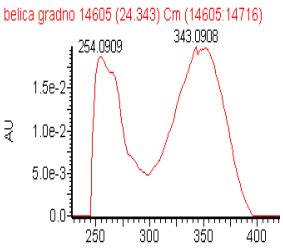
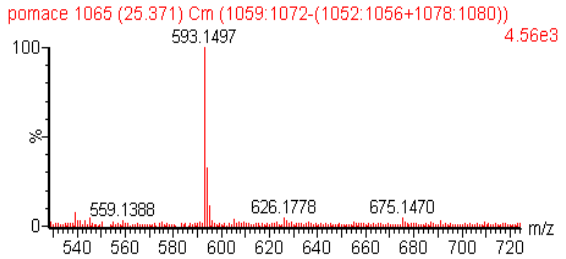
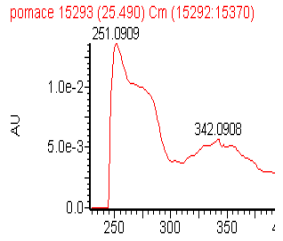
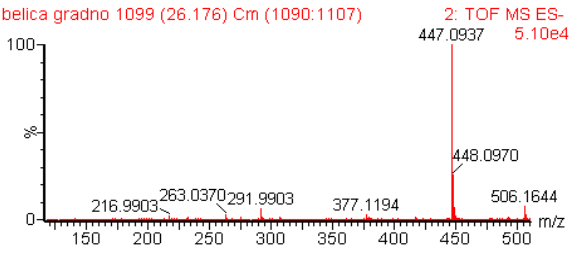
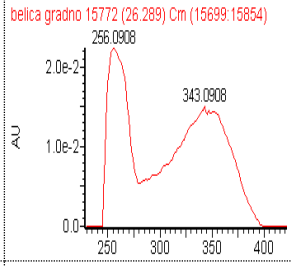
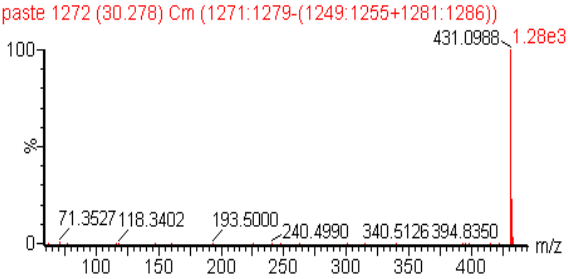
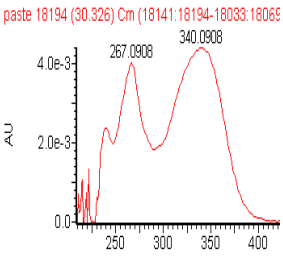
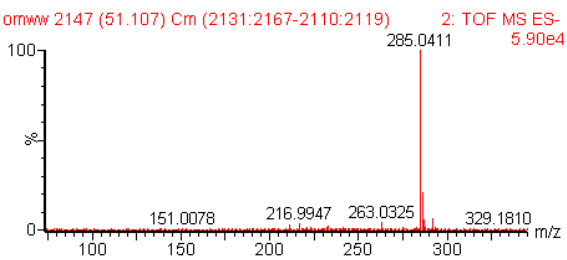
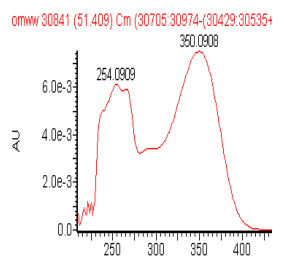
ANNEXES

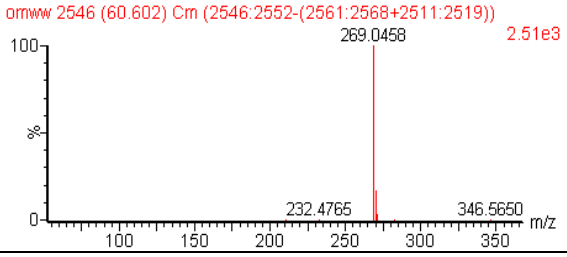
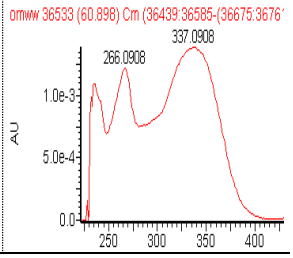
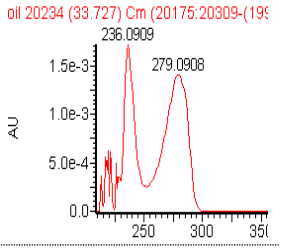
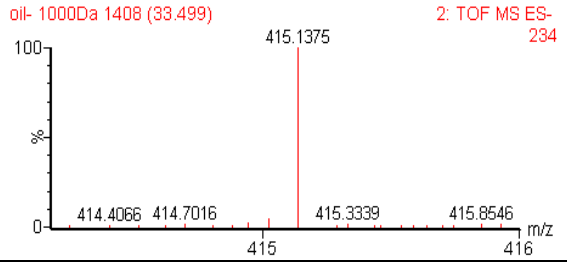
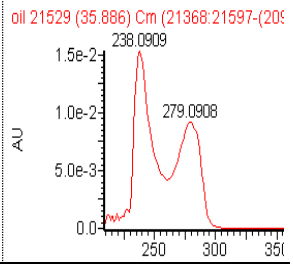
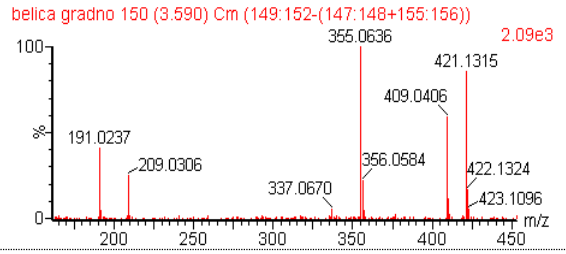
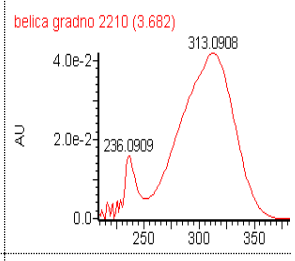
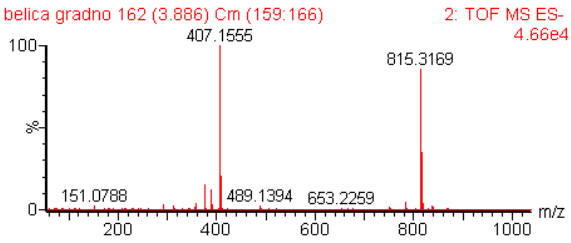
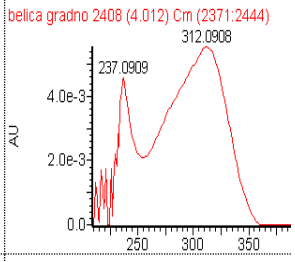
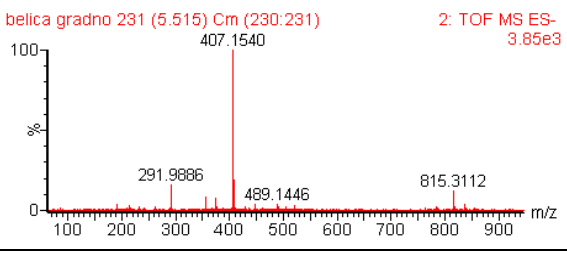
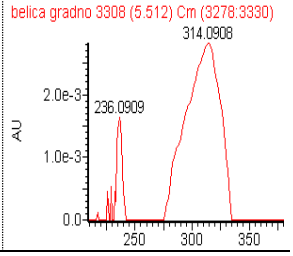
Annex A MS and UV-Vis spectra of olive phenols quantified in the doctoral thesis

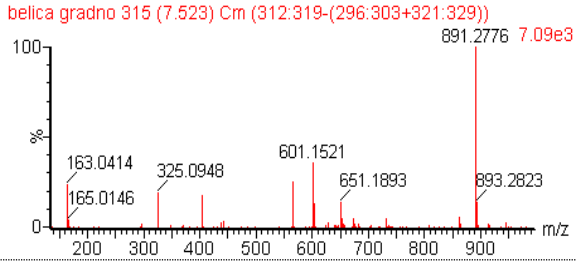
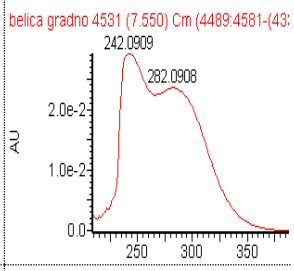
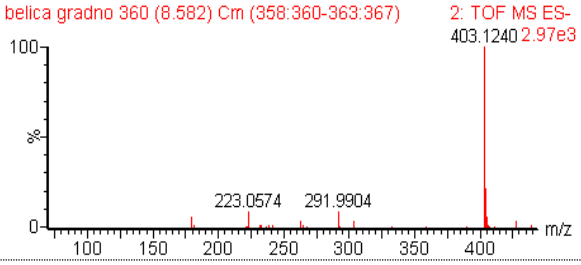
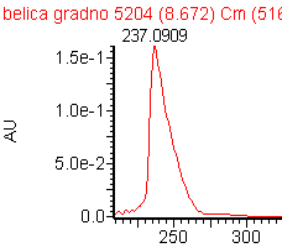
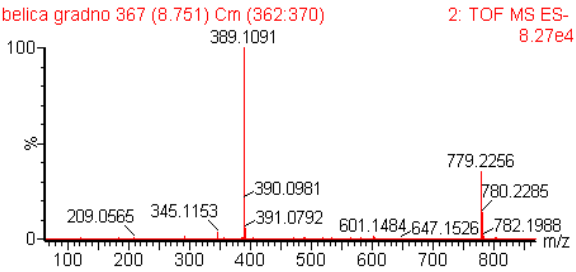
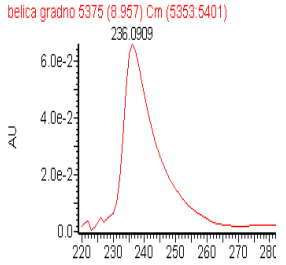
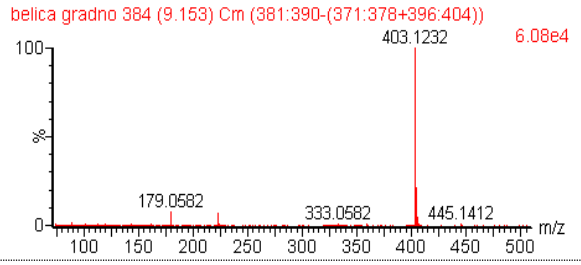
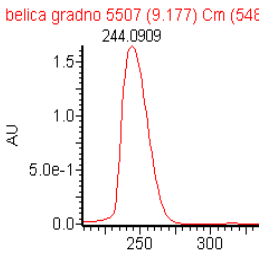
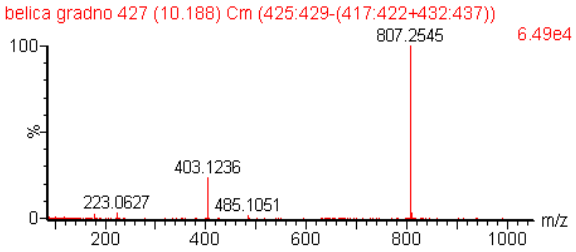
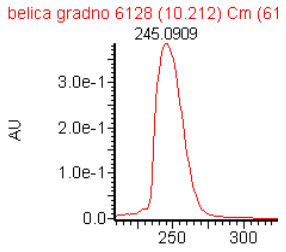
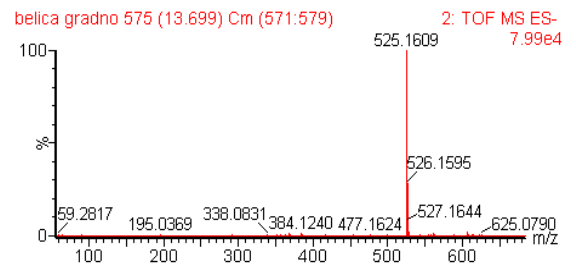
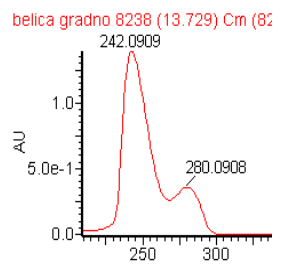
Class/Phenolic compound	MS spectrum at soft (TOF2) ionisation conditions	UV-Vis spectrum
Simple phenols		
Hydroxytyrosol glucoside m/z_{theor} 315.1080 (Table 6)		
Hydroxytyrosol-1-β-glucoside m/z_{theor} 315.1080 (Table 6)		
Hydroxytyrosol m/z_{theor} 153.0552 (Table 6)		
Tyrosol glucoside m/z_{theor} 299.1131 (Table 6)		
Tyrosol m/z_{theor} 137.0603 (Table 6)	Low ionisation	
Hydroxytyrosol acetate m/z_{theor} 195.0657 (Table 6)		

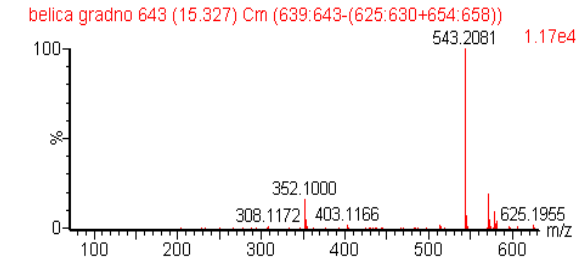
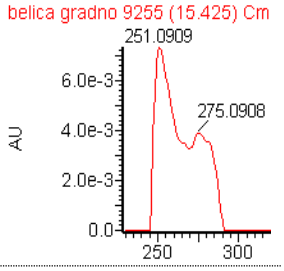
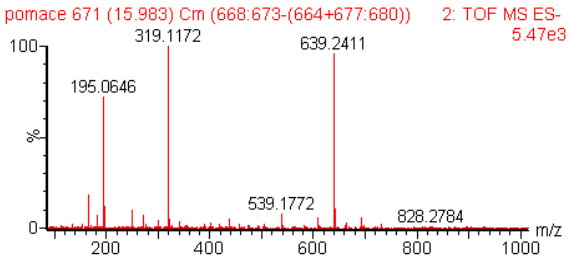
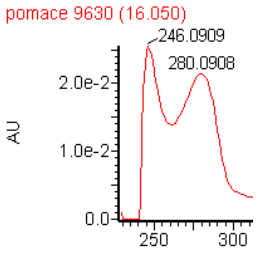
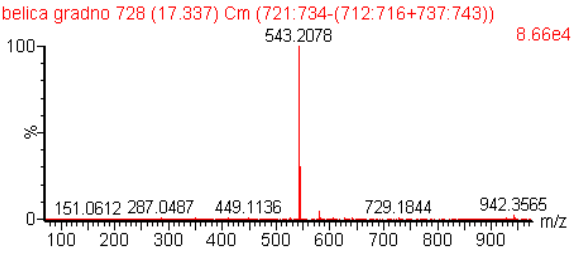
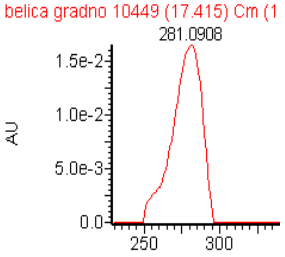
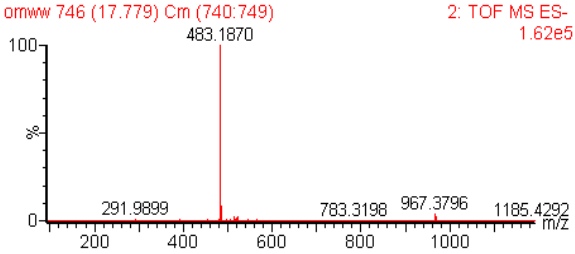
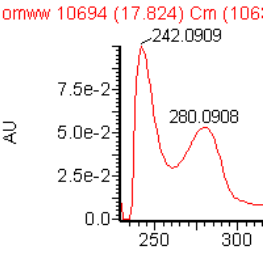
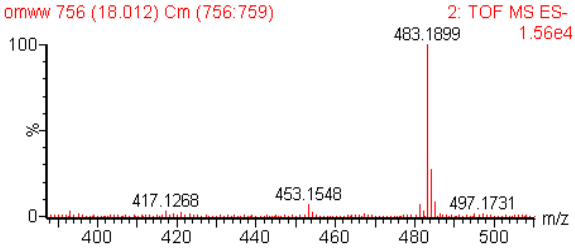
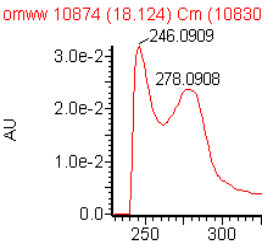
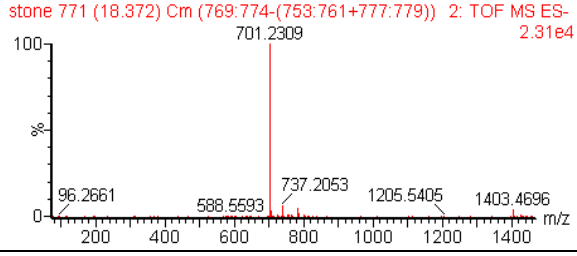
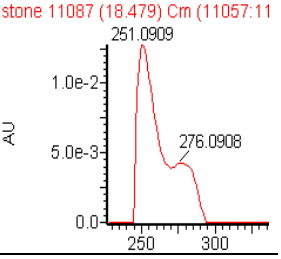
Class / Phenolic compound	MS spectrum at soft (TOF2) ionisation conditions	UV-Vis spectrum
Benzoic acids		
Vanillic acid *m/z* $_{theor}$ 167.0344 (Table 6)	Low ionisation	
Vanillin *m/z* $_{theor}$ 151.0395 (Table 6)		
Cinnamic acids		
p-Coumaric acid *m/z* $_{theor}$ 163.0395 (Table 6)		
β-OH verbascoside isomer 1 *m/z* $_{theor}$ 639.1925 (Table 6)		
β-OH verbascoside isomer 2 *m/z* $_{theor}$ 639.1925 (Table 6)		
β-Methyl-OH verbascoside isomer 1 *m/z* $_{theor}$ 653.2082 (Table 7)		

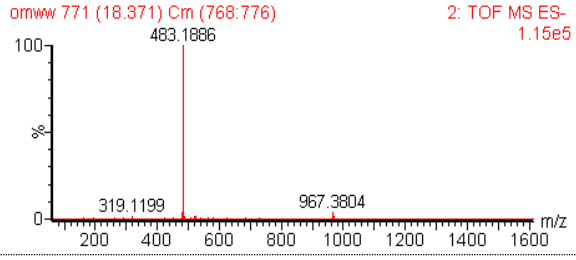
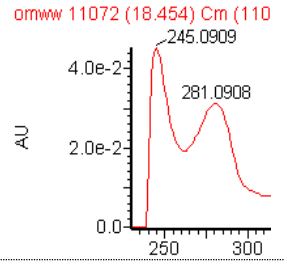
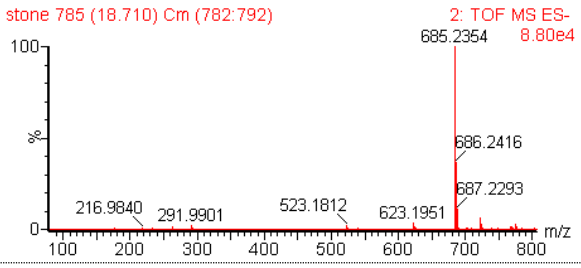
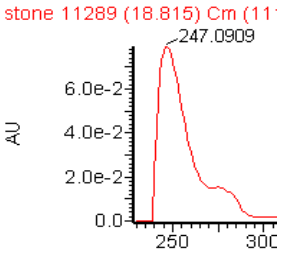
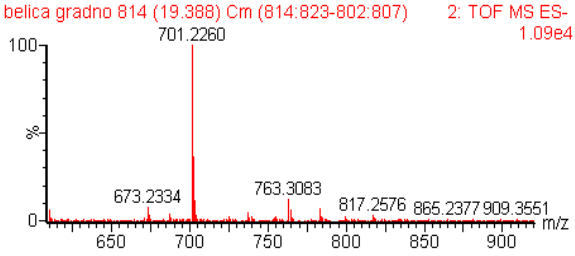
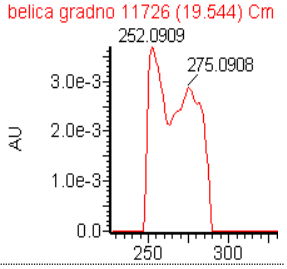
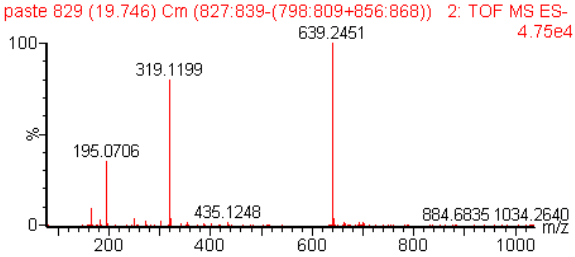
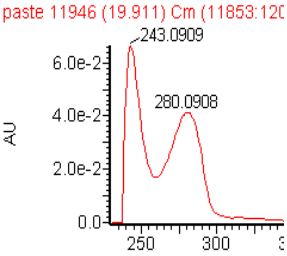
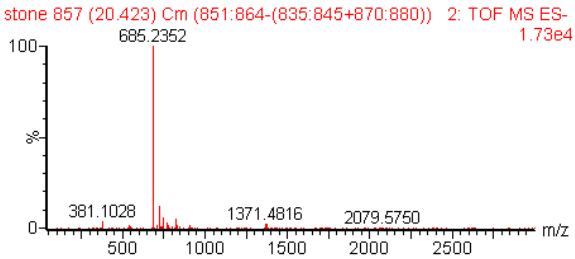
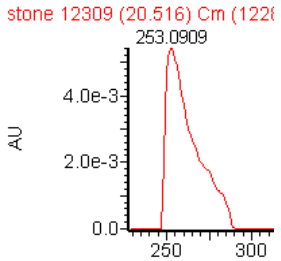
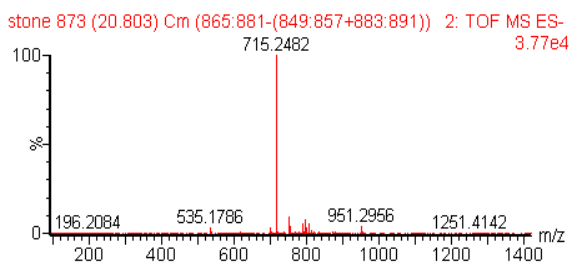
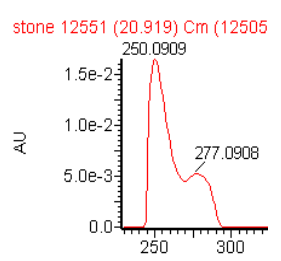
Class / Phenolic compound	MS spectrum at soft (TOF2) ionisation conditions	UV-Vis spectrum
Cinnamic acids *(Cont)*		
β-Methyl-OH verbascoside isomer 2 *m/z* $_{theor}$ 653.2082 (Table 7)		
Verbascoside *m/z* $_{theor}$ 623.1976 (Table 6)		
Flavonoids		
Rutin *m/z* $_{theor}$ 609.1456 (Table 6)		
Luteolin-4',7-*O*-diglucoside *m/z* $_{theor}$ 609.1456 (Table 6)		
Luteolin-7-*O*-glucoside *m/z* $_{theor}$ 447.0927 (Table 6)		
Luteolin-4'-*O*-glucoside *m/z* $_{theor}$ 447.0927 (Table 6)		

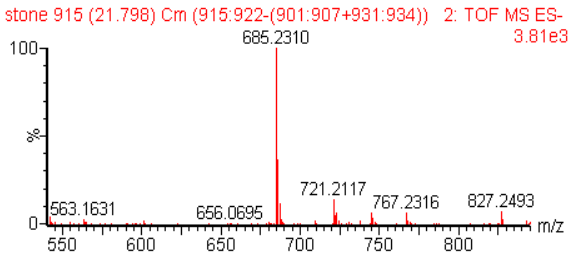
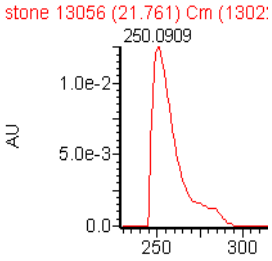
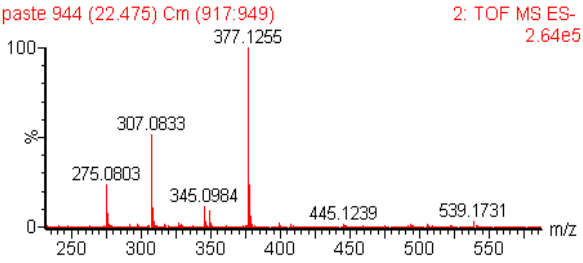
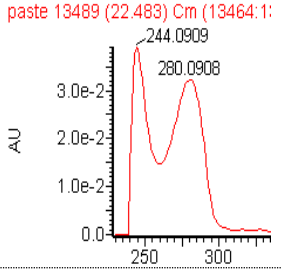
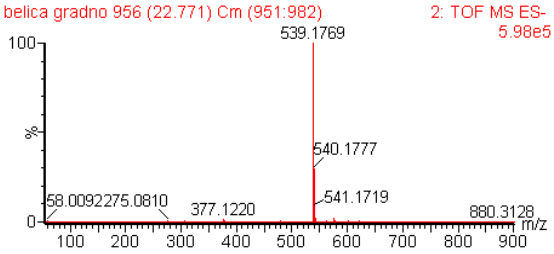
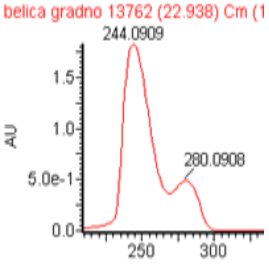
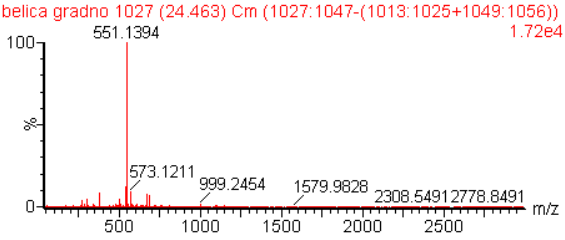
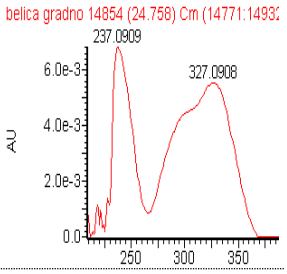
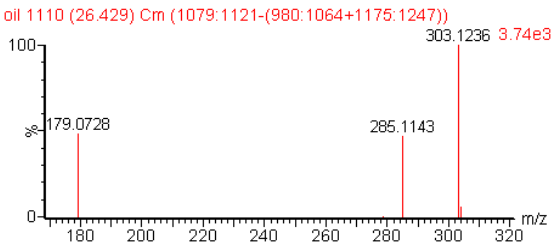
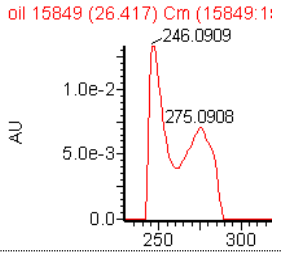
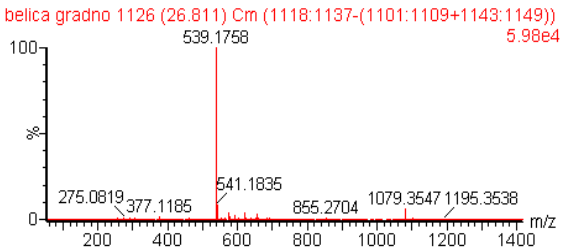
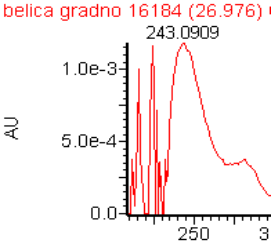
Class / Phenolic compound	MS spectrum at soft (TOF2) ionisation conditions	UV-Vis spectrum
Flavonoids *(Cont)*		
Luteolin -3'-*O*-glucoside *m/z* $_{theor}$ 447.0927 (Table 6)		
Luteolin rutinoside isomer 1 *m/z* $_{theor}$ 593.1506 (Table 6)		
Luteolin rutinoside isomer 2 *m/z* $_{theor}$ 593.1506 (Table 6)		
Quercitrin *m/z* $_{theor}$ 447.0927 (Table 6)		
Apigenin-7-*O*-glucoside *m/z* $_{theor}$ 431.0978 (Table 6)		
Luteolin *m/z* $_{theor}$ 285.0399 (Table 6)		

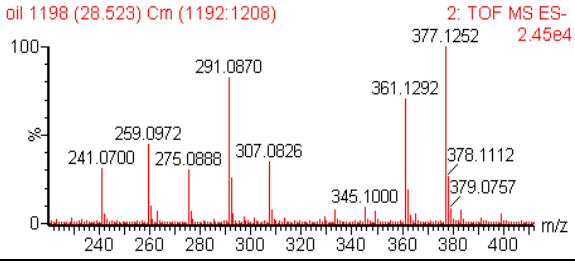
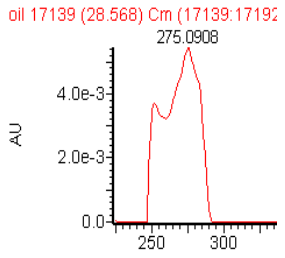
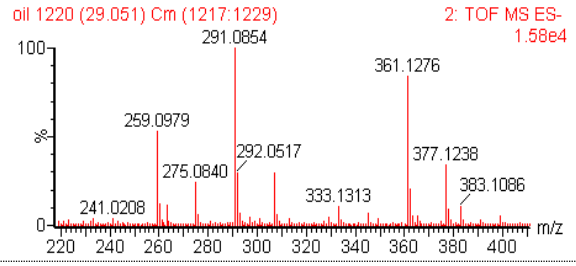
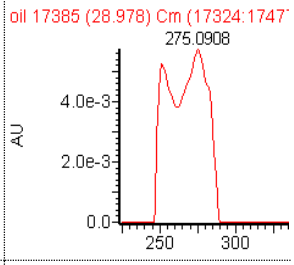
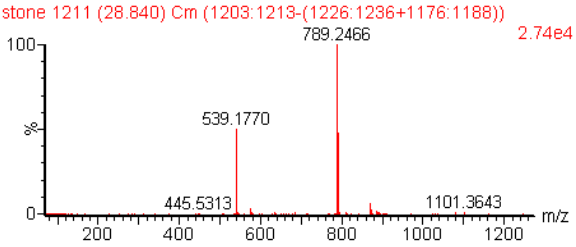
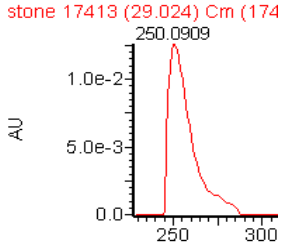
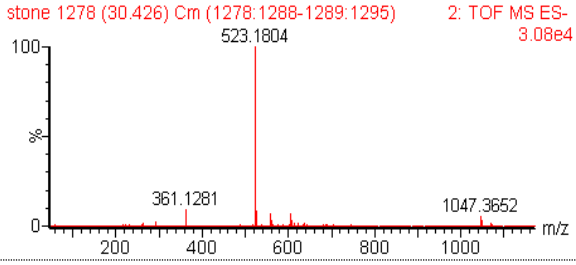
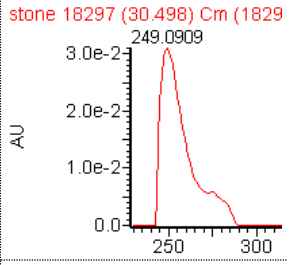
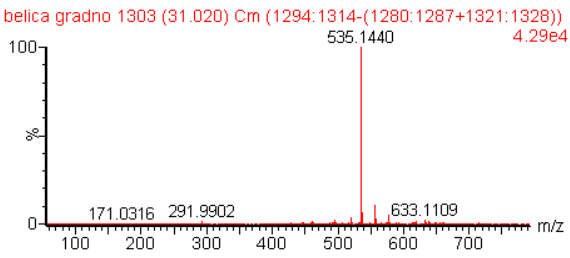
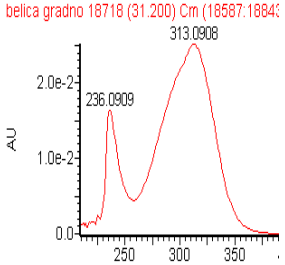
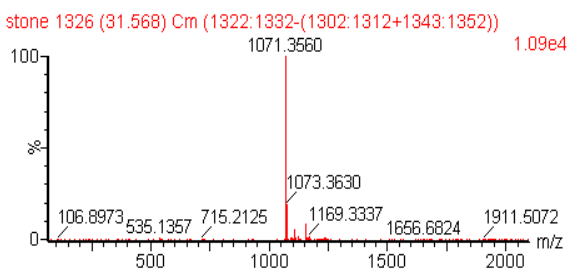
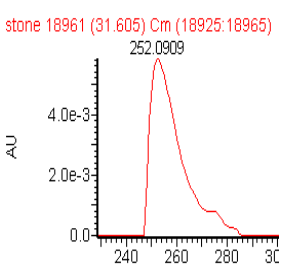
Class / Phenolic compound	MS spectrum at soft (TOF2) ionisation conditions	UV-Vis spectrum
Flavonoids *(Cont)*		
Apigenin *m/z* $_{theor}$ 269.0450 (Table 6)		
Lignans		
Pinoresinol *m/z* $_{theor}$ 357.1338 (Table 6)	Low ionisation	
Acetoxypinoresinol *m/z* $_{theor}$ 415.1393 (Table 6)		
Secoiridoids		
Unknown A (Table 7)		
Unknown 408 MW compound 1 *m/z* $_{theor}$ 407.1553 (Table 7)		
Unknown 408 MW compound 2 *m/z* $_{theor}$ 407.1553 (Table 7)		

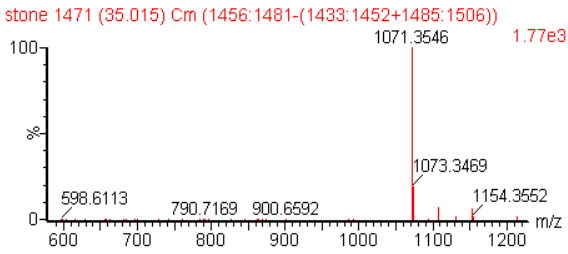
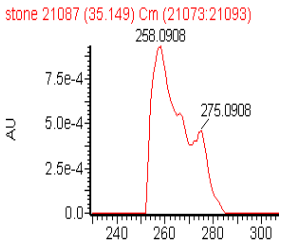
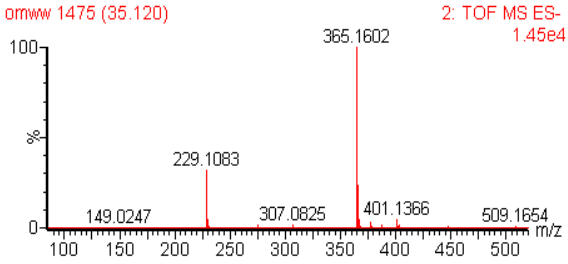
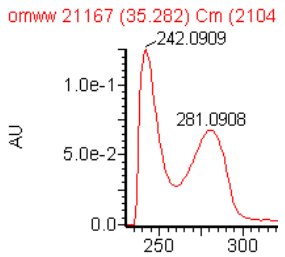
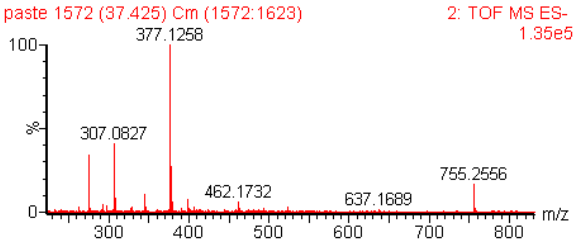
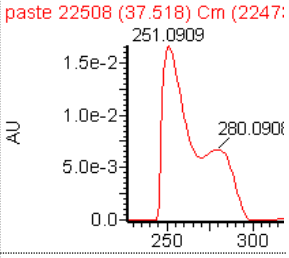
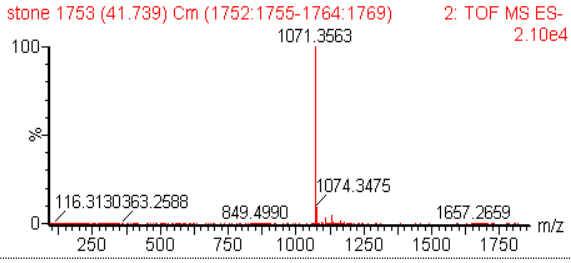
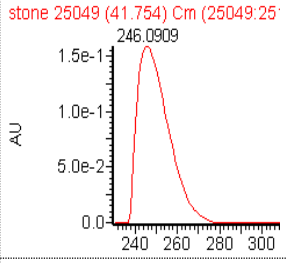
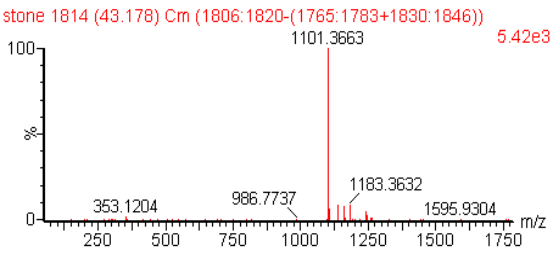
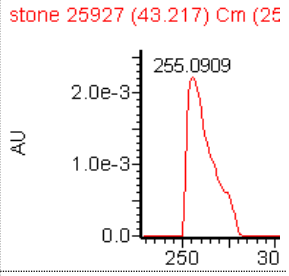
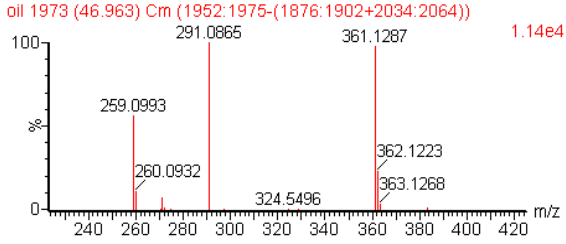
Class / Phenolic compound	MS spectrum at soft (TOF2) ionisation conditions	UV-Vis spectrum
Secoiridoids *(Cont)*		
Unknown B (Table 7)		
Elenolic acid glucoside isomer 1 *m/z* $_{theor}$ 403.1240 (Table 6)		
Secologanoside *m/z* $_{theor}$ 389.1084 (Table 6)		
Elenolic acid glucoside somer 2 *m/z* $_{theor}$ 403.1240 (Table 6)		
Elenolic acid glucoside isomer 3 *m/z* $_{theor}$ 403.1240 (Table 6)		
Demethyoleuropein *m/z* $_{theor}$ 525.1608 (Table 6)		

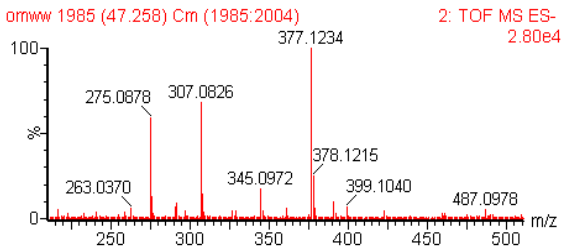
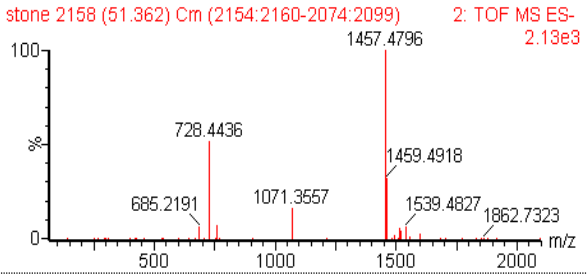
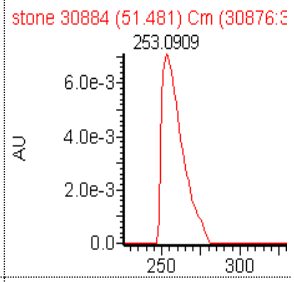
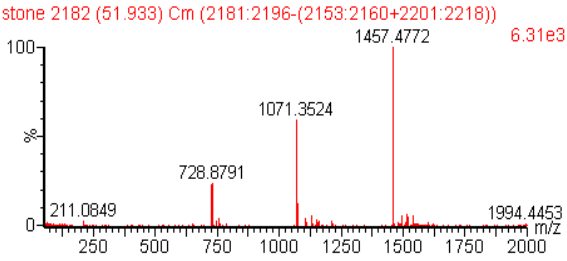
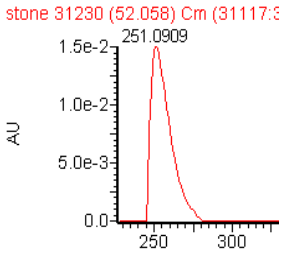
Class / Phenolic compound	MS spectrum at soft (TOF2) ionisation conditions	UV-Vis spectrum
Secoiridoids *(Cont)*		
Dihydro-oleuropein isomer 1 *m/z* $_{theor}$ 543.2078 (Table 6)		
3,4-DHPEA-EDA isomer 1 *m/z* $_{theor}$ 319.1182 (Table 6)		
Dihydro-oleuropein isomer 2 *m/z* $_{theor}$ 543.2078 (Table 6)		
Unknown 484 MW isomer 1 (Table 7)		
Unknown 484 MW isomer 2 (Table 7)		
Oleuropein diglucoside isomer 1 *m/z* $_{theor}$ 701.2293 (Table 6)		

Class / Phenolic compound	MS spectrum at soft (TOF2) ionisation conditions	UV-Vis spectrum
Secoiridoids *(Cont)*		
Unknown 484 MW isomer 3 (Table 7)		
Nüzhenide isomer 2 *m/z* $_{theor}$ 685.2344 (Table 6)		
Oleuropein diglucoside isomer 2 *m/z* $_{theor}$ 701.2293 (Table 6)		
3,4-DHPEA-EDA isomer 2 *m/z* $_{theor}$ 319.1182 (Table 6)		
Nüzhenide isomer 3 *m/z* $_{theor}$ 685.2344 (Table 6)		
Methoxynüzhenide isomer 2 *m/z* $_{theor}$ 715.2449 (Table 7)		

Class / Phenolic compound	MS spectrum at soft (TOF2) ionisation conditions	UV-Vis spectrum
Secoiridoids *(Cont)*		
Nüzhenide isomer 4 *m/z* $_{theor}$ 685.2344 (Table 6)		
Oleuropein aglycone isomer 1 *m/z* $_{theor}$ 377.1236 (Table 6)		
Oleuropein *m/z* $_{theor}$ 539.1765 (Table 6)		
Caffeoyl-6'-secologanoside *m/z* $_{theor}$ 551.1401 (Table 6)		
p-HPEA-EDA *m/z* $_{theor}$ 303.1232 (Table 6)		
Oleuroside *m/z* $_{theor}$ 539.1765 (Table 6)		

Class / Phenolic compound	MS spectrum at soft (TOF2) ionisation conditions	UV-Vis spectrum
Secoiridoids *(Cont)*		
Oleuropein + ligstroside aglycone *m/z* $_{theor}$ 377.1236 *m/z* $_{theor}$ 361.1287 (Table 6)		
Oleuropein + ligstroside aglycone *m/z* $_{theor}$ 377.1236 *m/z* $_{theor}$ 361.1287 (Table 6)		
Unknown C (Table 7)		
Ligstroside *m/z* $_{theor}$ 523.1816 (Table 6)		
Comselogoside *m/z* $_{theor}$ 535.1452 (Table 6)		
Nüzhenide 11-methyl oleoside isomer 1 *m/z* $_{theor}$ 1071.3557 (Table 6)		

Class / Phenolic compound	MS spectrum at soft (TOF2) ionisation conditions	UV-Vis spectrum
Secoiridoids *(Cont)*		
Nüzhenide 11-methyl oleoside isomer 2 *m/z* $_{theor}$ 1071.3557 (Table 6)		
Acetal of 3,4-DHPEa.EDA *m/z* $_{theor}$ 365.1600 (Table 6)		
Oleuropein aglycone isomer 2 *m/z* $_{theor}$ 377.1236 (Table 6)		
Nüzhenide 11-methyl oleoside isomer 4 *m/z* $_{theor}$ 1071.3557 (Table 6)		
Methoxynüzhenide 11-methyl oleoside isomer 2 *m/z* $_{theor}$ 1101.3662 (Table 7)		
Ligstroside aglycone isomer 2 *m/z* $_{theor}$ 361.1287 (Table 6)		

Class / Phenolic compound	MS spectrum at soft (TOF2) ionisation conditions	UV-Vis spectrum
Secoiridoids *(Cont)*		
Oleuropein aglycone isomer 3 *m/z* $_{theor}$ 377.1236 (Table 6)		
Nüzhenide di(11-methyl oleoside) isomer 1 *m/z* $_{theor}$ 1457.4770 (Table 6)		
Nüzhenide di(11-methyl oleoside) isomer 2 *m/z* $_{theor}$ 1457.4770 (Table 6)		

Annex B Published scientific papers

B1 Ultrasound-assisted solid liquid extraction of olive fruit (*Olea europaea*) phenolic compounds ("Reprinted from Ultrasound-assisted solid liquid extraction of olive fruit (*Olea europaea*) phenolic compounds, 123, Jerman T., Trebše P., Mozetič Vodopivec B., Food Chemistry, 175–182, Copyright (2010) with permission from Elsevier, licence number: 3396370369297").

Food Chemistry 123 (2010) 175–182

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Ultrasound-assisted solid liquid extraction (USLE) of olive fruit (*Olea europaea*) phenolic compounds

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ARTICLE INFO

Article history:
Received 20 April 2009
Received in revised form 27 December 2009
Accepted 1 April 2010

Keywords:
Phenolic compounds
Olive fruit (*Olea europaea*)
Ultrasound-assisted solid liquid extraction (USLE)
Optimisation
HPLC-DAD-FLD-MS/MS

ABSTRACT

A new method of ultrasound-assisted solid liquid extraction (USLE) of olive fruit phenols is described. Phenolics were extracted using high intensity probe ultrasonication and analysed by HPLC-DAD-FLD-MS/MS. Four USLE parameters – sonication time (4, 15, 20, 30 min), temperature (25, 45 °C), solvent composition (80%, 100% methanol) and extraction steps (1–5) were studied and optimised on the basis of nine major olive fruit phenols. A three-step extraction of 20 min with pure methanol (25 mL) at 45 °C was needed for sufficient phenol recoveries (94.1–98.7%) from 1.5 g of freeze-dried olive fruits. The proposed USLE method was more efficient in comparison to US bath and agitation, with up to 33% and 80% enhancement in the case of oleuropein, respectively. In addition, the overall method provided high selectivity, precision and sensitivity with LODs/LOQs ranging from 0.66–4.92 μg g^{-1} and 2.00–14.77 μg g^{-1} of olives DW, respectively.

1. Introduction

Olive fruit biophenols have been recognised as a potential nutraceutical targets for the food and pharmaceutical industries. Due to great importance associated with their wide range of bioactivities, there is a growing interest in developing of methods for their extraction, detection as well as quantification (Obied, Karuso, Prenzler, & Robards, 2007a). Numerous analytical procedures have been proposed for determination of olive fruit phenolic compounds employing various extraction, separation and quantification techniques (Ryan & Robards, 1998). In general, sample extraction procedure is regarded as a bottleneck of analytical method with a significant impact on the accuracy of results. Since conventional solvent extractions usually results in lower efficiencies (Chen et al., 2007) a complete quantitative extraction of polyphenols from a complex olive fruit matrix still presents a challenging analytical problem.

In recent years, various novel extraction techniques have been developed and the use of ultrasound sonication has opened up some great expectations with a promising results. Ultrasound-assisted extraction utilises acoustic cavitation to cause molecular movement of solvent and sample, offering advantages like improved efficiency, reduced extraction time, low solvent consumption and high level of automation as compared to conventional extraction techniques (Chen et al., 2007; Luque-García & Luque de Castro, 2003). Lately, a great number of studies have been published with different applications of US-assisted extraction to both organic and inorganic analytes in a wide variety of solid samples. The use of ultrasound has been also tested for the extraction of phenols from different plant materials employing various combinations of US power and frequency. Both, high and low power sonications have shown to be an efficient extraction tool providing higher phenol recoveries in comparison to conventional extraction methods. The use of high power ultrasonication (400–450 W) at 20–22 kHz was tested for plant matrices like red raspberries (Chen et al., 2007) and olive leaves (Japón-Luján, Luque-Rodríguez, & Luque de Castro, 2006), while lower US power (100–250 W) with 20–50 kHz was applied for polyphenols extraction from strawberries (Herrera & Luque de Castro, 2005), coconut shell powder (Rodrigues & Pinto, 2007), wheat bran (Wang, Sun, Cao, Tian, & Li, 2008) and others. Best to our knowledge, this kind of US extraction acceleration has not been used for the phenols extraction from complex olive fruits, known as a challenging matrix for analyte isolation (Ryan & Robards, 1998).

As seen from the literature, two common devices for an ultrasound application are employed in extractions, namely bath and the probe system. Although ultrasound baths are more widely used, an ultrasonic homogeniser (probe) offers an advantage of providing direct and therefore more efficient cavitation in the solution (Priego-Capote & Luque de Castro, 2004).

Abbreviation: USLE, ultrasound-assisted solid liquid extraction.

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doi:10.1016/j.foodchem.2010.04.006

In this paper, a new USLE method of phenols from olive fruit matrix using high intensity probe ultrasonication system has been described for the first time. The proposed method was optimised in terms of extraction time, temperature, solvent composition and the number of extraction steps needed for a sufficient phenols recoveries. The influence of high power probe sonication on extraction efficiency and chemical composition of phenol extracts was evaluated and compared to more commonly used source of US energy, the ultrasound bath and conventional extraction employing agitation, already known and well described for olive fruit phenols determination. In addition, the extract's major phenolics were identified and quantified by HPLC-DAD-FLD-MS/MS analysis and the overall method was characterised in terms of selectivity, precision, efficiency and sensitivity.

2. Material and methods

2.1. Plant material

Olive fruit samples (*Olea europaea*) Oblica *cv.* (ca. 5 kg) of green-reddish skin coloration were harvested on November 2007 at the end of their maturation period (RI = 3) in Croatian island, Hvar. The harvest maturity stage was determined using a subjective evaluation of olive fruits skin and pulp colours according to Morelló, Romero, Ramo, and Motilva (2005). All fruits were immediately frozen with liquid nitrogen and freeze-dried in a Kambič LIO-5P lyophilisator (Semič, Slovenia). The olive pulp was separated from the kernel, grounded into homogeneous powder with the aid of liquid nitrogen and stored at −25 °C until use.

2.2. Chemicals and solvents

Methanol (HPLC grade) and glacial acetic acid were purchased from Sigma Aldrich Company Ltd. (Gillingham, GB), while phenol standards of hydroxytyrosol, caffeic acid, *p*-coumaric acid, oleuropein, verbascoside, quercitrin, luteolin-7-O-glucoside and rutin were obtained from Extrasynthese (Genay, France). Double deionised water (DI) used in the experiments was purified on a Millipore Milli Q Plus Ultra-pure water system (Billerica, MA, USA).

2.3. Extraction methods of phenols from olive fruit sample

2.3.1. USLE – with ultrasonic homogeniser (probe)

For the experimental set up a 100 W and 30 kHz frequency LABSONIC®M ultrasonic homogeniser SARTORIUS (Gottingen, Germany) with amplitude of 100% was used. Freeze-dried olive fruit sample (1.5 g) was placed into PE centrifuge tube (50 mL, conical bottom) containing 25 mL of methanol and directly sonicated after different USLE parameters with a titanium tip probe (i.d. 3 mm) immersed 3 cm into solution. Four main variable extraction parameters were examined in the following order: sonication time (4, 10, 20 and 30 min), sonication temperature (0 °C, 25 °C), solvent composition (80%, 100% methanol) and extraction steps (varying from 1 to 5). While the efficiency of five consecutive extraction steps was tested for each single step, the influence of time, temperature and solvent concentration was evaluated based on two-step extraction. The phases after each extraction step were separated by centrifugation (9000 rpm, 5 min) using an Eppendorf centrifuge model 5804 (Hamburg, Germany) and combined methanol supernatants were evaluated for the extraction efficiency employing HPLC analysis.

2.3.2. USLE – with ultrasound bath

Extractions were carried out in a SONIS 4 GT ultrasound bath Iskra PIO (Šentjernej, Slovenia) operating at 30 kHz with providing power of 400 W. Phenolics were extracted using optimised extraction procedure obtained by ultrasonic homogeniser already described (Section 2.3.1). Olive fruit freeze-dried powder (1.5 g) was sonicated in 25 mL of pure methanol (20 min, at 25 °C). The extraction was repeated three times during each the temperature of homogenate varied from 43 to 45 °C. The homogenates of each extraction step were centrifuged (Eppendorf, 9000 rpm, 5 min), supernatants decanted, merged and diluted with methanol to 100 mL. Prepared extracts were put in a screw-capped dark glass container and stored in freezer (−25 °C) until further HPLC analysis.

2.3.3. Conventional extraction method – with agitation

For the purpose of comparison, the extraction employing only freeze-dried olive sample agitation in methanol was performed as well. Briefly, 1.5 g of freeze-dried olive sample was extracted (3 × 20 min) with 25 mL of methanol (25 °C) while, agitating, using a VIBROMIX 313 EVT (Železniki, Slovenia). Combined methanolic extracts were quantitatively transferred into 100 mL volumetric flask, diluted with methanol and stored as explained above (Section 2.3.2) prior HPLC determination.

The aliquots of methanolic extracts were concentrated (5–10 times) using a Heidolph LABOROTA 4000 rotary evaporator (Schwabach, Germany) at 35 °C. After successive methanol evaporation, a dry residue was re-dissolved in 1 mL of acidic HPLC eluent (H_2O/CH_3COOH, 95:5, v/v), filtered through 0.45 μm PTFE CHROMAFIL®O filters (Macherey–Nagel, Dűren, Germany) and analysed by HPLC within 2 h after preparation.

2.4. HPLC-UV/VIS(DAD)-FLD and LC-MS/MS analysis

The combination of reverse-phase HPLC-UV/VIS(DAD)-FLD and LC-MS/MS was employed to determine the composition of olive fruit phenols in acidic filtrates using the same gradient system and HPLC column; Waters Spherisorb ODS-2, (5 μm, 250 × 4.6 mm) attached to Supelco security guard (10 × 4.1 mm). Chromatographic phenols separation at flow rate of 1 mL min^{-1} was achieved by gradient elution consisted of solvent A (H_2O/CH_3COOH, 95:5, v/v) and HPLC grade methanol as B, similar to the one of Morelló et al. (2005) with some modifications in solvent pH and gradient mode employed as follows: 5% B (0 min), 10% B (3 min), 25% B (18 min), 29% B (19 min), 30% B (24 min), 31% B (30 min), 35% B (31 min), 45% B (41 min), 55% B (51 min), 65% B (61 min), 100% B (67 min) and 5% B (70 min).

HPLC-UV/VIS(DAD)-FLD analysis were carried out using a HP 1100 liquid chromatograph (Agilent Technologies, Santa Clara, USA) equipped with a diode-array UV/VIS(DAD) detector (190–600 nm) and fluorescence (FLD) detector ($\lambda_{excitation/emission}$ 280/330 nm). The sample volume of 20 μL was injected and DAD signals were recorded at 280 nm (simple phenols, secoiridoids), 320 nm (hydroxycinnamic acids) and 365 nm (flavonoids) at 25 °C with a 15 min re-equilibration between individual runs. Phenols identifications were performed with a tandem mass spectrometry analysis (LC-MS/MS) using Perkin Elmer Series 2000 (Schelton, CT, USA) linked to 3200 Q TRAP LC/MS/MS system equipped with electro spray ion (ESI) source from Applied Biosystems/MDS Sciex (Foster City, CA, USA). Mass spectrometric scans were performed after 15 μL injection of phenol acidic extract in positive and negative ion modes, scanning from *m/z* 100 to *m/z* 1200 in 1 s. An ESI source voltage of 4500 V was applied and the Turbo Ion Spray temperature was kept at 400 °C.

Identification of phenolics in olive fruit samples was obtained by comparison of retention times (R_t), UV–VIS, FLD and ESI-MS spectra with those of authentic standards when available while the tentative identity of other compounds was confirmed by comparison of UV–VIS and ESI-MS^2 spectra with those from literature

Table 1
Screening data of major phenolic compounds in olive fruit samples (*Olea europaea cv.* Oblica).

Peak No.	Phenolic compounds	HPLC-DAD-FLD				ESI-LC-MS		
		R_t (min)	λ_{max} (nm)	Fluorescence	Calibration equation	MW (M)	Major ESI$^-$ peaks $[M-H]^-$	Major ESI$^+$ peaks $[M-H]^+$
I	Hydroxytyrosol glucoside	7.3	236, **278**	No	$y = 17.104x - 10.419$ $R^2 = 0.9998$	316	315, 153, 121	317, 155
II	Demethyloleuropein	25.3	**242**, 280	No	$y = 4.3503x + 41.59$ $R^2 = 1.0000$	526	525, 389, 319, 183	549, 527, 381, 347, 137
3	Oleuropein	37.3	**242**, 280	No	$y = 4.3503x + 41.59$ $R^2 = 1.0000$	540	539, 377, 307, 275, 223	563, 379, 361, 287, 225, 165, 137
4	Verbascoside	28.4	240, **332**	No	$y' = 29.297x - 96.444$ $R^2 = 0.9999$	624	623, 461, 161	647, 523, 471, 325, 163
V	Caffeoyl-6′-secologanoside	39.0	232, **328**	No	$y' = 90.109x - 94.524$ $R^2 = 1.0000$	552	551, 507, 389, 281, 251, 179, 161	553, 163
6	Quercitrin	43.1	**256**, 350	No	$y' = 31.201x - 38.051$ $R^2 = 0.9998$	448	447, 301	449
VII	Comselogoside	45.1	238, **314**	No	$y' = 159.75x - 66.176$ $R^2 = 0.9997$	536	535, 491, 389, 345, 265, 163, 145	559, 537, 476, 309, 287, 165, 147
8	Luteolin-7-O-glucoside	39.9	255, **348**	No	$y'' = 35.177x - 328.66$ $R^2 = 0.9997$	448	447, 285	449, 287
9	Rutin	40.6	**256**, 356	No	$y'' = 28.594x + 721.45$ $R^2 = 0.9919$	610	609, 301	633, 611, 465, 303, 165

Roman numbers: non-commercially available phenol standards; arabic numbers: commercially available phenol standards. y – detection at 280 nm, y' – detection at 320 nm, y'' – detection at 365 nm. Maximum UV band indicated in bold.

(Obied, Bedgood Jr., Prenzler, & Robards, 2007b; Savarese, De Marco, & Sacchi, 2007).

Quantification of identified phenolic compounds was carried out by external 6-point calibration (from 5 to 6000 μg mL^{-1}) with authentic standards when available, while hydroxytyrosol glucoside, demethyloleuropein, caffeoyl-6′-secologanoside and comselogoside were expressed as hydroxytyrosol, oleuropein, caffeic and *p*-coumaric acid equivalents, respectively (Table 1).

Standard stock solutions were prepared by dissolving standards (1–6 mg) in a 1 mL HPLC grade methanol. Calibration concentrations were prepared by complete evaporation of methanol from aliquots (vacuum, 35 °C) that were in turn re-dissolved in HPLC eluent (H_2O/CH_3COOH, 95:5, v/v) in the range as expected for each compound in olive fruits; 5–500 μg mL^{-1} (hydroxytyrosol, quercitrin, caffeic and *p*-coumaric acid), 25–1000 μg mL^{-1} (verbascoside, luteolin-7-O-glucoside, rutin) and 75–6000 μg mL^{-1} (oleuropein). All standard solutions were filtered prior subjected to HPLC analysis.

2.5. USLE method characterisation

The selectivity of the method was assessed as reported by Abad-García et al. (2007). Chromatograms of sample extracts were compared to those of blank and standards available, considering R_t, peak shape and spectral purity of each chromatographic peak.

The precision of the method was evaluated by measuring phenols peak areas of the same sample extract (25 °C) with intra- and inter-batch precision estimations – within one ($n = 3$) and between days within a two-week period ($n = 3$), while the sample between analysis was stored in freezer (−25 °C).

A standard addition method with spiked olive matrices was used for the evaluation of phenols extraction efficiencies in terms of phenol recoveries. The olive matrix was prepared by extracting phenols from freeze-dried olive samples using proposed USLE method (3 × 20 min; 44 ± 2 °C; pure methanol) in order to reduce the matrices phenol content to trace amounts. The remaining crude material (olive matrix) considered as blank in recovery and sensitivity studies (15 g) was dried ($T = 40$ °C) and analysed by HPLC for a potential phenols presence. Then, known amounts of phenols through successive dilutions of methanol standard mix solution (mg mL^{-1}) consisting of oleuropein (3.36), verbascoside (0.25), rutin (1.10), luteolin-7-O-glucoside (3.70), quercitrin (1.30) and caffeic acid (2.96) were added to dried olive matrix and submitted to a complete USLE procedure proposed. Samples were analysed in triplicates before and after standard additions and recoveries were expressed as means ± RSD (%).

The sensitivity of the method was evaluated as described by De Sousa et al. (2009) where LODs and LOQs were calculated from y-intercept standard deviations (S_b) and slopes (a) of calibration curves prepared by adding aliquots of methanol standard mix solution to olive matrix in the concentration ranges (μg g^{-1} DW) close to LOQs expected for each phenol: oleuropein (6.2–62.0), verbascoside (2.5–25.0), rutin (1.1–11.0), luteolin-7-O-glucoside (3.7–37.0) and quercitrin (1.2–12.5). The parameters were calculated by using equations: LOD = 3.3 × Sb/a and LOQ = 10 × Sb/a.

2.6. Statistical analysis

All experimental results were performed at least in triplicate ($n \geqslant 3$) and the data are expressed as means ± SD. The statistical significances of process parameters were evaluated by analysis of variance (ANOVA) using STATGRAPHICS Plus 4.0 (Manugistics Inc., Rockville, MD). Duncan's multiple range test (MRT) was used to discriminate among the means at 95% of confidence level, where P values < 0.05 were regarded as significant, P values < 0.01 as very significant and P values > 0.05 as not significant.

3. Results and discussion

3.1. Determination of olive fruit phenolic compounds

3.1.1. Identification and quantification of major phenols in olive fruit samples

The HPLC-DAD profile (Fig. 2) showed several peaks corresponding to different phenolics, among which nine of them were identified using a RP-HPLC coupled with UV/VIS(DAD), FLD and ESI-MS/MS detector under chromatographic conditions described (Section 2.4). This kind of systematic identification approach provides a full range of analytical data needed to screen complex natural matrices such are olive fruits and related products (Obied et al., 2007b). In addition to UV/VIS(DAD) and MS detection commonly used in phenols HPLC analysis, the compounds were as-

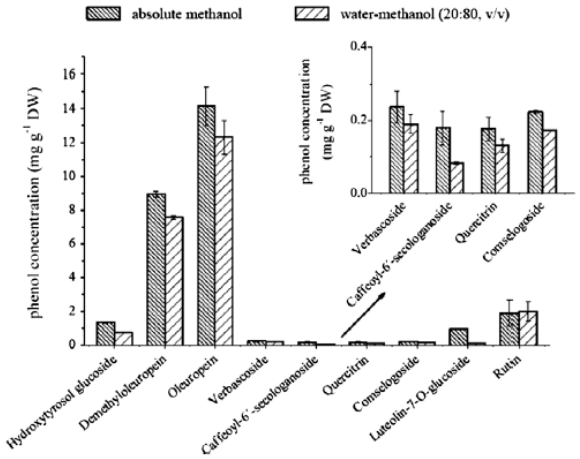

Fig. 1. The effect of solvent composition on olive fruit phenol yields.

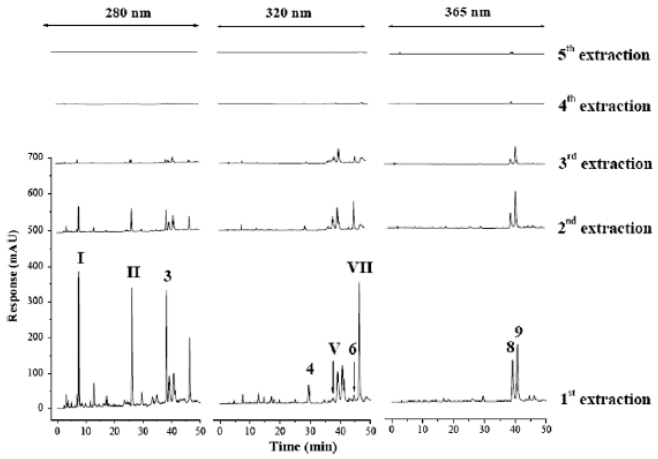

Fig. 2. The effect of sequential extraction steps on olive fruit phenol yields (the number of peaks are referring to Table 1).

signed with FLD as already proposed by others (Selvaggini et al., 2006) mainly due to higher sensitivity and selectivity in comparison to DAD. The $\lambda_{excitation/emission}$ 280/330 nm was chosen according to Ryan, Robards, and Lavee (1999) since they present the conditions of maximum fluorescence in olive extracts (Obied et al., 2007b). All screening results of nine main phenolics identified in *O. europaea* Oblica *cv.* are presented in Table 1.

Five phenolic compounds: oleuropein, verbascoside, quercitrin, luteolin-7-O-glucoside and rutin were identified by comparison of screening data with authentic standards, while a tentative identity of others was based on UV–VIS and ESI-MS/MS spectra by comparison with the data from literature. Identification of unknown phenolics was primarily based on the search of main pseudomolecular ion in both negative $[M-H]^-$ and positive $[M-H]^+$ ion ESI, and further confirmed by the MS/MS fragmentation profile of their $[M-H]^-$ ions.

The mass spectra of peak I (R_t = 7.3) displayed major signals at *m/z* 315, 154 and 121 in the negative ESI, while in the positive at *m/z* values of 317 and 155, corresponding to that of hydroxytyrosol glucoside previously found in olive fruit extracts (Obied et al., 2007b; Savarese et al., 2007). Moreover, the compound had λ_{max} at 278 and 236 nm, quite similar to hydroxytyrosol (λ_{max} = 280 and 240 nm) suggesting the presence of hydroxytyrosol derivative. In spite of already defined isomers of hydroxytyrosol glucosides

Table 2
Olive fruit phenol contents (mg g^{-1} DW) in different extraction times.

Phenolic compounds	Phenol extraction yield (mg g^{-1} DW)				*P* value
	4 min	10 min	20 min	30 min	
Hydroxytyrosol glucoside	0.175 ± 0.021^{d}	0.916 ± 0.033^{c}	1.352 ± 0.048^{a}	1.201 ± 0.071^{b}	<0.01
Demethyloleuropein	1.352 ± 0.078^{e}	8.423 ± 0.143^{c}	8.936 ± 0.158^{b}	9.334 ± 0.156^{a}	<0.01
Oleuropein	1.523 ± 0.027^{b}	14.154 ± 1.141^{a}	14.161 ± 1.138^{a}	13.834 ± 1.016^{a}	<0.01
Verbascoside	0.043 ± 0.002^{c}	0.225 ± 0.034^{ab}	0.237 ± 0.045^{ab}	0.274 ± 0.047^{a}	<0.01
Caffeoyl-6′-secologanoside	0.029 ± 0.001^{c}	0.109 ± 0.017^{b}	0.179 ± 0.047^{a}	0.176 ± 0.039^{a}	<0.01
Quercitrin	ND	0.124 ± 0.028^{ab}	0.177 ± 0.032^{a}	0.140 ± 0.028^{a}	<0.01
Comselogoside	0.037 ± 0.001^{d}	0.210 ± 0.002^{c}	0.223 ± 0.004^{a}	0.216 ± 0.002^{b}	<0.01
Luteolin-7-O-glucoside	0.110 ± 0.011^{c}	0.635 ± 0.034^{b}	$0.714 \pm 0,032^{a}$	0.688 ± 0.024^{a}	<0.01
Rutin	0.150 ± 0.007^{b}	1.638 ± 0.397^{a}	1.912 ± 0.774^{a}	1.878 ± 0.536^{a}	<0.01

All values are means ± SD of triplicates expressed as mg g^{-1} DW. Values marked with the same letter are not significantly different; α (error probability) = 5%. *P* values < 0.05 significant; $P < 0.01$ very significant; $P > 0.05$ not significant. ND: not detected. Extraction conditions: 44 ± 2 °C; pure methanol, two-step extraction.

Table 3
Comparison of different extraction methods; USLE – with homogeniser, US bath and agitation on olive fruit phenol content (mg g^{-1} DW).

Phenolic compounds	Phenol extraction yields (mg g^{-1} DW)			*P* value
	Ultrasonic homogeniser[f]	Ultrasound bath[g]	Agitation[h]	
Hydroxytyrosol glucoside	1.333 ± 0.130^{a}	1.316 ± 0.126^{ab}	1.101 ± 0.068^{b}	>0.05
Demethyloleuropein	9.889 ± 0.146^{a}	7.965 ± 0.588^{b}	7.452 ± 0.220^{b}	<0.01
Oleuropein	16.563 ± 1.720^{a}	12.278 ± 1.115^{b}	9.208 ± 0.317^{c}	<0.01
Verbascoside	0.284 ± 0.035^{a}	0.176 ± 0.060^{b}	0.159 ± 0.048^{b}	<0.05
Caffeoyl-6′-secologanoside	0.176 ± 0.010^{a}	0.150 ± 0.014^{b}	0.124 ± 0.011^{c}	<0.01
Quercitrin	0.166 ± 0.034^{a}	0.154 ± 0.011^{ab}	0.108 ± 0.018^{b}	<0.05
Comselogoside	0.233 ± 0.030^{a}	0.207 ± 0.034^{ab}	0.173 ± 0.021^{b}	>0.05
Luteolin-7-O-glucoside	0.748 ± 0.041^{a}	0.558 ± 0.034^{b}	0.519 ± 0.032^{b}	<0.01
Rutin	2.342 ± 0.433^{a}	1.434 ± 0.324^{b}	1.360 ± 0.087^{b}	<0.05

All values are means ± SD of triplicates expressed as mg g^{-1} DW. Values marked with the same letter within the row are not significantly different; α (error probability) = 5%. *P* values < 0.05 significant; $P < 0.01$ very significant; $P > 0.05$ not significant. ND: not detected. Extraction conditions: 20 min; pure methanol, three-step extraction, T^{f} = 44 ± 2 °C; T^{g} = 44 ± 1 °C; T^{h} = 25 ± 1 °C.

found in olive-related samples (Obied et al., 2007b), obtained data did not allow us to define the glycosidation position, therefore peak I can only be tentatively identified as hydroxytyrosol glucoside, respectively.

The compound eluting at 25.3 min (peak II) showed an intense ion at *m/z* 525 with three fragments at *m/z* values of 389, 319 and 183 in NIM, respectively. This MS/MS fragmentation profile is consistent with the scheme suggested by Savarese et al. (2007) for demethyloleuropein found in olive pulp phenolic extracts Pisciottana *cv*. Furthermore, the compound presented UV–VIS spectral characteristics closely related to that of oleuropein (λ_{max} of 242 and 280 nm) suggesting that peak II is a derivative of oleuropein, like demethyloleuropein known as oleuropein glucosylated derivative formed during olive fruit maturation (Ryan, Antolovich, Prenzler, Robards, & Lavee, 2002).

Peak V with R_t = 28.4 min had an absorbance spectra with λ_{max} at 328 and 232 nm, and a shoulder at 304 nm. Its ESI MS spectrum exhibited an intense $[M-H]^-$ ion at *m/z* 551, while a much lower peak at *m/z* 553 was observed in the positive (PIM) mode. The product ion scan spectra of *m/z* 551 showed various fragment ions at *m/z* values of 507, 389, 281, 251, 179 and 161, respectively. Since a compound with similar mass fragmentation profile and UV–VIS characteristics was previously identified as caffeoyl-6′-secologanoside (Obied et al., 2007b), the peak V was tentatively assigned as caffeoyl-6′-secologanoside as well.

The negative ESI-MS spectra of peak VII eluting at 45.1 min was characterised by an intense ion at *m/z* 535 and several other fragments of lower intensity (Table 1) that are in accordance with the fragmentation of comselogoside (Obied et al., 2007b). This compound has been recently discovered in Australian oil mill waste (OMW) and olive fruit Frantoio extracts (Obied et al., 2007a) but never reported for *O. europaea* Oblica *cv*. before. The peak's absorbance spectra was similar to the one reported by Obied et al. (2007a) with a little shift in absorption maximum (238/314 nm *vs*. 225/309 nm) most probably due to a different solvent used during UV/VIS(DAD) detection (aqueous acetic acid *vs*. aqueous methanol).

HPLC-DAD quantitative analysis of *O. europaea* Oblica *cv*. revealed a high phenol content for most of the olive fruit phenols identified. Oleuropein and demethyloleuropein were the major phenols present accounting more than 85% of all phenols quantified, followed by rutin, hydroxytyrosol glucoside, luteolin-7-O-glucoside, verbascoside, comselogoside, caffeoyl-6′-secologanoside and quercitrin (Table 3, Fig. 1). The levels of individual phenols (mg g^{-1} DW) are comparable with previous reports (Ryan et al., 2002; Vinha et al., 2005), depending on the olive fruit cultivar analysed, its origin and other factors usually influencing phenols content.

3.2. Optimisation of USLE – with ultrasonic homogeniser (probe)

Several operational variables can affect the process of ultrasound extraction such as the probe position, ultrasound radiation amplitude, percent of duty cycle of ultrasound exposure, sonication time and temperature, solvent composition and solid to liquid ratio (Herrera & Luque de Castro, 2005).

Preliminary studies were carried out in order to test the US probe working conditions for phenols extraction assistance from freeze-dried olive fruit sample – the US power, frequency, amplitude, duty cycle and probe position. First, the sample:solvent ratio 1:17 (g mL^{-1}) was chosen according to literature, where the ratios between 1:10 (Boskou et al., 2006) and 1:20 (Ryan, Lawrence, Prenzler, Antolovich, & Robards, 2001) have been widely reported for the olive fruit phenol extractions from freeze-dried samples.

The intra-house ultrasonic homogeniser LABSONIC®M used in our experiments operates at frequency 30 kHz with a maximum power output of 100 W regulated by the choice of sonication amplitude (1–100%). Since parameters, such as sonication amplitude, duty cycle and probe position turned out not to significantly influence the extraction efficiency of phenols from dried olive leave samples (Japón-Luján et al., 2006), the highest values of radiation amplitude (100%) providing max US power (100 W) as well as of duty cycle (100%) were chosen initially for the experiments. Continuous sonication (100% duty cycle) corresponds to permanent power output of US energy during extraction, allowing a constant pulse flow into extraction solution. Moreover, the intensity of ultrasound transmitted to the medium is directly related to the vibration amplitude of probe, producing greater number of cavitation bubbles and therefore increased extraction efficiency of analytes present in the sample (Dash et al., 2005). The position of titanium sonotrode, immersed 3 cm deep into solution, was chosen using recommendations of instrument producer (Sartorius) as well as the inner diameter of sonication probe (3 mm) selected based on extraction solvent volume (25 mL). The preliminary trials under these experimental conditions (30 kHz, 100 W, 100% amplitude and duty cycle) did not affect phenols stability tested on methanol standard mix solution (Section 2.5) and were therefore used for further USLE extraction optimisation.

In the present paper, four main variable extraction parameters influencing US extraction efficiency were optimised, namely sonication time and temperature, extraction solvent composition and the number of extraction steps. The influence of each parameter was evaluated separately on the basis of extraction efficiency keeping other variables at constant values. The highest phenol recovery was considered as optimal and was further implemented in the optimisation process of other subsequent parameters. After obtaining optimal values for all operational parameters, the proposed optimised method was referred as USLE method.

3.2.1. Effect of extraction time on extraction yields of olive fruit phenols

The influence of sonication time ranging from 4 to 10, 20 and 30 min on phenolics concentration is shown in Table 2. Statistical evaluation (ANOVA) suggested that phenol extraction yields were highly time-dependent. In fact sonication time was the most significant factor ($P < 0.01$) as already reported by Wang et al. (2008) in the case of wheat bran material. Our extraction studies showed that phenols recoveries increased with the time of sonication, reaching maximum at 20 min for the majority of olive fruit phenols analysed. The extraction efficiencies were low during first 4 min of sonication indicating that more time is needed for ultrasonic cell walls disruption releasing phenols from the cell constituents. Longer sonication times of 10 and 20 min improved extraction efficiencies and hence increased the rate of extraction, while prolonged application of 30 min did not benefit in phenol yields to large extent. When compared to 30 min of extraction, more than 60% phenolics were extracted during first 10 min, while sonication of 20 min provided more than 95% recovery for eight out of nine phenols analysed. It is worth noting that benefit by applying US energy has come from the combination of both, time and temperature, since the temperature of extraction medium increased together with the rise of sonication time, as evidenced also in our experiment. Obtained results are in agreement with Rostagno, Palma, and Barroso (2007), who clearly indicated that 20 min of sonication time was sufficient enough to extract phenolics from soy beverages. On contrary to Herrera and Luque de Castro (2005) we have not observed any phenol degradation at longer US applications (20, 30 min) and since 30 min was not superior in phenol yields to large extent, a sonication time of 20 min was selected for all subsequent experiments.

3.2.2. Effect of extraction temperature on extraction yields of olive fruit phenols

The temperature in phenol extraction has to be chosen very carefully, since the process may be affected in a non-desirable way. It is true that higher temperatures (40 °C) may increase the solubility of phenolics (Japón-Luján et al., 2006), but too high (60 °C) may cause their degradation (Rostagno et al., 2007).

In this study, the effect of temperature on phenol extraction efficiency was examined as follows: (1) with cooling system and (2) without (25 °C). Other parameters during extraction were controlled and kept constant using conditions: 20 min, pure methanol and a two-step extraction. As already stated above (Section 3.2.1), the application of US energy increased the temperature of extraction medium. This mechanism was thoroughly described by Luque-García and Luque de Castro (2003). The same phenomena was observed in our experiment where the temperature of homogenate during extraction performed at 25 °C significantly increased and exceeded 40 °C after 20 min of ultrasonic exposure (44 ± 2 °C). In order to test the possibility of phenols degradation, a new set up equipped with cooling system has been employed. The temperature of homogenate was controlled and kept constant (25 ± 1 °C) throughout sonication (20 min) by performing extraction in an ice-water bath (0 °C). Quantitative results of both experiments were compared and statistically significant ($P < 0.01$) differences in phenol amounts were observed. Clearly, the increase in homogenate temperature up to 40 °C and more (44 ± 2 °C) enhanced the extraction efficiency, resulting in higher recoveries for all nine phenolics analysed. The phenols increases ranged from 12% (hydroxytyrosol glucoside) to 142% in the case of caffeoyl-6′-secologanoside. As seen from the literature, temperatures up to 50 °C increases the number of cavitation nucleus formed (Filgueiras, Capelo, Lavilla, & Bendicho, 2000) responsible for acoustic cavitation resulting in enhanced mass transfer and therefore better access of solvent to cell components (Toma, Vinatoru, Paniwnyk, & Mason, 2001). Since higher temperatures could induce extraction solvent evaporation and consequently influence final phenol concentrations, all methanolic extracts were reconstituited after each extraction to the same final volume with methanol (Section 2.3.2). The extracts phenol composition was not altered by higher temperatures (44 ± 2 °C) since no differences was observed when compared to that of agitation (25 ± 1 °C). No indication of phenol degradation and chemical modifications was apparent as already reported by Japón-Luján et al. (2006) for the similar experimental temperatures (40 °C) in phenols extraction from olive leaves. Hence, the experimental set up without cooling system applied (44 ± 2 °C) was chosen in the further development of USLE method optimisation process.

3.2.3. Effect of solvent composition on extraction yields of olive fruit phenols

Various extraction solvents such as methanol, ethanol, acetone or mixtures of these with water have been proposed for solid liquid extractions of phenols from olive fruit samples. As most of the published studies examined the influence of methanol and water–methanol mixture (20:80, v/v), the extraction efficiency of both solvents were tested in USLE method as well. Nevertheless, methanol is a solvent in which no hydrogen peroxide neither large proportions of free radicals are formed due to cavitation when exposed to sonication, which could induce chemical degradation of phenols present (Paniwnyk, Beaufoy, Lorimer, & Mason, 2001). Other parameters during extraction were controlled and kept constant using conditions: 20 min, 44 ± 2 °C and a two-step extraction. The quantitative results presented in Fig. 1 showed highly significant ($P < 0.01$) differences in phenol recoveries obtained with methanol and its aqueous mixture (80%). Pure methanol was superior to 80% methanol for all phenolics measured, with the excep-

tion of rutin. The yield increase, attributed to suitable polarity of absolute methanol, ranged in average from 15% (oleuropein) up to 116% (caffeoyl-6′-secologanoside), except in the case of rutin where 5% of decrease was observed. Inspite of lower polarity of rutin, a methanol/water seemed to be more efficient extraction solvent in comparison to absolute one. This is probably due to the presence of more hydrophilic sugar moiety attached to the quercetin part of molecule as already reported (Kreft, Fabjan, & Yasumoto, 2006), where higher rutin extraction efficiencies were observed in water–methanol solvent. However, since pure methanol gave better results for the majority of phenols present, we decided to employ it as an extraction solvent for USLE olive fruit phenols extraction. Moreover, it allowed us more efficient evaporation to complete dryness (35 °C) in the pre-concentration step prior HPLC analysis.

3.2.4. Effect of sequential extraction steps on extraction yields of olive fruit phenols

The use of two to three extraction steps, with up to five (Boskou et al., 2006) have been reported in the literature for the quantitative determination of olive fruit phenol content. Therefore, the effect of five sequential extraction steps on the phenol extraction yield from freeze-dried olive fruit sample was tested in USLE method as well. Other parameters during extraction were controlled and kept constant using conditions: 20 min, 44 ± 2 °C and pure methanol. All five consecutive extractions were performed on the same sample matrix by re-extracting a solid olive residue after centrifugation, using the same volume of fresh solvent (25 mL) under identical extraction conditions. Methanol extracts from each extraction step were analysed and evaluated separately for the phenol yield recovery. The HPLC-DAD chromatograms (Fig. 2) suggested that three steps are enough for phenolics extraction from freeze-dried olive fruit sample, confirmed by the calculations as well. A comparison to phenols summation of five extraction steps reveled that majority of phenolics were on average recovered by the first extraction step (85%), followed by the second (9%) and the third (4%), respectively. Only two (verbascoside, caffeoyl-6′-secologanoside) out of nine phenols analysed were fully extracted after two subsequent extractions, while the rest, with the exception of rutin, were fully recovered in three steps. Therefore, we decided that a three-step extraction is sufficient enough for a quantitative recovery of main olive fruit phenolics since only rutin (2% of total amount) was detected in the 4th extraction step, meaning that a three-step extraction offers 98% yield of rutin, which will be taken into account in the future quantifications.

3.3. USLE method (US probe) versus US bath and agitation

The optimised USLE method with ultrasonic homogeniser (3 × 20 min, 44 ± 2 °C, pure methanol) was compared to other more commonly used extraction methods employing US bath and a room temperature agitation under the same operational parameters mentioned above. Recovery data of nine olive fruit phenolics obtained from three different extraction methods are presented in Table 3.

The statistical evaluation clearly demonstrated that US application accelerated the extraction process compared to that of agitation. Ultrasound enhanced the extraction rate by disrupting plant cell walls leading to increased diffusion of cell contents into extraction solution. The beneficial effects of ultrasonic waves are attributed to the formation of expansion-compression cycles in extracting media leading to generation of strong liquid jets rupturing the cells (Luque-García & Luque de Castro, 2003). Along with dried plant material swelling and hydration enhancement (Toma et al., 2001), they contribute to mass transfer as well as to more efficient solvent penetration to cell material. The use of US homogeniser was not only superior to classic extraction using agitation but also to US bath as confirmed by statistical comparison ($P < 0.05$) of average phenol recoveries (Table 3). Direct sonication with a probe system was more efficient than indirect employing US bath as already reported by Vinatoru et al. (1997) for other plant matrices like marigold. In a direct US application system, the energy of probe unit is directly focused on a localised sample zone thereby providing more efficient cavitation into extracting solution (Luque-García & Luque de Castro, 2003). Increased extraction yields are caused by a larger phenols diffusion out of cell constituents, either by rupturing additional cells or by accelerating their extraction from the inner parts of the olive fruit matrix. Lower efficiency of US bath to US homogeniser could be explained by its indirect US energy transfer into extraction medium. Moreover, US bath is characterised by a decline of power during sonication time as well as by the lack of US energy uniform distribution (Luque-García & Luque de Castro, 2003). This could also be a reason for lower extraction yields, in spite of higher ultrasonic power (400 W) applied with bath, *versus* probe (100 W). To summarise; comparing different extraction methods, the USLE employing ultrasonic homogeniser resulted in the highest recoveries for all nine olive fruit phenols measured. In the case of oleuropein (the most abundant olive fruit phenol), recoveries were on average higher with up to 33% and 80% in comparison to US bath and agitation, respectively. Even though USLE optimisation was based on the quantification of nine phenols only, we concluded that method proposed offers a sufficient phenol recovery from olive fruit freeze-dried matrix, since no peaks in extracts chromatograms were observed after (1) the 4th and 5th extraction step as well as (2) HPLC analysis of olive matrix solid leftover, known as blank in recovery and sensitivity studies.

3.4. Characterisation of USLE method proposed

The selectivity of the method was satisfactory with a good peak separation and resolution allowing quantification of all major phenols present in acidic extract of olive fruit samples. No interfering peaks were observed in the blank chromatograms at measured wavelengths 280, 320 and 365 nm. The peak purity and degree of match with the standard spectra varied from 92% (verbascoside) to 99% (rutin) and the peaks R_t showed a high compliance with standards as well.

The results of within-day repeatability ($n = 3$), expressed as percentage of RSD, varied between 0.8 (demethyloleuropein) and 3.1 (comselogoside), while between days from 0.6 (hydroxytyrosol glucoside) to 6.4 (verbascoside), indicating that method's precision was satisfactory.

The method's extraction efficiency was evaluated by recovery assay using a standard addition method with internal and external calibration over range of 1.10–3.36 mg g^{-1} DW allowing high phenol recoveries (%): 98.7 ± 2.1 (oleuropein), 96.9 ± 1.5 (luteolin-7-O-glucoside), 96.0 ± 2.7 (caffeic acid), 94.3 ± 3.8 (rutin), 94.2 ± 4.5 (verbascoside) and 94.1 ± 7.8 (quercitrin), respectively.

The sensitivity of method was evaluated by determining the limits of detection (LODs) and limits of quantification (LOQs) as described (Section 2.5). High correlation coefficients were obtained for all standards ($R^2 > 0.98$) indicating good linearity response of the method proposed with LODs/LOQs (μg g^{-1} DW) equal to: 2.77/8.38, 4.92/14.92, 1.48/4.48, 0.66/2.00 and 1.85/5.62 for oleuropein, verbascoside, rutin, luteolin-7-O-glucoside and quercitrin, respectively.

4. Conclusion

The potential use of US energy as a powerful tool for extraction assistance of olive fruit phenols was demonstrated for the first

time. A new method of USLE using high intensity probe ultrasonication was developed and fully optimised with the aim of improving phenol recoveries. The rate of extraction was shown to be a function of time, temperature, solvent composition and the number of extraction steps since they all significantly influenced the yields of phenols analysed. The optimal conditions for phenols extraction from a freeze-dried olive fruit material (1.5 g) obtained by ultrasonic probe consisted of a three-step extraction of 20 min sonication time, with pure methanol (25 mL) at 45 °C. The method described offers a sufficient phenol extraction from a complex olive fruit matrix without any degradations or chemical modifications observed. Compared to US bath and agitation methods, the proposed USLE was shown to be simpler and more efficient, providing results of high selectivity, precision and sensitivity. Therefore, could be used as an important extraction technique as a sample preparation step prior quantitative determination of olive fruit phenols by means of HPLC, with a possible application for other plant matrices as well.

Acknowledgments

We thank Slovenian Research Agency for financial support and Mrs. Valda Vodopivec for olive fruit (*cv.* Oblica) samples from island Hvar, Croatia.

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B2 Ultrasonic extraction of phenols from olive mill wastewater: Comparison with conventional methods ("Reprinted from Ultrasonic extraction of phenols from olive mill wastewater: Comparison with conventional methods, 59, Jerman Klen T., Mozetič Vodopivec B., Journal of Agricultural and Food Chemistry, 12725–12731, Copyright (2011) with permission from American Chemical Society").

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Ultrasonic Extraction of Phenols from Olive Mill Wastewater: Comparison with Conventional Methods

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ABSTRACT: Recovery of phenols from olive mill wastewater (OMWW) was studied, comparing five sample preparation methods: filtration, solid-phase (SPE), liquid−liquid (LLE) and ultrasonic (US)-assisted extraction of liquid and solid (freeze-dried) OMWW. Results showed that ultrasonication is a good alternative to conventional solvent extractions, providing higher recoveries at both levels of individual and total phenol yields. Sonication of liquid OMWW in organic solvent was more efficient vs its nonassisted counterpart (agitation), but did not provide a representative phenol chromatogram due to ethyl acetate use. By contrast, the US-assisted extraction of freeze-dried OMWW (3 × 20 min) in 100% methanol (1.5 g/25 mL, w/v) offered the highest qualitative−quantitative phenol yields without any US-induced alterations. Moreover, freeze-drying is an excellent preservation of initial liquid OMWW, holding a great potential for delayed analysis. This study is also the first report that Slovenian OMWW may be utilized as a valuable source of phenols, especially hydroxytyrosol and tyrosol.

KEYWORDS: ultrasound, phenols, olive mill wastewater, sample preparation, method comparison

INTRODUCTION

World-wide interests in natural product research for drug-discovery purposes have greatly expanded since the 1980s[1] with particular emphasis on recovery of value-added compounds from plant materials and/or their processing products. Concepts like these have also found application in various agri-food waste matrices, such as those of wine, olive and other food-processing industries,[2] with the aim to benefit from both an economic and ecological perspective. In particular, olive mill wastes have been widely studied as sources of different bioactive phenols[1,3−9] increasing attention for their wide range of health benefits, such as antioxidant, antimicrobial, antiviral and other activities. On the other hand, the same compounds have been reported as serious environmental pollutants with limited biodegradability[6−9] that, along with expansion of waste production, pose an economic and environmental burden on olive oil industry's sustainable development.[3]

Currently, commercial olive oil production is carried out using both continuous (centrifugation) and batch (traditional press) approaches. Although the quantity and type of olive byproduct largely depend on the extraction system used for oil production, the process typically generates, next to olive oil (20%), the two problematic wastes known as olive cake (30%) and olive mill wastewater (50%).[8] In particular, the latter has been of major concern from both a quantitative and polluting load perspective. Indeed, olive mill wastewater (OMWW) is up to 200 times more polluted than domestic sewage with high organic loads, such as sugars, tannins, polyalcohols, pectins, lipids and phenols,[8] making its treatment extremely difficult. However, the significance of the problem arises mainly from its high phenolic content,[7−9] most of which are hydrophilic in nature, and thus remain in the water when partitioned during oil processing.[10] On the other hand, this has offered producers great potential for recovery of phenols and added value to this problematic waste. In fact, the phenols recovered from OMWW have become important targets for the food, cosmetic and pharmaceutical industries, especially as benefits of their bioactivity has been proven at all levels of in vitro,[4,5,9,11] ex vivo[12] and in vivo[13] studies.

Consequently, a number of reports have appeared in the literature endeavoring OMWW's dephenolization with the aim to reduce its toxicity[14,15] and/or obtain its phenol yields.[4−7,9,16−19] Their vast field of commercial applications is now widely documented and covered by several patents, varying from numerous solvent extractions, chromatographic separations, up to integral processes and membrane technologies.[7] By contrast, phenol recovery studies at the laboratory scale have been only sparingly investigated, utilizing the two basic extraction techniques of solid-phase (SPE) and liquid−liquid (LLE) extractions with different organic solvents and conventional methods of manual or mechanical agitation.[4−6,9,11,16−19]

Most recently, ultrasound (US) has been accepted as a timid alternative to conventional and other accelerated-type extractions, providing higher recovery of analyte with lower solvent consumption and/or faster analysis.[20] To the best of our knowledge, US potential has not been yet investigated for OMWW phenol extraction assistance from either solid or liquid samples, although previously proven as very effective for this purpose.[20,21] Thus, in view of the analytical challenge and commercial interest for their efficient recovery, three common methods of sample filtration, solid-phase and liquid−liquid extractions were compared to US-assisted extractions of liquid and solid OMWW matrices for the first time. In addition, the Slovenian OMWW was investigated as a potent source of natural antioxidants never reported before.

MATERIALS AND METHODS

Olive Mill Wastewater (OMWW). Two liters of fresh multivarietal OMWW were collected in November 2009 from a local three-phase

Received: July 13, 2011
Accepted: November 4, 2011
Revised: November 3, 2011
Published: November 04, 2011

olive mill plant (Pieralisi) located in Dobrovo (Slovenia) and immediately transferred to the laboratory, where it was further homogenized and equally divided. One half was acidified with HCl (pH = 2.0) and defatted with *n*-hexane (3 × 15 mL) according to De Marco et al., 2007,[9] while the other half remained in its crude (nonacidified) form. Then, both fractions were again divided. One was immediately frozen with liquid nitrogen, freeze-dried in a Kambič LIO-5P lyophilizator (Semič, Slovenia) and stored at −25 °C until analysis, while the other was transferred into screw-capped, dark glass containers and stored in a freezer until analysis. The dry-matter measurements of the freeze-dried sample were performed gravimetrically,[11] providing the basis for methods comparison using liquid OMWW.

Chemicals and SPE Sorbents. Phenolic standards of hydroxytyrosol, tyrosol, oleuropein, luteolin, luteolin-7-*O*-glucoside, apigenin, apigenin-7-*O*-glucoside, protocatechuic and homovanillic acid, verbascoside, quercitrin and rutin were obtained from Extrasynthese (Genay, France), while ferulic, benzoic, chlorogenic, gallic, *p*-hydroxybenzoic and *p*-coumaric acids were purchased from Sigma-Aldrich Co. (Steinheim, Germany). All standards were prepared in methanol that was, in turn, evaporated using rotary evaporation at 35 °C. The dried residue was then redissolved to its original volume with acidic water, i.e., H_2O/CH_3COOH (95:5, v/v), in the range as expected for each compound in the OMWW sample: 5−500 μg mL^{-1} (hydroxytyrosol, tyrosol, caffeic, chlorogenic, vanillic, protocatechuic, benzoic, ferulic, *p*-hydroxybenzoic and *p*-coumaric acids), 25−1000 μg mL^{-1} (verbascoside, rutin, apigenin, apigenin-7-*O*-glucoside, quercitrin and luteolin), and 5−6000 μg mL^{-1} (oleuropein).

Methanol (HPLC grade), glacial acetic and hydrochloric acids (12.1 M), ethyl acetate and diethyl ether were obtained from Sigma-Aldrich Co. (Steinheim, Germany), while Folin−Ciocalteu reagent (Merck) was obtained from a local supplier.

SPE sorbents were provided by different suppliers: HLB Oasis (60 mg/3 mL) and Sep-Pak (820 mg/1.6 mL) from Waters Corp. (Milford, MA, USA), Strata X (200 mg/6 mL) from Phenomenex (Aschaffenburg, Germany), Isolute C18 (500 mg/3 mL) from Biotage (Hertford, U.K.), and Discovery DPA-6S (500 mg/6 mL) from Sigma-Aldrich Co. (Steinheim, Germany).

Phenol Extraction Methods. *US-LLE: Ultrasound-Assisted Liquid−Liquid Extraction.* A LABSONIC M ultrasonic probe (100 W, 30 kHz) from SARTORIUS (Gottingen, Germany) was used for phenols extraction assistance, where the liquid sample of OMWW (10 mL) was placed into a PE centrifuge tube and directly sonicated for different extraction parameters in the following order: sonication time (5, 10, 15, 20 min), solvent type (ethyl acetate vs diethyl ether) and extraction steps[1−5] using 10 mL of solvent under US conditions (100% amplitude/duty cycle, titanium 3Ø mm sonotrode) primarily optimized elsewhere.[21]

LLE-Agitation: Liquid−Liquid Agitation. For comparison purposes, an optimized US-LLE was compared to its nonassisted counterpart based on LLE-agitation with VIBROMIX 313 EVT (Železniki, Slovenia) using identical extraction conditions, i.e., 3 ×10 min with ethyl acetate (10 mL), respectively.

Preliminary SPE Sorbent Testing. Five commercially available SPE cartridges, namely, Oasis HLB, Strata X, Isolute C18, Discovery DPA-6S and Sep-Pak C18, were tested for extraction efficiencies on fifteen olive phenol standards using their mix solution in methanol: (1) hydroxytyrosol, (2) protocatechuic acid, (3) tyrosol, (4) *p*-hydroxybenzoic acid, (5) homovanillic acid, (6) chlorogenic acid, (7) *p*-coumaric acid, (8) benzoic acid, (9) ferulic acid, (10) oleuropein, (11) rutin, (12) apigenin-7-glucoside, (13) quercitrin, (14) luteolin and (15) apigenin, in the concentration ranges expected in OMWW samples.[9,11,16−19] First, an acidic standard-mix solution was prepared using rotary evaporation at 35 °C, where the methanol was removed and dried residue redissolved to its original volume with acidic water (HCl, pH = 2.0). Then, prior to sample loading (1.5 mL), the cartridges were preconditioned with methanol, followed by acidic water (HCl, pH = 2.0), using the volumes suggested by the producers. Finally, the loaded cartridges were washed with *n*-hexane (10 mL) in order to remove the nonpolar fraction (in case of highly fatty extracts), and the phenols were eluted with methanol (2 × 5 mL) that were further analyzed by HPLC−DAD−FLD analysis.

SPE: Solid-Phase Extraction. Based on preliminary results of SPE sorbent testing, an HLB Oasis sorbent (60 mg/3 mL) was employed for further OMWW phenol extraction, where the cartridge was primarily conditioned with methanol (2 mL) and acidic water (HCl, pH = 2.0, 2 mL), followed by OMWW percolation (1.5 mL) using a Visiprep vacuum manifold from Sigma-Aldrich Co. (Steinheim, Germany). Afterward, the loaded sample was washed with *n*-hexane (10 mL) and the phenols were eluted with methanol (2 × 5 mL), further analyzed by HPLC−DAD−FLD and total phenol (TP) analysis.

USLE: Ultrasound-Assisted Solid−Liquid Extraction. Phenols were extracted using an optimized extraction procedure according to a previously published method.[21] Freeze-dried OMWW (1.5 g) was sonicated for 20 min using 25 mL of pure methanol. The extraction was repeated three times, and combined methanol extracts were quantitatively transferred into a 100 mL volumetric flask, diluted to volume with methanol and stored at −25 °C until further HPLC−DAD−FLD and TP analyses.

HPLC−DAD−FLD and LC−MS Analyses. Prior to chromatographic analysis, an aliquot of extract (10 mL) was evaporated to dryness using rotary evaporation (Heidolph, Schwabach, Germany), then redissolved in 1 mL of HPLC eluent (H_2O/CH_3COOH, 95:5, v/v), and further filtered through 0.45 μm PTFE filters (Macherey-Nagel, Düren, Germany).

HPLC−DAD−FLD. An HP 1100 liquid chromatograph (Agilent Techn., Santa Clara, CA, USA), coupled to DAD (190−600 nm) and FLD ($\lambda_{excitation/emission}$ = 280/330 nm) detectors, was used for individual phenol determination. 20 μL of extract was injected, and DAD signals were recorded at 280 nm (simple phenols, benzoic acids, phenol secoiridoids), 320 nm (cinnamic acids), 365 nm (flavonoids) and 520 nm (anthocyanins), using gradient elution on Luna PFP (5 μm, 250 mm ×4.6 mm) attached to a security guard (4 × 3 mm), both from Phenomenex (Macclesfield, U.K.), under analytical conditions described previously.[21]

LC−MS. Phenol identification was confirmed by LC−MS/MS analysis using Perkin-Elmer series 2000 (Shelton, CT, USA) linked to a 3200 Q TRAP LC/MS/MS system equipped with an ESI ion source from Applied Biosystems/MDS Sciex (Foster City, CA, USA). Mass spectrometric scans were performed after 15 μL injection of OMWW extracts in negative ionization (NIM) mode, scanning in Q3 from *m*/*z* 50 to *m*/*z* 1000 in 1 s. The ESI conditions were as follows: capillary temperature 400 °C, capillary voltage −4500 V, declustering potential (DP) −30 V and entrance potential (EP) −30 V, respectively.

OMWW phenols were primarily identified by comparison of t_R, UV−vis, FLD and ESI-MS spectra with those of authentic standards when available, while the tentative identity of others was confirmed by comparison of UV−vis and ESI-MS spectra with those from the literature and our database.[1,6,9,11,16−19,21,22] Hydroxytyrosol and tyrosol were quantified with calibration with authentic standards at 280 nm and chlorogenic acid at 320 nm, respectively. Other phenols, hydroxytyrosol glucoside, vanillin, demethyligstroside, nüzhenide and *p*-HPEA-DEDA, were expressed as hydroxytyrosol, vanillic acid and oleuropein equivalents at 280 nm, while β-hydroxy verbascoside, isoacteoside, caffeoyl-6′-secologanoside and comselogoside were expressed as verbascoside, caffeic and *p*-coumaric acid equivalents at 320 nm in μg mL^{-1} of OMWW.

Total Phenol (TP) Analysis. The total phenol content of extracts was determined colorimetrically at 765 nm using Folin−Ciocalteu reagent, according to Ough and Amerine (1988),[23] and results were expressed as gallic acid (GAE) equivalents in μg mL^{-1} of OMWW.

Statistical Analysis. All experiments were performed at least in triplicate ($n \geq 3$), and the results were expressed as means ± SD. Statistical significances among parameters were evaluated by ANOVA using STATGRAPHICS Plus 4.0 (Manugistics Inc., Rockville, MD, USA) and MRT test at 95% confidence level, where P values <0.05 were regarded as significant, P values <0.01 as very significant and P values >0.05 as insignificant.

RESULTS AND DISCUSSION

Identification of OMWW Phenols. The HPLC−DAD chromatograms of OMWW extracts showed several peaks (Figure 1), although not all of them could be identified. In fact, among fifteen major phenols only three could be confirmed with authentic standards (peaks 4, 6 and 7). Nine were tentatively identified based on UV−vis, FLD and MS spectral characteristics (peaks 3, 8−15), while the identity of three (peaks 1, 2 and 5) remained inconclusive (Table 1). However, in general, our OMWW phenol profile agrees with previous reports for other OMWW matrices,[6,9,11,16−19,24] dominated by the same phenol classes of simple phenols (hydroxytyrosol glucoside, hydroxytyrosol, tyrosol), benzoic acids (vanillin), cinnamic acids (chlorogenic acid, β-hydroxy verbascoside, isoacteoside) and phenol secoiridoids (demethyl-ligstroside, nüzhenide, *p*-HPEA-DEDA, caffeoyl-6′-secologanoside, comselogoside), respectively.

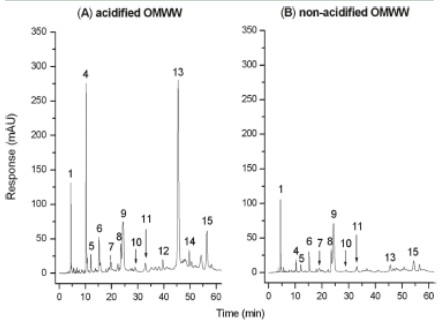

Figure 1. HPLC−DAD chromatograms of phenol extracts from (A) acidified (pH = 2.0, HCl) and (B) nonacidified (crude) OMWW at 280 nm. Peak numbers refer to Table 1.

OMWW Sample Handling. In many studies, the details of sample handling prior to extraction are omitted, and although an immediate analysis is always preferable, it is rarely achievable, and thus the preservation and storage of the sample is often unavoidable.[4] Consequently, different sample preparations prior to OMWW analysis have been proposed in the literature, varying from those of untreated samples[2,4] up to their acidification with HCl[6,9,11] or freeze-drying.[5,17,19]

In an effort to avoid or minimize any of the sample artifacts, the influence of acidification and *n*-hexane lipid removal was tested primarily on an OMWW qualitative−quantitative phenol profile, according to a previous report.[9] As seen from results, the acidified sample (pH = 2.0, HCl) presented a much richer quantitative phenol profile vs the nonacidified one (Figure 1), with increases up to 36% (in TP yields) or even more (i.e., 97% in HPLC−DAD individuals sum), while no differences between extracts were observed at the qualitative level. These results are in agreement with a previous report, demonstrating increased phenol solubility and/or their better cell-wall release under acidic conditions.[9] However, by contrast to the above, no hydrolysis of complex phenols was observed since all of the peaks were substantially increased in the acidic sample, suggesting that the addition of acid neither modifies nor degrades any of the phenols analyzed. Similarly, *n*-hexane washing has not markedly influenced its TP yields, as the differences between defatted (1840.0 ± 74.4 μg GAE mL^{-1}) vs non-defatted (1798.0 ± 103.4 μg GAE mL^{-1}) extracts were low and insignificant ($P > 0.05$), confirming both sample handlings (acidification and *n*-hexane washing) as useful pretreatment methods prior to OMWW phenol analysis.

Table 1. UV/Vis, FLD and MS Spectral Characteristics of the Main OMWW Phenols

	HPLC−DAD−FLD			ESI-LC−MS			
peak	t_R (min)	λ_{max} (nm)	$\lambda_{ex/em}$ 280/330 (nm)	MW (M)	major $[M-H]^-$	compound	standard
1	4.5	234, 280[a]	yes		191, 151	unknown	no
2	7.1	236, 284	yes		583, 511, 468,	unknown	no
3	8.1	236, 278	no	316	315, 153, 121	hydroxytyrosol glucoside	no
4	10.2	236, 280	yes	154	153, 123, 95	hydroxytyrosol	yes
5	11.2	238, 276	yes		407, 347, 181	unknown	no
6	15.1	236, 276	yes	138	137	tyrosol	yes
7	18.1	247, 305, 328	no	354	353, 191, 179	chlorogenic acid	yes
8	23.2	262, 292	weak	152	151	vanillin	no
9	24.8	236, 280	no	510	509, 405, 373, 191, 151	demethylligstroside	no
10	28.8	240, 300sh, 332	weak	640	639, 621, 461, 179	β-hydroxy verbascoside	no
11	33.1	234, 308, 332	no	624	623, 513, 461, 161	isoacteoside	no
12	39.5	246, 290sh, 332	no	685	685, 523, 299	nüzhenide	no
13	45.4	238, 278	weak	304	303, 285, 179, 165, 156	*p*-HPEA-DEDA	no
14	49.7	232,296sh, 328	no	552	551, 507, 389, 281, 161	caffeoyl-6′-secologanoside	no
15	56.7	238, 314	no	536	535, 491, 389, 345, 265, 145	comselogoside	no

[a] Maximum UV band indicated in bold.

Table 2. Optimization of US-LLE Extraction Conditions Based on Total Phenol (TP) Analysis

US-LLE parameters	TP extraction yields (μg GAE mL^{-1})					*P*-value[d]
Extraction time[a]	5 min	10 min	15 min	20 min	30 min	< 0.01
	1463.6 ± 92.0β	1840.0 ± 74.4α	1821.1 ± 53.8α	1844.4 ± 64.3α	1839.3 ± 71.3α	
Extraction solvent[b]	Ethyl acetate			Diethyl ether		< 0.01
	1840.0 ± 74.4α			1151.7 ± 71.8β		
Extraction steps[c]	n = 1	n = 2	n = 3	n = 4	n = 5	< 0.01
	1144.0 ± 59.2α	718.6 ± 26.2β	213.6 ± 7.7γ	91.1 ± 3.7δ	26.9 ± 1.6χ	

[a] Extraction conditions: ethyl acetate, 2-step extraction. [b] Extraction conditions: 2 × 10 min. [c] Extraction conditions: 10 min, ethyl acetate. [d] Values in the same row marked by the same letter ($\alpha, \beta, \gamma, \delta, \chi$) are not significantly different; confidence level = 95%; $P < 0.05$, significant; $P < 0.01$, very significant; $P > 0.05$, insignificant.

Optimization of US-LLE Extraction Conditions and Its Comparison to Other Methods: LLE-Agitation, SPE, Direct OMWW Filtrate Analysis, and USLE. Since olive phenols comprise a diverse spectrum of phenol constituents with high structural diversity and physicochemical behavior, a complete recovery from the matrix presents a difficult task.[1] In view of the analytical challenge to obtain the highest recoveries for most of the phenols present in OMWW, five different methods were tested and compared based on individual and TP analyses (Table 3).

First, an US-assisted extraction was applied to a liquid OMWW sample, where a solvent:sample ratio of 1:1 (v/v) was chosen based on previous reports,[9,11] while the US probe working conditions (100% amplitude and duty cycle) remained as previously reported.[21] Then, the three main LLE variables of sonication time (5, 10, 15, 20, 30 min), solvent type (ethyl acetate, diethyl ether) and extraction steps[1−5] were optimized, evaluating each variable independently, while keeping others at constant values. The one providing the highest yield was considered as optimal and further implemented in the optimization process of others until the final (optimal) US extraction conditions were obtained, referred to as the US-LLE method.

As demonstrated by the results, all variables have significantly ($P < 0.01$) influenced the TP yields in the US-LLE optimization study (Table 2), confirming their importance in overall OMWW analysis. First, the impact of sonication time (5, 10, 15, 20, 30 min) on phenol recoveries was examined, using two-step extraction with ethyl acetate, where a 10 min sonication had resulted in maximum TP yields (1840.0 ± 74.4 μg GAE mL^{-1}), while no further increases were observed at longer sonications (i.e., 15, 20, 30 min). Then, a solvent extraction study was conducted, comparing ethyl acetate vs diethyl ether according to previous reports,[6,17] where the former was almost 2 times more efficient (60%), confirming its superior extracting power for isolation of phenols from aqueous matrices.[6] Finally, the influence of five sequential extraction steps was assayed to maximize phenol recoveries, where the TP yield has decreased gradually with each extraction step by 52.1%, 32.7%, 9.7%, 4.2% and 1.2% on average, confirming that five-step extraction is efficient enough for the quantitative OMWW phenol analysis. Therefore, the optimized US-LLE method consisted of 5 × 10 min extraction with ethyl acetate (10 mL) using high-intensity probe ultrasonication of the liquid sample (10 mL), respectively.

Second, an optimized US-LLE was compared to its nonassisted counterpart based on LLE-agitation using identical extraction conditions (5 × 10 min) with ethyl acetate (10 mL), respectively. As expected, the amount of phenols recovered by US was significantly greater than those by agitation (Table 3), observed at both levels of individual (61% HPLC sum) and TP (20%) analysis. This can be attributed to either mechanical and/or chemical effects greatly facilitating the phenol transfer rates between two immiscible phases (i.e., aqueous OMWW−ethyl acetate) through superagitation, mixing and stirring, US abilities that break down the fruit's remaining constituents, hence liberating the phenols into extraction solvent.[20]

Third, an US-LLE was compared to a solid phase (SPE) extraction. Since there has been no systematic examination of the use of different SPE sorbents in existing literature, prior to this comparison, five commercially available SPE sorbents with good retention characteristics toward polar compounds[25] (Oasis HLB, Strata X, Isolute C18, Discovery DPA-6S and Sep-Pak) were tested and compared in terms of main olive phenol standard recoveries (Figure 2). As evident from the results, the maximum recoveries were achieved with HLB Oasis, varying from 73.4 ± 3.8% (apigenin) up to 101.3 ± 0.3% (tyrosol), followed by Isolute C18 and Strata X, which both retained phenols in high percentage as well (88% and 85% on average). By contrast, Sep-Pak and Discovery DPA-6S sorbents provided much lower phenol yields (79% and 76% on average) in spite of their higher SPE capacities (bed masses 820 and 500 mg) when compared to others, i.e., HLB Oasis (60 mg) or Strata X (200 mg). Moreover, the Waters HLB Oasis was found to provide the highest repeatability with RSDs below 6.8% (apigenin), while in the case of other sorbents the values exceeded even 20% or more (Sep-Pak, chlorogenic acid). However, based on these tests, an HLB Oasis SPE sorbent was chosen for further OMWW phenol analysis. Unfortunately, in the real OMWW sample this sorbent did not achieve efficient phenol recoveries. In fact, two out of fifteen phenols analyzed were not retained by this sorbent, while others showed poor recoveries. The reason for that could lie in matrix interferences, such as sugars, pectins, polyalcohols, etc., which disturb extraction of phenols. All of these compounds are indeed known to react with the Folin−Ciocalteu reagent, which explains a relatively high TP content (935.3 ± 98.3 μg GAE mL^{-1}) of SPE extracts when compared to US-LLE, respectively.

Fourthly, the US-LLE chromatograms were compared to those obtained after direct OMWW filtrate analysis. Surprisingly, the latter presented a much richer qualitative−quantitative HPLC phenol profile (Figure 3), indicating a nonsufficient US-LLE method previously utilized. The amount of phenols in filtrate was on average higher by 67% (in TP yields), or even more, i.e., 133% in HPLC individuals sum, when compared to US-LLE (Table 3), suggesting that some of phenols have remained in the aqueous

Table 3. Comparison of Methods Efficiencies in Terms of OMWW Phenol Recoveries (μg mL^{-1})

	liquid OMWW					
phenol recoveries	US-LLE[d]	LLE-agitation[e]	SPE	filtration	freeze-dried OMWW: USLE[f]	*P*-value[g]
hydroxytyrosol glucoside	nd[c]	nd	3.0 ± 0.5 γ	137.6 ± 2.7 β	367.6 ± 18.2 α	<0.01
hydroxytyrosol	100.3 ± 11.1 γ	29.6 ± 1.45 δ	19.8 ± 5.8 δ	331.2 ± 7.7 β	360.3 ± 4.7 α	<0.01
tyrosol	30.3 ± 2.0 β	23.8 ± 1.4 γ	22.7 ± 3.2 γ	114.2 ± 4.5 α	115.3 ± 5.1 α	<0.01
chlorogenic acid	9.8 ± 0.1 γ	1.0 ± 0.2 δ	6.0 ± 0.9 $\gamma\delta$	43.7 ± 6.6 α	33.5 ± 0.4 β	<0.01
vanillin	3.3 ± 0.1 γ	1.9 ± 0.2 δ	11.3 ± 0.5 α	nd	7.5 ± 0.4 β	<0.01
demethylligstroside	244.8 ± 6.0 γ	218.6 ± 8.5 δ	310.9 ± 26.5 β	412.5 ± 9.8 α	312.4 ± 8.3 β	<0.01
β-hydroxy verbascoside	6.7 ± 0.6 γ	3.5 ± 0.3 γ	nd	150.8 ± 8.2 α	125.1 ± 3.9 β	<0.01
isoacteoside	18.0 ± 0.1 γ	13.4 ± 0.6 γ	11.1 ± 1.6 γ	76.7 ± 9.6 α	68.6 ± 2.0 β	<0.01
nüzhenide	32.2 ± 0.4 β	nd	nd	392.9 ± 7.7 α	389.1 ± 6.7 α	<0.01
p-HPEA-DEDA	1065.9 ± 13.5 γ	623.7 ± 9.0 δ	174.6 ± 19.7 χ	1830.6 ± 20.7 α	1422.1 ± 20.6 β	<0.01
caffeoyl-6′-secologanoside	2.5 ± 0.1 γ	1.2 ± 0.2 γ	1.1 ± 0.2 γ	21.0 ± 1.3 β	24.2 ± 3.2 α	<0.01
comselogoside	7.6 ± 0.2 δ	4.5 ± 0.5 χ	18.2 ± 1.9 γ	29.0 ± 0.9 α	25.6 ± 1.4 β	<0.01
sum TP: HPLC[a]	1521.3 ± 34.0 γ	944.5 ± 23.2 δ	578.6 ± 60.9 χ	3540.1 ± 79.7 α	3251.1 ± 74.6 β	<0.01
TP: FC[b]	2247.4 ± 92.4 β	1870.7 ± 94.1 γ	935.3 ± 98.3 δ	3755.7 ± 230.5 α	3608.2 ± 113.2 α	<0.01

[a] HPLC−DAD results in μg GAE mL^{-1} of OMWW. [b] Folin−Ciocalteu total phenols (TP) results in μg GAE mL^{-1} of OMWW. [c] Not detected. [d] Extraction conditions: 5 × 10 min, methanol, US probe. [e] Extraction conditions: 5 × 10 min, methanol, agitation. [f] Extraction conditions: 3 × 20 min, methanol, US probe. [g] Values in the same row marked by the same letter ($\alpha, \beta, \gamma, \delta, \chi$) are not significantly different; confidence level = 95%; $P < 0.05$, significant; $P < 0.01$, very significant; $P > 0.05$, insignificant.

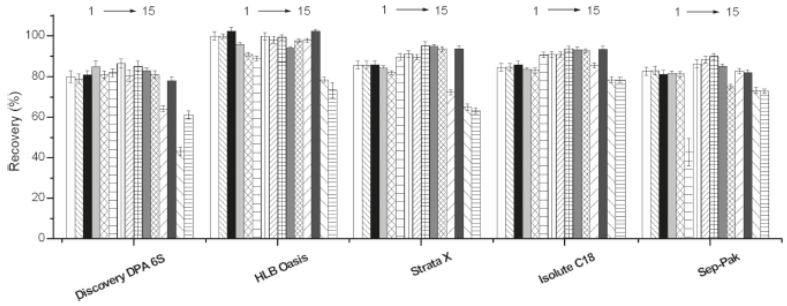

Figure 2. Recoveries of 15 phenolic compounds on different SPE sorbents: 1, hydroxytyrosol; 2, protocatechuic acid; 3, tyrosol; 4, *p*-hydroxybenzoic acid; 5, homovanillic acid; 6, chlorogenic acid; 7, *p*-coumaric acid, 8, benzoic acid; 9, ferulic acid; 10, oleuropein; 11, rutin; 12, apigenin-7-*O*-glucoside; 13, quercitrin; 14, luteolin; 15, apigenin.

residue after extraction was finished. Moreover, the presence of two new major phenols detected (peaks 2 and 3) indicated a nonappropriate extraction solvent choice (i.e., ethyl acetate) apparently not favorable for all the phenols present in the OMWW sample. In fact, the latter was already reported as selective toward only low and medium molecular weight phenols,[5] while not for glycosidal ones, like hydroxytyrosol 4-β-D-glucoside found in crude OMWW before.[24]

Consequently, a new extraction method had to be employed, using a solvent of higher selectivity toward a wider range of phenols, e.g., methanol, often reported as the best extracting medium for most phenols.[1] However, as the use of methanol requires nonaqueous matrices, a liquid OMWW sample was subjected to freeze-drying preservation, and the phenols were finally extracted using USLE previously developed for olive fruit phenol isolation from freeze-dried sample.[21] Figure 3 demonstrates both the qualitative and quantitative differences observed among phenol profiles of the three methods tested (US-LLE, filtration and USLE). While the US-LLE profile differed qualitatively from both USLE and filtration, it did not differ between the latter two. In fact, both of them presented similar phenol profiles with the same peaks detected, suggesting that USLE offers a representative and complete extraction of all the phenols present in OMWW, although sometimes reported as being lost during the extraction procedure.[5,24] However, no statistical differences in TP yields were observed between USLE and filtration (Table 3), while on an individual level, only three phenols, namely, hydroxytyrosol glucoside, hydroxytyrosol and caffeoyl-6′-secologanoside, were significantly enhanced by US, most likely due to their US-induced release from olive plant material still largely contained

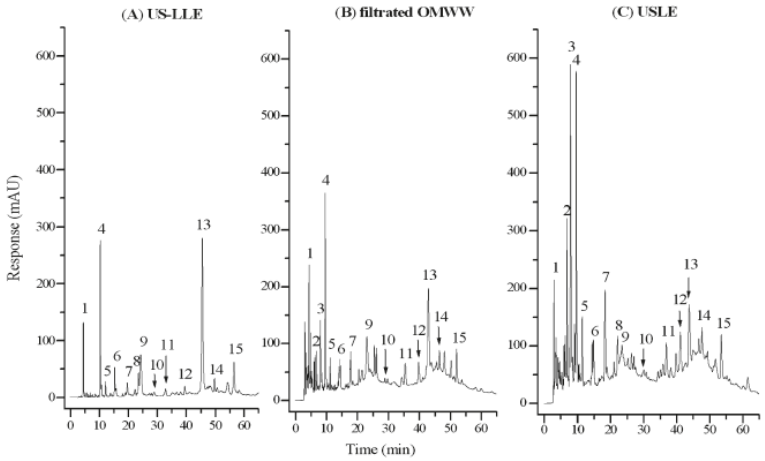

Figure 3. HPLC−DAD chromatograms of OMWW phenolic compounds from (A) US-LLE extract, (B) filtrated OMWW and (C) USLE extract at 280 nm. The peak numbers refer to Table 1.

in the OMWW. In fact, the dry matter content of OMWW analyzed was relatively high (9.5%) in comparison to Azbar et al. (2004) (3%),[26] while low when compared to the data of Lesage-Meessen et al. (2001) (20−24%),[11] respectively.

Since the fidelity of the phenol profile between the starting material and the final extract is the measure of analytical success,[1] only two out of five methods tested, USLE and direct filtrate analysis, could be considered for OMWW phenol analysis. Generally, the extraction of phenols from the matrix has been a prerequisite to quantification. Although sometimes complicated and time-consuming, it offers advantages, such as the preconcentration of analyte and removal of interfering matrix components.[1] This can prolong the lifetime of analytical columns used, in addition to a higher sensitivity and chromatographic resolution obtained.[27] However, referring to our study, the main advantage of USLE over direct filtrate analysis is the sample preparation step, since the USLE method is based on a freeze-dried sample analysis. Indeed, the combination of sample freezing and lyophilization is one of the best sample-handling techniques[1] that guarantees the stability of a complex matrix such as OMWW prior to delayed phenol analysis.[19] High water content in a liquid sample provides a medium for several chemical and biochemical reactions, therefore, the need for phenol stabilization is of great importance in order to avoid and/or minimize any of the potential artifacts.[1] Nevertheless, the USLE method proposed holds greater potential for practical applications since most of the olive mill wastewaters produced worldwide are dried in large evaporation lagoons,[26] resulting in the dry solid leftovers, the matrix upon which USLE is based.

Phenol Content of Slovenian OMWW. The quantitative analysis confirmed Slovenian OMWW as a rich source of several phenolic compounds, especially when compared to other wastewater samples from different European Union (EU) olive fruit-producing countries. Interestingly, the TP yield obtained (3608.2 μg GAE mL^{-1}) was significantly higher than those reported for Portuguese (243 μg GAE mL^{-1}),[14] Greek (691 μg GAE mL^{-1}) [4] or Italian (980 μg GAE mL^{-1}) [15] samples, but lower than those for Spanish (5780 μg GAE mL^{-1}),[28] respectively. However, at the level of individual phenol analysis, two phenols were predominant in Slovenian OWWW, hydroxytyrosol and tyrosol, coincidentally associated with the highest antioxidant activities[1] and, hence, of the highest commercial value. Moreover, their concentrations were much higher than those found in other commercial OMWWs analyzed within the 2001 EU project of natural antioxidants from olive oil processing wastewaters.[19] The content of hydroxytyrosol found in Slovenian OMWW (360.3 μg mL^{-1}) was superior to Spanish (36.0 μg mL^{-1}) and Italian (131.0 μg mL^{-1}) levels, while absent in French and Portuguese samples. However, tyrosol was found in all four samples, but likewise in much lower concentrations (Spain; 41.0 μg mL^{-1}, Italy; 29.0 μg mL^{-1}, France; 5.0 μg mL^{-1}, Portugal; 99 μg mL^{-1}) in comparison to Slovenian OMWW (115.3 μg mL^{-1}), indicating its high potential for the phenol screening hits utilization.

In conclusion, the double nature of OMWW phenols (natural antioxidants vs environmental pollutants) creates not only an economical and ecological challenge in their high and efficient recovery but also a difficult analytical task owing to their chemical diversity and matrix complexity. Within this context, US-assistance has proven to be a good alternative to conventional solvent extraction techniques for both liquid and solid OMWW matrices with several advantages, including increased extraction yields, faster kinetics and simplicity. While the US extracting efficiency in aqueous OMWW has been limited due to water nonmiscible solvent use, its application to freeze-dried solid OMWW has shown no limitations. In fact, the USLE method proposed, combined with the preliminary freeze-drying step of liquid OMWW, is based on methanol's high extracting abilities,

allowing analysis of all OMWW phenols without any US-induced alterations. The freeze-drying step also ensures higher stability of the liquid sample in cases of delayed analysis, as well as a reduction of its volume which eliminates the related storage problems. Compared with other novel extraction techniques such as microwave-assisted extraction, the US setup is cheaper and easier to operate,[29] while compared to advanced membrane separations, it is more economically feasible and holds potential for individual application.[12] Indeed, the cost−benefit analysis of US-assisted extraction has shown a rapid return on investment (4 months);[30] therefore the main disadvantages of this method arise from its high solvent consumption and its limited capacity if not operated in a continuous mode.

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Funding Sources

This work was funded by Slovenian Research Agency (ARRS) and World Federation of Scientists (WFS).

ABBREVIATIONS USED

OMWW, olive mill wastewater; US-LLE, ultrasound-assisted liquid−liquid extraction; SPE, solid-phase extraction; USLE, ultrasound-assisted solid−liquid extraction; TP, total phenols

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Food Chemistry 134 (2012) 2481–2488

Contents lists available at SciVerse ScienceDirect

Food Chemistry

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Analytical Methods

Optimisation of olive oil phenol extraction conditions using a high-power probe ultrasonication

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ARTICLE INFO

Article history:
Received 14 June 2011
Received in revised form 25 October 2011
Accepted 15 April 2012
Available online 24 April 2012

Keywords:
Ultrasound assisted liquid–liquid extraction (US-LLE)
Optimisation
Extra virgin olive oil (EVOO)
Phenols

ABSTRACT

A new method of ultrasound probe assisted liquid–liquid extraction (US-LLE) combined with a freeze-based fat precipitation clean-up and HPLC–DAD-FLD-MS detection is described for extra virgin olive oil (EVOO) phenol analysis. Three extraction variables (solvent type; 100%, 80%, 50% methanol, sonication time; 5, 10, 20 min, extraction steps; 1–5) and two clean-up methods (*n*-hexane washing vs. low temperature fat precipitation) were studied and optimised with aim to maximise extracts' phenol recoveries. A three-step extraction of 10 min with pure methanol (5 mL) resulted in the highest phenol content of freeze-based defatted extracts (667 μg GAE g^{-1}) from 10 g of EVOO, providing much higher efficiency (up to 68%) and repeatability (up to 51%) vs. its non-sonicated counterpart (LLE-agitation) and *n*-hexane washing. In addition, the overall method provided high linearity ($r^2 \geqslant 0.97$), precision (RSD: 0.4–9.3%) and sensitivity with LODs/LOQs ranging from 0.03 to 0.16 μg g^{-1} and 0.10–0.51 μg g^{-1} of EVOO, respectively.

1. Introduction

The past few decades has witnessed an amazing increase in ultrasound (US) energy application into various fields of science and technology, ranging from those of biomedical diagnosis up to the food, material and analytical chemistry. In particular the latter has gained significance due to the development of new US based and US assisted detection systems supported by recent advances in US electronic/transducer designs as well as their commercial availability. Its vast field of analytical applications is now widely documented (Luque de Castro & Priego-Capote, 2007a, 2007b; Priego-Capote & Luque de Castro, 2007), and clearly testifies to its advantages over classical sample preparation techniques in facilitating and/or accelerating operations such as digestion, leaching, homogenisation, emulsification, extraction and others.

Most recently US has also found applications in the phenol analysis of various plant materials, as seen from several US assisted extraction integrations into analytical processes. Although US induced high temperatures and pressure release could alter some phenol structure modification during extraction, and hence cause some considerations in phenol analysis (Jerman, Trebše, & Mozetič Vodopivec, 2010), the latter have instead gained popularity as evident from an increasing number of recent publications. In fact, US assisted extractions are continuously proposed in phenol analysis of different plant matrices such as strawberries, red raspberries, grape seeds and olive fruits (Chen et al., 2007; Ghafoor, Choi, Jeon, & Jo, 2009; Herrera & Luque de Castro, 2005; Jerman et al., 2010) due mainly to advantages in terms of higher efficiency, lower solvent consumption and faster extraction. Such assistance is typically provided by high-power US baths and/or probe-type sonicators (100–450 W) operated at low frequencies (20–50 kHz) as seen in the analytical literature. Although US probes are less frequently used in comparison to baths, the former are far more suitable for analytical use, not only due to higher reproducibility and construction flexibility (Luque de Castro & Priego-Capote, 2007a), but mainly due to a higher extracting power as already previously proved (Jerman et al., 2010).

Olive oil phenols are one of the most intensively studied groups of natural food antioxidants, increasing attention for their wide range of bioactivities beneficial for human health (Obied, Bedgood, Prenzler, & Robards, 2007). Although many reports have been dedicated to olive oil phenol analysis (Carrasco-Pancorbo et al., 2006; Fu et al., 2009; Suárez, Maciá, Romero, & Motilva, 2008; Torre-Carbot et al., 2005) studies aimed at their efficient recoveries have been rare (Bendini et al., 2003; Montedoro, Servili, Baldioli, & Miniati, 1992; Pirisi, Cabras, Falqui Cao, Migliorini, & Muggelli, 2000). In fact, most have utilised only two basic extraction techniques – a solid-phase (SPE) extraction employing diol, C8 or C18 sorbents (Bendini et al., 2003; Carrasco-Pancorbo et al., 2006; Fu et al., 2009; Pirisi et al., 2000; Torre-Carbot et al., 2005) or liquid–liquid (LLE) extraction using different organic solvents and conventional methods of

Abbreviations: US-LLE, ultrasound assisted liquid–liquid extraction; EVOO, extra virgin olive oil; TP, total phenols.

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0308-8146/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodchem.2012.04.096

manual or mechanical agitation (Brenes, Romero, García, Hidalgo, & Ruiz-Méndez, 2004; Kalua, Allen, Bedgood, Bishop, & Prenzler, 2005; Montedoro et al., 1992; Suárez et al., 2008). However, several examples from the literature illustrate that conflicting results can be obtained under different extraction/analysis conditions used (Pirisi et al., 2000) making foodstuffs' quality data comparison on a global level difficult or even disputable, especially where dietary intake/availability is of paramount importance.

Currently, the US baths/probes are part of almost every analytical lab, but unfortunately rarely optimised when implemented into analytical methods. Few reports have already utilised the US assisted extraction in order to facilitate phenols from solid olive matrices like olive fruits (Jerman et al., 2010) and leaves (Japón-Luján, Luque-Rodríguez, & Luque de Castro, 2006), but to our best knowledge, its potential has not been yet fully exploited for extra virgin olive oil (EVOO) matrices. Although liquids seem less likely to require energy for analyte acceleration (Priego-Capote & Luque de Castro, 2007) they can benefit in many aspects, such as, improved efficiency, emulsification and/or homogenisation (Luque de Castro & Priego-Capote, 2007a). Thus, in view of identifying optimal extraction conditions guiding US assisted isolation of bioactive phenols, the present study was undertaken with aim to maximise phenols recovery from EVOO sample, and hence investigate the US probe's potential for LLE acceleration in high fatty matrices. In addition, the efficiencies of two clean-up methods for extracts lipids removal (*n*-hexane washing vs. low temperature fat precipitation) were tested and compared in terms of phenol yields.

2. Materials and methods

2.1. Samples

A multi-varietal extra virgin olive oil (EVOO), used for optimisation studies (1 L), was obtained from a commercial two-phase olive mill plant located in Hvar (Gdinj, Croatia) in November 2009, while refined olive oil (ROO), used as blank in recovery and sensitivity studies, was purchased in a local store. Other four EVOO samples used in US-LLE application study (Section 3.7) were obtained from different locations: oil A, from Istrska belica olives (Guci, Slovenia); oil B, from Leccino olives (Goriška Brda, Slovenia); oil C, from Istrska belica/Leccino (60/40%) olives (Plavje, Slovenia) and oil D, from Oblica/Leccino/Itrana (60/30/10%) olives (Hvar, Croatia).

2.2. Chemicals and solvents

Methanol (HPLC grade), 2,2-diphenyl-1-picrylhydrazil (DPPH), *n*-hexane and glacial acetic acid were purchased from Sigma–Aldrich Co. Ltd. (Gillingham, GB), while phenol standards of hydroxytyrosol, tyrosol, oleuropein, apigenin, luteolin and gallic acid were obtained from Extrasynthese (Genay, France) and Folin–Ciocalteu (Merck) from a local supplier. High-quality water (Millipore Q Plus 18 Ω; Billerica, MA, USA) was used to prepare both, the water/methanol solvent mixtures and a mobile chromatographic phase.

2.3. Extraction conditions

2.3.1. US-LLE with ultrasonic probe

A high-power LABSONIC® M ultrasonic probe (100 W, 30 kHz) from SARTORIUS (Gottingen, Germany) was used for the EVOO phenols extraction assistance. Extra virgin olive oil (10 g) was placed into a PE centrifuge tube, dissolved in *n*-hexane (10 mL) and directly sonicated for different extraction parameters in the following order: solvent type (50%, 80%, 100% methanol), sonication time (5, 10, 20 min) and extraction steps (1–5) using 5 mL of solvent under US working conditions (100% amplitude/duty cycle, titanium 3∅ mm sonotrode) primarily optimised elsewhere (Jerman et al., 2010). While the efficiency of five consecutive extraction steps was evaluated for each extraction step, the influence of solvent type and sonication time was assessed based on a three-step extraction. The phases after each extraction step were separated by centrifugation (9000 rpm, 2 min) using an Eppendorf centrifuge model 5804 (Hamburg, Germany) and combined methanol supernatants were quantitatively transferred into 25 mL volumetric flask, defatted prior dilution with methanol, and evaluated for the extraction efficiency employing HPLC-DAD-FLD analysis. In addition, a comparison between capped vs. non-capped extraction PE tubes was carried out in order to test the possibility of EVOO phenol losses (e.g. evaporation) during sonication process.

2.3.2. LLE-agitation

For the purpose of comparison, the extraction employing only liquid–liquid (LLE) mechanical agitation (VIBROMIX 313 EVT, Železniki, Slovenia) was performed (as control), where 10 g of EVOO was dissolved in *n*-hexane (10 mL) and extracted using optimised extraction conditions obtained by US probe; 3 × 10 min with 5 mL of pure methanol. The homogenates of each extraction step were centrifuged (Eppendorf, 9000 rpm, 2 min), supernatants decanted, merged and defatted prior dilution with methanol to 25 mL. Prepared extracts were put in a screw-capped dark glass containers and stored in freezer (−25 °C) until further HPLC analysis.

2.4. Clean-up of extracts

When extraction was completed, the methanol extracts were cleaned-up using two methods of lipids removal techniques; (a) washing with *n*-hexane (3 × 10 mL) according to Suárez et al. (2008) and (b) a low temperature fat precipitation (2–3 h at −25 °C or 20 min at −80 °C) according to Lentza-Rizos, Avramides, and Cherasco (2001). After extracts were defatted with a simple solvent decantation process, the remaining methanol solution containing phenols was reconstituted to 25 mL with methanol and analysed by HPLC-DAD-FLD analysis.

2.5. HPLC-DAD-FLD and LC–MS analysis

Prior to chromatographic analysis, the aliquot of extract (15 mL) was evaporated to dryness using rotary evaporation (35 °C, LABOROTA 4000; Heidolph, Schwabach, Germany) and re-dissolved in 1 mL of acidic HPLC eluent (H_2O/CH_3COOH, 95:5, v/v), further filtered through 0.45 μm PTFE filters (Macherey-Nagel, Düren, Germany).

2.5.1. HPLC-DAD-FLD

A Hewlett Packard 1100 liquid chromatograph (Agilent Technologies, Santa Clara, USA) equipped with UV/VIS diode-array (190–600 nm) and fluorescence (FLD) detectors ($\lambda_{excitation/emission}$ 280/330 nm) was used for individual phenols determination. The sample volume of 70 μL was injected and DAD signals were recorded at 280 nm, using gradient elution on Luna PFP (5 μm, 250 mm × 4.6 mm) attached to a security guard (4 × 3 mm) both from Phenomenex (Macclesfield, UK) under analytical conditions previously described (Jerman et al., 2010).

2.5.2. LC–MS

Phenols identification was confirmed with LC–MS/MS analysis using Perkin Elmer Series 2000 (Schelton, USA) linked to 3200 Q TRAP LC/MS/MS system equipped with electro spray ion (ESI) source from Applied Biosystems/MDS Sciex (Foster City, USA). All mass spectrometric scans were performed infusing 20 μL of EVOO extracts in a negative ion mode (NIM), scanning from *m/z* 50 to *m/z*

1000 in 1 s. The electrospray conditions were as follows: capillary temperature 400 °C, capillary voltage −3500 V, declustering potential (DP) −30 V and entrance potential (EP) −30 V, respectively.

EVOO phenols were identified using analytical approach as previously described (Jerman et al., 2010), i.e. by comparison of retention times (Rt), UV–VIS, FLD and ESI-MS^2 spectra with those of authentic standards when available, while the tentative identity of others was confirmed by comparison of UV–VIS spectra and ESI–MS^2 fragmentation profile of molecular $[M-H]^-$ ions with those from the literature and our database (Section 3.1). The simple phenols (hydroxytyrosol, tyrosol) were quantified based on a calibration curve of their authentic standards, while the secoiridoids (3,4-DHPEA-DEDA, *p*-HPEA-DEDA, ligstroside and oleuropein aglycone derivatives) and Peak 4 were expressed as oleuropein equivalents in $\mu g\ g^{-1}$ of olive oil.

2.6. Total phenol (TP) analysis

The extracts total phenol content was determined colorimetrically at 765 nm using Folin–Ciocalteu reagent, according to Ough and Amerine (1988), and results were expressed as gallic acid (GAE) equivalents in $\mu g\ g^{-1}$ of olive oil.

2.7. Method characterisation

The method was characterised in terms of main analytical quality parameters according to our previous study (Jerman et al., 2010).

The precision in terms of within-laboratory repeatability was evaluated by measuring the phenol peak areas of the same EVOO extract within-one-day ($n = 3$) and between days over two-week period time ($n = 3$), while the sample between analyses was stored in freezer (−25 °C).

The linearity range of the method proposed was evaluated by linear regression analysis of spiked ROO matrices with five phenolic standards, i.e. hydroxytyrosol, tyrosol, oleuropein, apigenin and luteolin. Calibration curves were prepared by serial dilution of their methanolic stock solutions over the typical concentration range consistent with the levels of these phenols in EVOO sample.

The extraction efficiency was evaluated in terms of phenol recoveries using standard addition method over the range defined in the linearity study. Five phenolic standards (hydroxytyrosol, tyrosol, oleuropein, apigenin, luteolin) were added to ROO through successive dilutions of their methanolic mix solution and extracted by US-LLE proposed ($n = 3$). All samples were analysed in triplicates before and after additions and recoveries were expressed as means ± RSD (%).

The method's sensitivity evaluation was based on LODs and LOQs calculation, using the signal-to-noise ratio criteria of 3 and 10, respectively. Calibration curves of five phenolic standards (hydroxytyrosol, tyrosol, oleuropein, apigenin, luteolin) were prepared by serial dilution of their methanolic stock solution and aliquots added to ROO in the concentration ranges close to LOQs expected for each phenol.

2.8. Statistical analysis

All experiments were performed at least in triplicate ($n \geqslant 3$) and the results were expressed as means ± SD. Statistical significances among parameters were evaluated by analysis of variance (ANOVA) using STATGRAPHICS Plus 4.0 (Manugistics Inc., Rockville, MD) and a Duncan's multiple range test (MRT) was used to discriminate between the means at 95% confidence level, where P values < 0.05 were regarded as significant, P values < 0.01 as very significant and P values > 0.05 as insignificant.

3. Results and discussion

3.1. Phenols identification

The HPLC-DAD profile of EVOO extracts, prepared by optimised US-LLE method (Section 3.2), revealed the presence of 13 peaks (Fig. 1), however, only twelve could be identified, while the identity of one remained inconclusive (Table 1). As the Peaks 1, 2, 12 and 13 were easily identified with the help of authentic standards as hydroxytyrosol (Rt = 10 min), tyrosol (Rt = 15 min), luteolin (Rt = 70 min) and apigenin (Rt = 71 min), the following discussion is mainly limited to those not confirmed with standards, but identified based on a screening data comparison with existing literature.

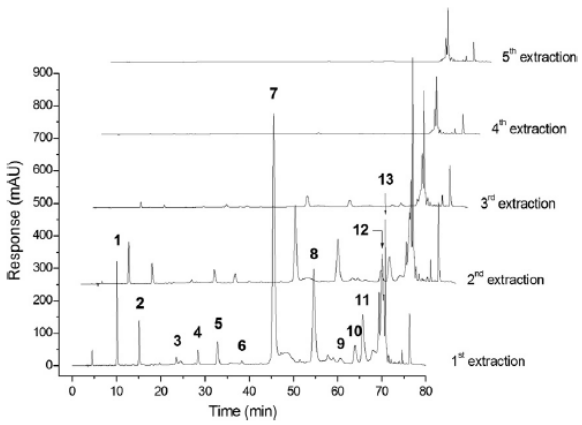

Fig. 1. HPLC-DAD phenol profile of EVOO extracts at 280 nm. Peak identification is shown in Table 1.

Table 1
UV/VIS, FLD and MS spectral characteristics of main phenols from EVOO extract.

Peak	HPLC-DAD-FLD			ESI-LC–MS		Tentative identification	Standard
	Rt (min)	λ_{max} (nm)	$\lambda_{ex/em}$ 280/330 (nm)	MW (M)	Major $[M-H]^-$ (m/z)		
1	10	236, **280**[a]	Yes	154	153, 123, 95	Hydroxytyrosol (3,4 DHPEA)	Yes
2	15	236, **276**	Yes	138	137	Tyrosol (*p*-HPEA)	Yes
3	23	**262**, 294	Weak	336	335, 155, 151	Ligstroside derivative	No
4	28	236, **280**, 308	Weak		581, 505, 339, 241, 209, 165, 139, 127, 121	Unknown	No
5	33	236, 290sh, **310**	Weak	320	319, 195, 165, 155	3,4-Dihydroxyphenylethyl decarboxymethyl elenolic acid dialdehyde (3,4-DHPEA-DEDA)	No
6	39	236, 298sh, 324	No	378	377, 307, 275, 195, 155, 111	Oleuropein aglycone derivative	No
7	45	**238**, 278	Weak	304	303, 285, 179, 165, 156	4-Dihydroxyphenylethyl decarboxymethyl elenolic acid dialdehyde (*p*-HPEA-DEDA)	No
8	55	**272**, 298	Weak	378	377, 307, 275, 195, 155, 111	Oleuropein aglycone derivative	No
9	63	**238**, 276	Weak	378	377, 307, 275, 195, 155, 111	Oleuropein aglycone derivative	No
10	65	**236**, 280	Yes	378	377, 307, 275, 195, 155, 111	Oleuropein aglycone derivative	No
11	67	**236**, 280	Yes	378	377, 307, 275, 195, 155, 111	Oleuropein aglycone derivative	No
12	70	**251**, 348	No	286	285, 155	Luteolin	Yes
13	71	221, **263**, 335	No	270	269	Apigenin	Yes

[a] Maximum UV band indicated in bold.

Peak 3 eluting at 23 min was assigned as ligstroside derivative, based on its UV/VIS spectrum (λ_{max} 262, 294) and the fact that it gave a deprotonated molecule ion at m/z 335, in addition to a weak FLD signal ($\lambda_{ex/em}$ 280/330 nm) due to an aldehydic group in its open elenoic acid ring (Selvaggini et al., 2006; Suárez et al., 2008; Torre-Carbot et al., 2005).

The mass spectra of Peak 4 (Rt = 28 min) displayed various ions at m/z 581, 505, 339, 241, 209, 165, 139, 127 and 121, while its UV/VIS spectrum had an absorption maxima at 236, 280 and 308 nm. Best to our knowledge this type of spectral characteristics have not been reported yet in the literature, but could based on two strong signals (m/z 241, 137) and λ_{max} close to 240 nm fit to elenoic acid, suggesting its presence in the phenol structure. However, based on the data obtained, it was not possible to deduce its feasible structure for tentative identification, thus Peak 4 remained as an unknown.

Peak 5 (Rt = 33 min) was assigned as an oleuropein derivative in a decarboxylade form displaying a molecular ion at m/z 319 and fragments at m/z 195, 165 and 155. Although three different models of this derivative with the same mass molecule are reported in the literature (Carrasco-Pancorbo et al., 2006; Torre-Carbot et al., 2005), a weak FLD signal and UV–VIS characteristics are indicative of 3,4-dihydroxyphenylethyl decarboxymethyl elenolic acid dialdehyde or 3,4-DHPEA-DEDA (Montedoro et al., 1993; Obied et al., 2007; Selvaggini et al., 2006).

Likewise, Peak 6 (Rt = 39 min) was assigned as oleuropein derivative, however, here present in carboxylic form due to its molecular ion signal at m/z 377 (Torre-Carbot et al., 2005). Its product ion spectra showed various fragment ions at m/z 307, 275, 195, 155 and 111, which is consistent with previous MS^2 profiles for oleuropein aglycone derivatives (Bendini et al., 2003; Fu et al., 2009; Kalua et al., 2005; Obied et al., 2007).

The predominant compound at 45 min (Peak 7) showed an intense molecular ion at m/z 303 with four fragments at m/z 285, 179, 165 and 156. This MS fragmentation profile and UV–VIS/FLD characteristics are comparable with previous reports for 4-dihydroxyphenylethyl decarboxymethyl elenolic acid dialdehyde (*p*-HPEA-DEDA), commonly found in EVOO matrices (Brenes et al., 2004; Carrasco-Pancorbo et al., 2006; Kalua et al., 2005; Obied et al., 2007; Selvaggini et al., 2006; Suárez et al., 2008).

The ESI-MS spectra generated for Peaks 8, 9, 10 and 11, eluting at 55, 63, 65 and 67 min have shown the same molecular ion at m/z 377, confirming the presence of oleuropein derivatives in carboxylic forms (Torre-Carbot et al., 2005). As their fragmentation ions were almost identical, their further structural assignment was not possible, and were thus, as many times before, solely assigned as oleuropein aglycone derivatives (Bendini et al., 2003; Fu et al., 2009; Kalua et al., 2005; Obied et al., 2007; Pirisi et al., 2000; Suárez et al., 2008; Torre-Carbot et al., 2005).

In general, our EVOO phenol profile obtained agrees with previous reports for other EVOO matrices, dominated by the same phenol classes, namely secoiridoids (oleuropein and ligstroside derivatives), simple phenols (hydroxytyrosol and tyrosol) and flavonoids (luteolin and apigenin), while surprisingly lignans did not appear in the sample, which is in line with previous report (Torre-Carbot et al., 2005). However, although the presence of thirteen phenols was confirmed in EVOO extract, only eleven major phenols (hydroxytyrosol, tyrosol, ligstroside derivative, 3,4-DHPEA-DEDA, *p*-HPEA-DEDA, Peak 4 and five oleuropein aglycone derivatives) could be quantified, providing the basis for further US-LLE extraction optimisation study.

3.2. Optimisation of US-LLE extraction conditions

In US assisted liquid–liquid extraction several factors entailed optimisation, including one related to US and those characteristic for LLE (Luque de Castro & Priego-Capote, 2007a) Thus, the typical US related variables (probe position, amplitude and duty cycle) were primarily optimised according to previous report (Jerman et al., 2010), where the strong sonication conditions (100% amplitude and duty cycle) with 3∅ mm sonotrode has resulted in the highest phenol recoveries of spiked ROO matrices (>94%). In contrast to a previous report (Ruiz-Jiménez & Luque de Castro, 2003), the US here has avoided an EVOO emulsion formation, although forming finely dispersed droplets during sonication, however, returning immediately back to a heterogeneous liquid–liquid system after extraction was finished.

Once US related variables were fixed (100% amplitude and duty cycle, 3∅ mm probe), the three main LLE parameters, namely solvent type, sonication time and extraction steps were optimised based on recovery of eleven individual and total phenols using HPLC and Folin–Ciocalteu analysis. The influence of each variable was evaluated independently, while keeping others at constant values. The one providing the highest yield was considered as optimal

Table 2
The effect of extraction solvent type and sonication time on EVOO phenols extraction yields (μg g^{-1}).

Phenol compounds	EVOO phenol extraction yields (μg g^{-1})					
	Extraction solvent type[c]			Extraction time[d]		
	100%	80%	50%	5 min	10 min	20 min
Hydroxytyrosol	1.72 ± 0.08α[e]	1.65 ± 0.26α	1.55 ± 0.19α	1.64 ± 0.26α	1.72 ± 0.08α	1.72 ± 0.28α
Tyrosol	2.30 ± 0.11α	2.29 ± 0.1α	2.28 ± 0.12α	2.17 ± 0.16α	2.30 ± 0.11α	2.43 ± 0.17α
Ligstroside derivative	2.35 ± 0.31α	2.34 ± 0.30α	2.22 ± 0.13α	2.38 ± 0.12α	2.35 ± 0.31αβ	1.80 ± 0.35β
Unknown	5.59 ± 0.47α	5.57 ± 0.45α	5.37 ± 0.31α	4.32 ± 0.09β	5.59 ± 0.47α	5.83 ± 0.75α
3,4-DHPEA-DEDA	12.95 ± 0.30α	12.90 ± 0.24α	12.64 ± 0.56α	9.60 ± 0.04χ	12.95 ± 0.30α	11.26 ± 0.42β
Oleuropein aglycone derivative	2.08 ± 0.18α	2.07 ± 0.17 α	1.96 ± 0.02α	1.09 ± 0.19β	2.08 ± 0.18α	1.81 ± 0.41α
p-HPEA-DEDA	140.54 ± 1.82α	132.12 ± 2.19β	110.71 ± 2.09χ	70.59 ± 1.6 β	140.54 ± 1.82α	141.92 ± 3.93α
Oleuropein aglycone derivative	64.12 ± 0.02α	54.42 ± 3.28β	52.36 ± 2.58β	29.76 ± 3.02β	64.12 ± 0.02α	66.51 ± 3.27α
Oleuropein aglycone derivative	5.13 ± 0.17α	4.55 ± 0.32α	4.56 ± 0.54α	3.53 ± 0.61α	5.13 ± 0.17α	5.21 ± 0.59α
Oleuropein aglycone derivative	16.62 ± 2.67α	16.55 ± 2.59α	14.65 ± 1.06α	14.35 ± 0.21α	16.62 ± 2.67α	16.21 ± 0.51α
Oleuropein aglycone derivative	39.93 ± 6.73α	39.77 ± 6.54α	35.04 ± 2.82α	38.07 ± 1.15α	39.93 ± 6.73α	41.91 ± 2.49α
Sum TP-HPLC[a]	293.33 ± 13.13α	274.23 ± 16.46α	243.34 ± 10.42β	177.50 ± 7.54β	293.33 ± 13.13α	296.61 ± 13.17α
TP-FC[b]	667.09 ± 10.91α	633.90 ± 12.72β	598.26 ± 4.42χ	436.21 ± 17.53β	667.09 ± 10.91α	668.29 ± 12.58α

[a] HPLC-DAD results in μg g^{-1} of olive oil.
[b] Folin–Ciocalteu total phenols (TP) results in μg GAE g^{-1} of olive oil.
[c] Extraction conditions: 10 min, 3-step extraction.
[d] Extraction conditions: 100% methanol, 3-step extraction.
[e] Values marked with the same letter (α, β, χ) are not significantly different; confidential level = 95%.

and further implemented into an optimisation process for others till the final (optimal) US extraction conditions were obtained, referred to as the US-LLE method.

Firstly, a solvent study was conducted to select the best extraction solvent for EVOO phenols recovery, testing pure methanol and its water mixtures (80%, 50%) at volume:mass ratio (1:2, mL g^{-1}) according to previous reports (Kalua et al., 2005; Montedoro et al., 1992; Suárez et al., 2008). Although the use of methanol/water mixtures (80–60%, v/v) clearly predominates in existing literature due to superior yields obtained (Montedoro et al., 1992), our study revealed a different outcome. Surprisingly, the phenol yields decreased upon increasing the water level in a solvent, confirmed by both individual and TP analysis (Table 2). The maximum recoveries were obtained with pure methanol followed by 80% and 50% of its water mixtures. Although the differences were low and insignificant for most of phenols analysed (<5%, $P > 0.05$) an almost linear decline ($r^2 = 0.89$) was observed in the case of two predominating phenols; *p*-HPEA-DEDA and an oleuropein aglycone derivative (Peak 8), decreasing consistently along with a solvent polarity. This indicates to their rather lipophilic nature, supporting the hydrophobic EVOO phenols partition theory during oil processing (Rodis, Karanthanos, & Mantzavinou, 2002). However, as water additions (20–50%) to extracting media were apparently not in favour of phenols extractability, a pure (100%) methanol was chosen for further experiments.

Secondly, the EVOO phenols extractability was studied as a function of sonication time (5, 10, 20 min) and likewise the latter appeared to affect their yields as well (Table 2). The time increases up to 10 min provoked an almost linear rise in TP yields ($r^2 = 0.97$), however, no further increases were observed at longer sonications (20 min), suggesting that maximum recoveries were already attained before (i.e. 10 min). This type of behaviour is rather usual for phenols extractability, requiring a sufficient time to reach the analyte/solvent equilibrium (Chen et al., 2007) though its profound impact can be rather ascribed to a combined effect of time and temperature (Jerman et al., 2010) both promoting yields to greater extend. In fact, the US induced cavities are known to cause a temperature rise, although the latter is rapidly dissipated with no drastic changes in environmental conditions (Luque de Castro & Priego-Capote, 2007a). Our study of US time-temperature behaviour supports this observation, as the extracts heating has proceeded slowly, but consistently with US time increases, reaching maximum by the end of sonication, i.e. 38 ± 2 °C (10 min) or 44 ± 2 °C (20 min). Although the phenols extractability has been proved to increase with temperature rise (Jerman et al., 2010) their yields have not increased above 38 ± 2 °C (10 min), and thus a 10 min-sonication was chosen for further extraction optimisation.

However, a positive US time/TP yield correlation also suggests that EVOO phenols are rather stable under US conditions tested, although sometimes reported as the subject of thermal artefacts in other matrices (Herrera & Luque de Castro, 2005). This is further supported by our phenol recovery study as almost linear correlations ($r^2 \geqslant 0.97$) were obtained with spiked ROO standard additions. Moreover, no qualitative differences between sonicated vs. non-sonicated extract profiles were observed, suggesting that phenols are not modified nor degraded under US probe exposure. Nevertheless, the evaporation study confirmed that EVOO phenols are not lost during sonication, as the HPLC-DAD yield differences between capped vs. non-capped PE tubes were almost negligible (1.7%), respectively.

Finally, the influence of five sequential extraction steps were assayed to maximise phenols recovery (Fig. 1), where a three-step extraction was efficient enough for the quantitative EVOO extraction, providing recoveries superior to 98%. As expected, the percentage of individual phenols sum recovered has decreased gradually with each extraction step by 73.0%, 20.0%, 5.0%, 1.7% and 0.3% on average.

Overall, the optimised US-LLE method thus consisted of dissolving 10 g of EVOO sample in *n*-hexane (10 mL) and its further sonication (3 × 10 min) with 5 mL of pure methanol under US probe conditions (100% amplitude and duty cycle, 3∅ mm probe).

3.3. Clean-up of extracts

Prior to final EVOO phenol analysis an additional clean-up step is typically required to remove the extracts' co-eluting lipid interferences, usually based on liquid–liquid partitioning with *n*-hexane, acetonitrile and other solvents (Brenes et al., 2004; Montedoro et al., 1992; Pirisi et al., 2000; Suárez et al., 2008) and/or their combinations with subsequent solid-phase (SPE) extraction (Fu et al., 2009; Pirisi et al., 2000; Torre-Carbot et al., 2005). However, a complete lipids removal without phenol losses from high fatty matrices (e.g. EVOO) is yet a challenging task, further complicated by the phenols double nature containing both – more and less polar constituents.

Table 3
Comparison of two extract's fat removal clean-ups; *n*-hexane washing and a freeze-based fat precipitation vs. full-fat extract on EVOO phenols yields (μg g^{-1}).

Phenol compounds	EVOO phenol yields (μg g^{-1})		
	Full-fat	Defatted (*n*-hexane washing)[c]	Defatted (freeze-based fat precipitation)[d]
Hydroxytyrosol	1.27 ± 0.20β[e]	1.16 ± 0.28β	1.72 ± 0.08α
Tyrosol	1.99 ± 0.13α	2.00 ± 0.27α	2.30 ± 0.11α
Ligstroside derivative	0.15 ± 0.16χ	1.18 ± 0.37β	2.35 ± 0.31α
Unknown	3.64 ± 0.11χ	4.78 ± 0.04β	5.59 ± 0.47α
3,4-DHPEA-DEDA	8.17 ± 0.29χ	10.08 ± 0.19β	12.95 ± 0.30α
Oleuropein aglycone derivative	0.46 ± 0.01χ	1.17 ± 0.14β	2.08 ± 0.18α
p-HPEA-DEDA	59.49 ± 4.28χ	101.01 ± 5.00β	140.54 ± 1.82α
Oleuropein aglycone derivative	23.51 ± 1.65χ	41.34 ± 1.70β	64.12 ± 0.02α
Oleuropein aglycone derivative	3.43 ± 0.37χ	4.09 ± 0.18β	5.13 ± 0.17α
Oleuropein aglycone derivative	9.08 ± 0.42χ	12.85 ± 2.80β	16.62 ± 2.67α
Oleuropein aglycone derivative	28.61 ± 5.83β	38.27 ± 4.67αβ	39.93 ± 6.73α
Sum TP-HPLC[a]	139.79 ± 13.44χ	217.93 ± 15.64β	293.33 ± 13.13α
TP-FC[b]	406.27 ± 11.92χ	516.91 ± 14.30β	667.09 ± 10.91α

[a] HPLC-DAD results in μg g^{-1} of olive oil.
[b] Folin–Ciocalteu total phenols (TP) results in μg GAE g^{-1} of olive oil.
[c] Conditions: 100% methanol, 3-step extraction.
[d] Conditions: 2–3 h freezing at −25 °C or 20 min at −80 °C.
[e] Values marked with the same letter (α,β,χ) are not significantly different; confidential level = 95%.

Thus, in an effort to obtain an ideally fat-free extract of all EVOO phenol representatives, the two lipids removal techniques were tested on methanolic extract obtained after US-LLE extraction, using (a) common *n*-hexane washing (Suárez et al., 2008) and (b) a low temperature fat precipitation (−25 °C or −80 °C) as previously proposed for pesticide isolation from olive oils (Lentza-Rizos et al., 2001). As seen from the results, both defatted extracts presented much richer quantitative phenol profile vs. non-defatted one (Table 3) with increases up to 56% (*n*-hexane washing) or even more i.e. 110% (freeze-based fat precipitation), while no differences between extracts were observed at the qualitative level. Although *n*-hexane washing has been one of the most commonly used clean-up strategies in EVOO phenol analysis to date (Brenes et al., 2004; Montedoro et al., 1992; Pirisi et al., 2000; Suárez et al., 2008), a freeze-based fat precipitation has shown to provide much higher phenol yields (>34% in individuals sum, >29% in TP yield), repeatability (RSD; 4.5% vs. 7.2%) as well as fat removal efficiency, however, determined solely on analyst's visual perception. Moreover, this straightforward approach is also easier, cheaper and simpler, with minimal impact on the environment, also safer for the analyst. In fact, this method is simply based on oil crystallization during extract freezing (2–3 h at −25 °C or 20 min at −80 °C), providing gravitational separation of fat (on the top) and remaining liquid (methanol) containing phenols (at the bottom) without additional solvent application. This can be ascribed to a partial coalescence of oil droplets leading to a clump formation, resulting in clear extracts, completely free of fat interferences.

Overall, our results confirmed the previous findings of Gilbert-López, García-Reyes, and Molina-Díaz (2009) study, emphasizing that efficient clean-up of extracts prior to its end-point analysis is of paramount importance, not only in order to prolong the column's and chromatographic system's lifetime, but also to obtain higher yields of desired analytes (e.g. phenols), since the lipid–matrix interferences appears to mask the signals of target compounds at detection point.

3.4. US-LLE versus LLE-agitation

Although manual or mechanical shaking of EVOO with suitable solvents can be effective for phenols extraction, the application of US assistance has higher potential for droplet disruptions and thus for better analyte extraction (Luque de Castro & Priego-Capote, 2007a). However, a comparison of US-LLE with its non-assisted counterpart confirmed the above, as the amount of phenols recovered by US was significantly greater than those by agitation (Fig. 2). Interestingly, the US-LLE yields were almost doubled (>68% in individuals sum, >57% in TP yields) when compared to a non-sonicated extracts (LLE-agitation) as well as of higher repeatability (4.5% vs. 8.7%). This can be attributed to either mechanical and/or chemical effects greatly facilitating the mass transfer rates between immiscible phases (i.e. oil-methanol) through super-agitation, mixing and stirring US abilities, especially significant at high-power US probe applications (Luque de Castro & Priego-Capote, 2007a). As the tip of a probe is directly placed into solution, the power is 100 times higher than in traditional US baths (Errekatxo et al., 2008), generating highly efficient microjets that disperse the liquid, and thus increase the interfacial contact areas for better phenols transfer *via* two main phenomena: (1) through the interphase of two immiscible phases and (2) between the film of an organic solvent and the other circulating phase (Luque de Castro & Priego-Capote, 2007a).

This type of US yield promotions has also been observed in other liquids for extracting aroma compounds and monoterpenoids from wine, aged brandies and aqueous/alcoholic wood extracts (Caldeira, Pereira, Clímaco, Belchior, & De Sousa, 2004; Hernanz Vila, Heredia Mira, Beltrán Lucena, & Fernández Recamales, 1999; Peña, Barciela, Herrero, & García-Martín, 2005), and though the latter (US-LLE) studies are scarce vs. solid–liquid system accelerations (Chen et al., 2007; Ghafoor et al., 2009; Herrera & Luque de Castro, 2005; Jerman et al., 2010), they have shown a high practical potential at both continuous and batch approaches. In fact, one of such a reported example (and the only one best to our knowledge) has been demonstrated for olive oil polyphenols extraction using a continuous US manifold coupled with spectrophotometric detection (Ruiz-Jiménez and Luque de Castro, 2003). However, the recovery conclusions were based solely on TP quantification, with no optimisation details of extraction solvent choice and time required for efficient EVOO phenols isolation.

3.5. Quantification of major EVOO phenols

Under optimal US-LLE conditions and the extracts' freeze-based fat removal at −25 °C, the maximum recovery in TP yields was calculated to be 667.09 ± 10.91 μg GAE g^{-1} which is within a range of previous reports (Bendini et al., 2003; Montedoro et al., 1992). However, as seen from the results, the class of secoiridoids (3,4-DHPEA-DEDA, *p*-HPEA-DEDA, ligstroside and oleuropein aglycone derivatives) were the predominant phenols present (98.6%) in

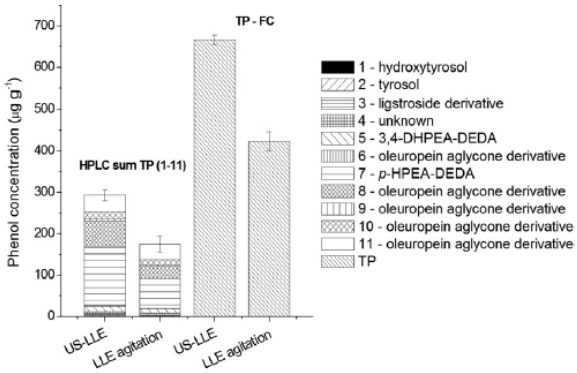

Fig. 2. Comparison of US-LLE with its non-assisted counterpart (LLE agitation) on EVOO phenols extraction yields (μg g^{-1}).

our EVOO sample, followed by phenyl alcohols (hydroxytyrosol and tyrosol, 1.4%) – both typically forming EVOO qualitative–quantitative phenol profiles), while flavonoids (apigenin and luteolin) were not quantified due to their elution at the end of HPLC analysis (Fig. 1). In spite of its several gradient programs testing, apigenin and luteolin were not eluted until the mobile phase reached 100% of methanol value (washing step), which unfortunately also triggered the elution of other interfering compounds contained in EVOO extract, while not in spiked ROO of US-LLE characterisation study. As the quantification of apigenin and luteolin using peak area integration approach would therefore not ensure an accurate phenol analysis in EVOO extract, the latter two were not included in any of the US-LLE optimisation and clean-up studies.

3.6. Method characterisation

A new method proposed, based on US probe assisted extraction (meOH, 3 × 5 mL, 10 min) and a low temperature fat precipitation at −25 °C, was evaluated in terms of main analytical quality parameters such as precision, linearity, recovery and sensitivity.

The results of method's precision evaluation, expressed as % of RSD, varied between 0.4 (oleuropein aglycone derivative) and 7.4 (ligstroside derivative) for run-to-run precision, while for the day-to-day from 0.5 (oleuropein aglycone derivative) to 9.3 (ligstroside derivative), indicating to a highly precise method, suitable for routine EVOO phenol analyses.

The linearity of the method was satisfactory with almost linear calibration curves obtained for all spiked ROO standard additions, where the correlation coefficients (r^2) over concentration range (μg g^{-1}) tested for individual phenols were as follows: 0.97 for hydroxytyrosol (0.23–20.00), 0.99 for tyrosol (0.20–20.00), 0.99 for oleuropein (1.00–300.00), 0.98 for apigenin (0.20–10.00) and 0.97 for luteolin (0.20–10.00), respectively.

The method's extraction efficiency was considered as good, allowing high phenol recoveries of spiked ROO matrices: 96.0 ± 4.4% for hydroxytyrosol, 99.1 ± 0.9% for tyrosol, 94.4 ± 1.5% for oleuropein, 97.3 ± 2.7% for apigenin and 97.8 ± 1.9% for luteolin, respectively.

The sensitivity of method was satisfactory, within the range expected for HPLC-DAD analysis. The results are comparable to previous reports (Selvaggini et al., 2006; Torre-Carbot et al., 2005), where the LODs/LOQs over the concentration range (μg g^{-1}) tested were equal to: 0.04/0.13 μg g^{-1} for hydroxytyrosol (0.23–0.75), 0.08/0.24 μg g^{-1} for tyrosol (0.20–0.60), 0.16/0.51 μg g^{-1} for oleuropein (1.00–6.20), 0.03/0.10 for apigenin μg g^{-1} (0.30–0.80) and 0.04/0.13 μg g^{-1} for luteolin (0.30–0.80), respectively.

Table 4
Phenols concentration (μg g^{-1}) of essayed EVOO samples determined by US-LLE[c] and a freeze-based fat precipitation (at −25 °C or −80 °C).

Phenol compounds	EVOO phenol yields (μg g^{-1})			
	A[d]	B[d]	C[d]	D[d]
Hydroxytyrosol	2.90 ± 0.28 δ[e]	4.74 ± 0.33β	3.50 ± 0.17 χ	13.26 ± 0.73α
Tyrosol	6.19 ± 0.16 αβ	4.36 ± 0.34β	2.52 ± 0.15 χ	6.99 ± 0.51α
Ligstroside derivative	1.11 ± 0.13 β	2.82 ± 0.20α	0.91 ± 0.13 β	0.14 ± 0.02 χ
Unknown	0.59 ± 0.04 χ	6.08 ± 0.62α	2.08 ± 0.31 β	5.99 ± 0.36α
3,4-DHPEA-DEDA	5.46 ± 0.31 β	4.93 ± 0.33 χ	7.22 ± 0.42α	5.48 ± 0.47β
Oleuropein aglycone derivative	0.59 ± 0.08α	0.21 ± 0.03β	0.22 ± 0.02β	0.29 ± 0.04β
p-HPEA-DEDA	44.34 ± 1.32δ	66.17 ± 2.34 χ	163.24 ± 2.10β	254.23 ± 4.03α
Oleuropein aglycone derivative	59.17 ± 0.75 β	32.94 ± 1.48 χ	108.86 ± 1.21α	108.44 ± 3.09α
Oleuropein aglycone derivative	2.03 ± 0.17 β	1.55 ± 0.12 χ	2.56 ± 0.45 β	3.35 ± 0.50α
Oleuropein aglycone derivative	6.21 ± 0.14 χ	12.46 ± 1.8α	11.97 ± 1.23α	8.31 ± 0.76β
Oleuropein aglycone derivative	41.60 ± 1.14α	41.44 ± 2.68α	45.96 ± 2.08α	27.43 ± 1.39β
Sum TP-HPLC[a]	170.19 ± 4.52	177.70 ± 10.27	349.04 ± 8.27	433.91 ± 11.90
TP-FC[b]	343.81 ± 51.41	455.98 ± 44.25	634.34 ± 59.16	910.14 ± 57.70

[a] HPLC-DAD results in μg g^{-1} of olive oil.
[b] Folin–Ciocalteu total phenols (TP) results in μg GAE g^{-1} of olive oil.
[c] US-LLE conditions: 100% methanol, 3-step extraction, 10 min.
[d] Letters (A, B, C, D) presents four different EVOO samples.
[e] Values marked with the same letter (α, β, χ, δ) are not significantly different; confidential level = 95%.

3.7. Application of US-LLE to other EVOO matrices

The optimised US-LLE method combined with a low temperature fat precipitation (at −25 °C) was used for the phenol analysis of four different EVOO samples in order to demonstrate its wider applicability for other EVOO matrices. Table 4 shows the results of individual and total phenol yields of different EVOOs essayed, where significant quantitative differences were observed among the samples analysed, ranging from 170.19 ± 4.52 up to 433.91 ± 11.90 μg g^{-1} at individual level (sum TP-HPLC) and from 343.81 ± 51.41 up to 910.14 ± 57.70 μg g^{-1} at TP level (TP-FC), while their phenolic profiles remained qualitatively similar. This may be ascribed to any or all of varietal, geo-climatical, agronomical and technological differences among the oils (see Section 2.1), suggesting that the method proposed could be applied to a wide range of EVOO samples.

4. Conclusions

The US probe super-agitation abilities has been once again proved to efficiently assist the phenols extraction, however, for the first time from a complex extra virgin olive oil matrices, demonstrating its high practical potential for LLE accelerations as well. The new US-LLE proposed (meOH, 3 × 5 mL, 10 min) combined with extracts' further freeze-based fat precipitation clean-up (2–3 h at −25 °C or 20 min at −80 °C) has shown to be much more efficient, precise, simpler and eco-friendlier in comparison to conventional LLE-agitation and *n*-hexane washing, providing a high-quality EVOO phenol analysis and could therefore serve as a good alternative to official IOC method (International Olive Council., 2009).

Acknowledgements

We thank Slovenian Research Agency (ARRS) and World Federation of Scientists (WFS) for their financial support.

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B4 The fate of olive fruit phenols during commercial olive oil processing: Traditional press *versus* continuous two- and three-phase centrifuge ("Reprinted from The fate of olive fruit phenols during commercial olive oil processing: Traditional press *versus* continuous two- and three-phase centrifuge, 49, Jerman Klen T., Mozetič Vodopivec B., LWT - Food Science and Technology, 267–274, Copyright (2012) with permission from Elsevier, licence number: 3396370210436").

LWT - Food Science and Technology 49 (2012) 267–274

Contents lists available at SciVerse ScienceDirect

LWT - Food Science and Technology

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The fate of olive fruit phenols during commercial olive oil processing: Traditional press *versus* continuous two- and three-phase centrifuge

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ARTICLE INFO

Article history:
Received 13 June 2011
Received in revised form
6 February 2012
Accepted 1 March 2012

Keywords:
Olives
Olive oil
Extraction technology
Phenols yields
HPLC-DAD/MS/MS

ABSTRACT

The fate of olive fruit phenols during commercial olive oil processing was investigated, comparing three available industrial scale extraction systems; traditional press *vs.* continuous two- and three-phase centrifuge in terms of phenols transfer rates and antioxidant potential (AOP) results from fruits to paste and its final products *i.e.* oil and wastes. The fruits, paste and wastes presented similar phenol composition, which differed significantly from that of oil, indicating that phenols are not only transferred but also transformed during oil processing. No qualitative differences in phenol profiles were observed between the systems, whereas significant ones at the quantitative level. Crushing and malaxation resulted in the highest phenols lost, since only 50–60% of total phenols were transferred from fruits to paste, while their further partition depended on the solubility nature of phenols between phases produced. Only 0.3–1.5% of available phenols were found in olive oil, while the rest ended up in wastes (>40%) depending on the system used. A two-phase centrifuge provided the highest transfer rate of phenols to oil (1.5%) with the highest AOP, followed by a traditional press (1.2%) and a three-phase centrifuge (0.5%) where, most of the phenols were flushed away with the wastewater produced (>30%).

1. Introduction

The regular consumption of olive oil has for decades been linked with the prevention of many oxidative stress-related diseases, supported by several clinical and research studies that have clearly testified to its wide range of healthy effects (Covas, 2007). Consequently, olive oil consumption has increased tremendously, reaching a global audience far beyond Mediterranean borders. Olive oil is now recognized as one of the healthiest lipid sources worldwide, due largely to its high phenol content proven to possess antioxidant, anti-inflammatory, anti-atherogenic, antibacterial and anticancer properties (Artajo, Romero, Suárez, & Motilva, 2007; Salvador, Aranda, Gómez-Alonso, & Fregapane, 2003).

Although olive oil is simply cold, pressed juice obtained from fresh ripe olives, its phenol composition differs greatly in comparison to fruits which contain different phenol representatives and yet those of the same classes, namely, simple phenols, phenolic acids, flavonoids, secoiridoids, lignans and others. While the type and quantity of olive fruit phenols depend on several factors, such as, cultivar and genetics, degree of maturation, climatic conditions and agriculture practices, the phenol profile of its corresponding oil depends mainly on the type of extraction technology used (Yorulmaz, Tekin, & Turan, 2011). Indeed, during olive oil processing, fruit phenols are transferred from paste (crushed fruits) to oil and resulting by-products (wastewater and pomace), depending on the relative polarities of phenols, the presence of surfactants, temperature, and the composition and relative amounts of resulting phases (Rodis, Karanthanos, & Mantzavinou, 2002). Others are newly formed through various enzymatic biotransformation-reaction pathways (Servili et al., 2004).

Currently, commercial olive oil production is carried out using both continuous (centrifugation) and batch (traditional press) approaches, although the former has been more widely used due to advantages in terms of higher capacity, shorter processing/storage time and reduced manpower costs (Issaoui et al., 2009). On the other hand, centrifugal systems face larger waste-disposal problems and produce oils of lower quality, especially in terms of phenols content (Gimeno, Castellote, Lamuela-Raventós, De la Torre, & López-Sabater, 2002). This supposition, however, is debatable since contradictory results of olive oil quality characteristics have been reported in literature, promoting the traditional press over the continuous two-phase (Torres & Maestri, 2006) or three-phase (Issaoui et al., 2009) centrifuges or the two-phase *vs.* three-phase systems (Angerosa & Di Giovacchino, 1996; De Stefano, Piacquadio, Servili, Di Giovacchino, & Sciancalepore, 1999; Gimeno

Abbreviation: USLE, Ultrasound-assisted solid liquid extraction.

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doi:10.1016/j.lwt.2012.03.029

et al., 2002), while all of the three existing systems have been rarely compared (Di Giovacchino, Sestili, & Di Vincenzo, 2002; Salvador et al., 2003). Nevertheless, these studies have only evaluated the effect of the extraction system on the oil phenol characteristics, while phenol content/composition information of the remaining by-products was left out or simply has been not reported.

Therefore, the aim of our study was to obtain a deeper insight into phenols distribution patterns during olive oil processing, comparing three commercially available olive oil extraction systems at industrial scale; traditional press *vs.* continuous two- and three-phase centrifuge, in terms of phenols fate assessment from fruits to paste and its final products *i.e.* oil and waste. To the best of our knowledge, this kind of systematic approach has never been used in determining the fate of olive fruit phenols fate during oil extraction while providing valuable information about the quality and quantity of natural antioxidants expected in our food, *i.e.* olive oil, as well as in its processing wastes, making it important from both an economical and ecological viewpoint.

2. Materials and methods

2.1. Olives and olive oil extraction

Olive fruits of Istrska belica *cv.* were harvested at the end of their maturation period ($R = 3$) in November 2010 from two Slovenian orchards located in Goriška Brda and Slovenian Istria, while fruits of Oblica *cv.*, Leccino *cv.*, and Itrana *cv.* ($R = 3$) were sampled from the Croatian island, Hvar (October, 2010). All fruits were stored overnight (4 °C) and processed to olive oil the next day using three commercially available extraction systems. Approximately 250 kg of fruits were used for each process, performed in duplicate ($n = 2$), respectively.

2.1.1. Traditional press with Pieralisi Simplex (Jesi, Italy)

Olive fruits (Istrska belica *cv.*) from Slovenian Istria were washed (0.1 L/kg of fresh fruits), crushed and slowly mixed for 50 min at room temperature. Then, the paste was diluted with water (0.2 L/kg of fresh fruits) prior to squeezing (300×10^5 pascal) and the liquid obtained (aqueous and oily) was separated by a standard decantation process, obtaining three final products: the oil and two wastes (pomace and wastewater).

2.1.2. Continuous two-phase centrifuge with Pieralisi Major 2 (Jesi, Italy)

A mixture of Croatian olive fruits (Oblica *cv.*, Leccino *cv.* and Itrana *cv.*: 80%: 15%: 5%) was washed before crushing (0.1 L/kg of fresh fruits), and then slowly malaxed for 50 min at room temperature without the addition of water. Then, the paste was separated by a horizontal centrifuge, obtaining two final products of oil and a wet pomace.

2.1.3. Continuous three-phase centrifuge with Pieralisi MF9 (Jesi, Italy)

Olive fruits (Istrska belica *cv.*) from Goriška Brda (Slovenia) were washed (0.1 L/kg of fresh fruits), crushed and slowly malaxed for 50 min at room temperature. Then, the paste was diluted with water (0.5 L/kg of fresh fruits) and the oil was extracted using a horizontal centrifuge. The liquid obtained (aqueous and oily) was separated by a discharge vertical centrifuge, producing three final products: the oil and two wastes (pomace and wastewater).

2.2. Sampling and sample pre-treatment

In-situ sampling was performed in triplicate ($n = 3$), collecting input (fruits) and output materials (paste in the middle of malaxation process, pomace, wastewater and oil) of approximately 1 kg or 1 L, respectively. The fruits, paste and pomace were immediately stored under liquid nitrogen, freeze-dried in a Kambič LIO-5P lyophilisator (Semič, Slovenia) and stored at −25 °C prior to analysis, while the wastewater (OMWW) was primarily acidified (HCl, pH = 2.0) and defatted with *n*-hexane prior to freeze-drying and storage at −25 °C, as previously described by De Marco, Savarese, Paduano, and Sacchi (2007). Olive oil samples were stored in the dark at room temperature until extracted. The dry matter measurements of freeze-dried samples were performed gravimetrically and expressed as *g* of dry weight per kg of fresh sample (Lesage-Meessen et al., 2001), providing the basis for the phenols transfer rate calculations.

2.3. Extraction of phenols

2.3.1. Olive fruits, paste, pomace and wastewater

Phenols were extracted according to a previously published USLE method (Jerman, Trebše, & Mozetič Vodopivec, 2010), where a freeze-dried sample (1.5 g) was sonicated (3×20 min) with 25 mL of methanol. The homogenates of each extraction step were centrifuged (6350*g*, 5 min) and combined supernatants diluted with methanol to 100 mL, further stored in screw-capped dark glass containers in a freezer (−25 °C) prior to analysis.

2.3.2. Olive oil

Extra virgin olive oil (10 g) was placed into a polyethylene (PE) centrifuge tube, dissolved in *n*-hexane (10 mL) and directly sonicated (3×10 min) with pure methanol (5 mL). Then, extracts were combined and defatted using a low temperature fat precipitation at −25 °C for 3 h, respectively. Afterwards, the remaining methanol solution containing phenols was reconstituted to 25 mL with methanol, and stored in screw-capped dark glass containers at −25 °C until analysis (Jerman Klen & Mozetič Vodopivec, in press).

2.4. HPLC-DAD-FLD and LC–MS analysis

Prior to chromatographic analysis, the aliquot of extract (10 mL, except oil; 15 mL) were evaporated to dryness using rotary evaporation (35 °C, LABOROTA 4000; Heidolph, Schwabach, Germany) and re-dissolved in 1 mL of acidic HPLC eluent (H_2O/CH_3COOH, 95:5, v/v) providing better resolution, symmetry of peaks and recoveries above 98% (Jerman et al., 2010). Finally, the re-dissolved samples were filtered through 0.45 μm PTFE filters (Macherey-Nagel, Düren, Germany) prior subjected to HPLC-DAD-FLD and LC–MS analysis.

2.4.1. HPLC-DAD-FLD

A Hewlett Packard 1100 liquid chromatograph (Agilent Techn., Santa Clara, USA) equipped with UV/VIS diode-array (190–600 nm) and fluorescence (FLD) detectors ($\lambda_{excitation/emission}$ 280/330 nm) was used for individual phenols determination. The extracts of 20 μL (except oil; 70 μL) were injected and DAD signals were recorded at 280, 320 and 365 nm. Two HPLC columns were tested in order to achieve maximum retention and separation characteristics of phenols from individual olive matrices. A Phenomenex Luna PFP (5 μm, 250 mm × 4.6 mm) attached to a security guard (4 × 3 mm) provided the best results for paste, pomace, wastewater and oil analysis, while the Waters Spherisorb ODS2 (5 μm, 250 mm × 4.6 mm) attached to a Supelco guard column (10 × 4.1 mm) was better for olive fruit analysis, under analytical conditions previously described (Jerman et al., 2010).

2.4.2. LC–MS

Phenols identification was confirmed with LC–MS/MS analysis using Perkin Elmer Series 2000 (Schelton, USA) linked to a 3200 Q TRAP LC/MS/MS system equipped with an electro spray ion (ESI) source from Applied Biosystems/MDS Sciex (Foster City, USA) as already described (Jerman et al., 2010; Jerman Klen & Mozetič Vodopivec, 2011).

Phenols were primarily identified by comparison of retention times, UV–VIS, FLD and ESI-MS spectra with those of authentic standards when available, while the tentative identity of others was confirmed by comparison of UV–VIS and ESI-MS^2 spectra with those from the literature and our database (Jerman et al., 2010; Jerman Klen & Mozetič Vodopivec, in press). Hydroxytyrosol, tyrosol, oleuropein, verbascoside, luteolin-7-*O*-glucoside and rutin were quantified based on a calibration curve of their authentic standards, while hydroxytyrosol glucoside, comselogoside and secoiridoids representatives (demethyloleuropein, ligstroside and its derivative, *p*-HPEA-DEDA, 3,4-DHPEA-DEDA, oleuropein aglycone derivatives) were respectively expressed as hydroxytyrosol, *p*-coumaric acid and oleuropein equivalents in g/kg of samples fresh weight (FW). Verbascoside and comselogoside were quantified at 320 nm, luteolin-7-*O*-glucoside and rutin at 365 nm, and all the other compounds at 280 nm.

2.5. Total phenol (TP) analysis

The extracts total phenol (TP) content was determined colorimetrically at 765 nm using Folin–Ciocalteu reagent, according to Ough and Amerine (1988), and results were expressed as gallic acid (GAE) equivalents in g/kg of samples FW.

2.6. DPPH assay

The antioxidant potential (AOP) of extracts was determined as DPPH free radical scavenging activity according to Obied, Bedgood, Mailer, Prenzler, and Robards (2008) with minor modifications as follows. A DPPH stock solution (~0.1 mmol/L) was prepared daily in methanol, and further diluted to 1.0 AU at 515 nm prior to use. Aliquots of tested extracts (20–200 μL) were adequately diluted to a volume of 200 μL and added to 1.5 mL of DPPH solution in glass cuvettes. The latter were capped, shaken and kept in the dark for 60 min, and then the absorbance was measured at 515 nm using methanol as a blank. The percentage of scavenged DPPH radicals was calculated according to Eq. (1):

$$\%\mathrm{DPPH}_{\mathrm{rem}} = \left[\left(A_0 - A_{\mathrm{sample}}\right)/A_0\right] \times 100 \quad (1)$$

where A_0 and A_{sample} stand for the absorbances of control and sample, respectively.

The concentration resulting in 50% inhibition was referred to as EC_{50} which was expressed as μg/mL of reaction mixture, and then the AOP was calculated using Eq. (2):

$$\mathrm{AOP} = 100/\mathrm{EC}_{50} \quad (2)$$

2.7. Statistical analysis

All analyses were performed in triplicate ($n = 3$), and the results were expressed as means ± SD. Statistical significances among parameters were evaluated by ANOVA using STATGRAPHICS Plus 4.0 (Manugistics Inc., Rockville, MD) and an MRT test at 95% confidence level, where P values <0.05 were regarded as significant, P values <0.01 as very significant and P values >0.05 as insignificant.

3. Results and discussion

3.1. The phenol profiles of olives and olive-derived samples

The chemical structures of main phenols identified in olives and olive-derived samples (paste, pomace, wastewater, oil) are shown in Fig. 1, while the HPLC-DAD phenol profiles of those obtained from a continuous three-phase centrifuge are shown in Fig. 2, providing some basic insights into the phenols' partition trail from fruits to paste and its final products, *i.e.* oil and waste. As seen from results, the phenols qualitative profile of fruits has slightly differed from that of paste and wastes, while significantly from that of oil, suggesting that phenols are not only transferred, but also transformed during oil processing. In general, the phenols followed a typical partitioning model, where the main fruit secoiridoids (Table 2), *i.e.* oleuropein, demethyloleuropein and ligstroside, were degraded during the crushing/malaxation operation, forming several secoiridoid aglycone derivatives according to the mechanism previously proposed (Servili et al., 2004). However, on contrary to Artajo et al. (2007), none of these secoiridoids were further confirmed in any of the olive-derived matrices, indicating their complete transformation through a complex biotransformation pathway. Similarly, none of the fruit flavonoids (luteolin-7-*O*-glucoside, rutin) and cinnamic acid esters (verbascoside, comselogoside) were found in olive oil, while they were present in all other matrices (paste, pomace and wastewater), indicating their preferred transfer to wastes without many alterations, such as hydrolysis and/or other degradation reactions. This is in line with previous reports (Artajo, Romero, & Motilva, 2006; Artajo et al., 2007) demonstrating that olive oil is mainly composed of secoiridiod aglycone derivatives, while luteolin-7-*O*-glucoside, rutin and verbascoside were only present in paste, pomace and wastewater, and not in the oil. By contrast, the simple phenols (tyrosol, hydroxytyrosol and its glucoside) have appeared in all matrices, although not with all of their class representatives. While hydroxytyrosol was found in all of the olive-derived matrices (paste, pomace, wastewater and oil), it was not detected in the starting fruits material, where only its glycosidic form was found. This suggests that the majority of fruits' hydroxtyrosol glucoside was degraded to hydroxytyrosol during crushing/malaxation, while a small part was also transferred to the paste, pomace and wastewater, but not to the oil. Based on this evidence, we can only speculate that the occurrence of hydroxytyrosol in all olive-derived matrices is not only result of its glucoside hydrolysis, but also a result of other phenol secoiridoids transformation pathways containing hydroxytyrosol in their molecular structures *e.g.* 3,4-DHPEA-DEDA and oleuropein aglycon (Servili et al., 2004).

However, this type of phenols qualitative behavior was also observed in samples of other systems *i.e.* traditional press and continuous two-phase centrifuge. Interestingly, the phenols partition pathway has similarly responded to all transfer/transformation trends, following the same phenol composition pattern as previously illustrated (Fig. 2) regardless of the phenol profile variations of input fruit materials (Table 2). This suggests, however, that the type of technology alone has no impact on the qualitative phenol profiles of individual matrices, which seems to be strictly dependent upon enzyme-activity but has, on the other hand, an important impact on their quantitative distribution between individual matrices, as further discussed in detail (3.3.).

3.2. Comparison of extraction systems

Olive oil processing, in general, consists of three operational steps: (*i*) olive crushing – where fruits are crushed to break down the cells and release the oil; (*ii*) malaxation – where paste (crushed

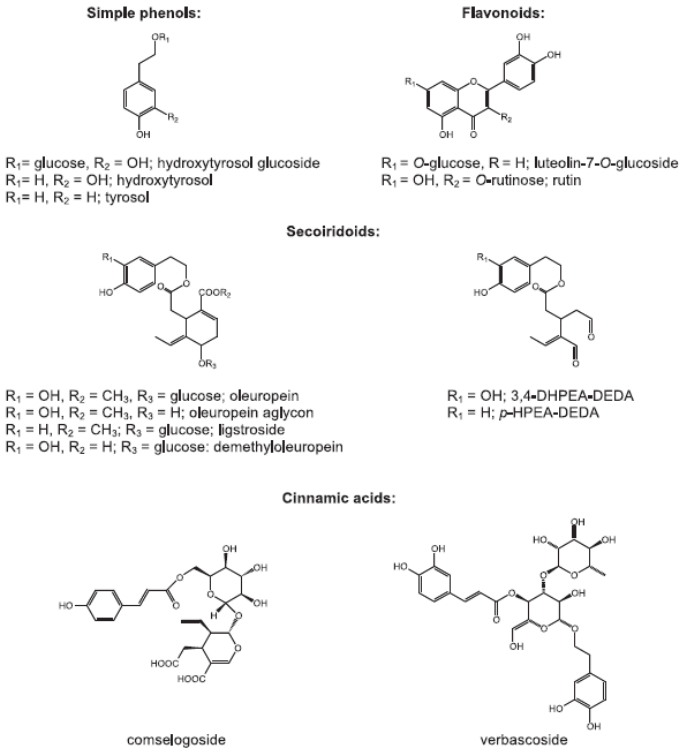

Fig. 1. Chemical structures of main phenols identified in olives and olive-derived samples.

fruits) is slowly mixed to increase the oil yield, and; (*iii*) oil separation – where oil is extracted/separated from the remaining wastes. Currently, there are three commercial olive oil extraction systems available on the market, differing in two main aspects; in the physical force used to recover oil (press or centrifuge) and; in the amount of water (if) added to the paste during oil extraction. This, however, has an important impact on the quantity and quality of products obtained, producing, in addition to oil, one or two streams of wastes (Di Giovacchino et al., 2002).

Thus, all extraction systems were compared primarily in terms of mass balance data and product dry matter characteristics (Table 1) in order to correctly evaluate the distribution rates of phenols between oil and resulting by-products. While all of them have resulted in comparable oil yields obtained (~0.2 L/kg of fruits FW), they, on contrary, produced different amounts of waste of different characteristics. Both, the press and continuous three-phase centrifuge have generated two by-products (wastewater and pomace) that required water additions, whereas the two-phase centrifuge produced only one *i.e.* pomace of high moisture (DW; 200 g/kg), requiring no added water due to improved centrifugation efficiency (De Stefano et al., 1999). Therefore, a continuous three-phase centrifuge has shown to be the most problematic from a polluting load perspective, producing the highest amount of wastes per kg of fruits FW (~0.9 L OMWW and ~0.5 kg pomace), followed by the traditional press (~0.7 L OMWW and ~0.4 kg pomace) and a two-phase centrifuge (~0.9 kg pomace), comparable to previous reports (Azbar et al., 2004; Di Giovacchino et al., 2002).

3.3. Olive fruit phenols transfer rates in different extraction systems

Fig. 3 demonstrates the phenols transfer rates between individual olive matrices of different extraction systems (traditional press *vs.* two- and three-phase centrifuge), with results expressed as a percentage of initial olive fruit phenols monitored at both levels of individual (HPLC-DAD) and total (Folin–Ciocalteu) phenol analyses. The results based on HPLC summation of individual peak responses were grouped into classes: simple phenols (tyrosol, hydroxytyrosol and its glucoside); secoiridoids (demethyloleuropein, oleuropein and its aglycone derivatives, *p*-HPEA-DEDA, 3,4-DHPEA-DEDA, ligstroside and its derivative); cinnamic acids (verbascoside and its derivative, comselogoside), and; flavonoids (luteolin-7-*O*-glucoside, rutin). This class grouping allows more comprehensive visualization of the phenols fate assessment during

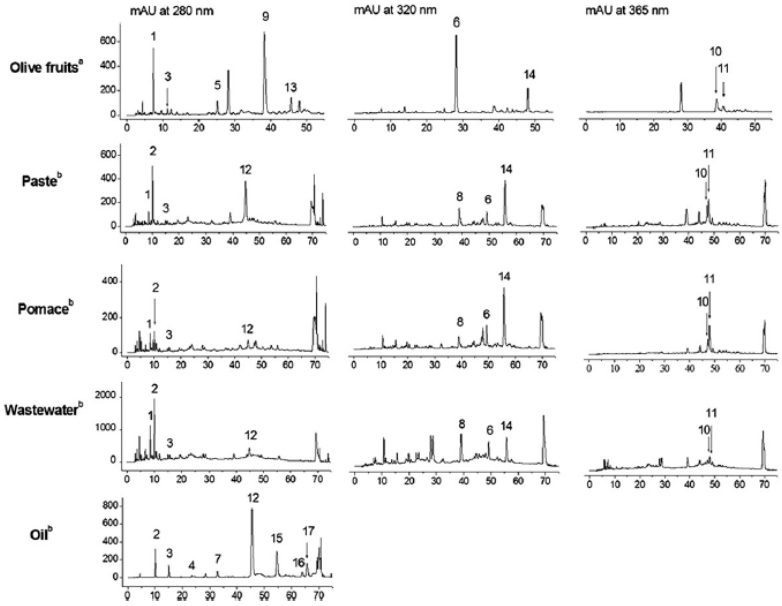

Fig. 2. HPLC-DAD phenol profiles of olive fruits, paste, pomace, wastewater and oil extracts from a continuous three-phase centrifuge monitored at 280, 320 and 365 nm on Spherisorb ODS2 (a) or Luna PFP (b) columns. Peak assignment: hydroxytyrosol glucoside (**1**), hydroxytyrosol (**2**), tyrosol (**3**), ligstroside derivative (**4**), demethyloleuropein (**5**), verbascoside (**6**), 3,4-HPEA-DEDA (**7**), verbascoside derivative (**8**), oleuropein (**9**), luteolin-7-*O*-glucoside (**10**), rutin (**11**), *p*-HPEA-DEDA (**12**), ligstroside (**13**), comselogoside (**14**), oleuropein aglycone derivatives (**15**–**18**).

oil processing. However, a high correlation ($R^2 = 0.954$) between both analyses was observed for all matrices, except in the case of wastewater, where the correlation was poor and insignificant ($P < 0.05$), indicating a false positive reaction of Folin–Ciocalteu reagent with other non-phenolic compounds (sugars, pectins, polyalcohols *etc.*) largely contained in the wastewater (De Marco et al., 2007).

Nonetheless, a significant phenol loss during oil processing was observed in all extraction systems, following the same gradual TP content decrease as follows: paste > wastes > oil. Our results showed that only 0.3–1.5% of available phenols were transferred to olive oil, while the rest ended up in wastes (>40%) depending on the extraction system used. This is consistent with previous report of Rodis et al. (2002) which demonstrated that phenolic fraction of olive oil comprises 1–2% of total fruit phenols, while the remaining 98% is lost with wastes; 53% with the wastewater and 45% with the pomace. This also suggests that olive fruit phenols are rather partitioned into wastes instead of oil, which is especially significant at systems requiring higher water additions such as a three-phase centrifuge. However, though the phenols transfer rates have differed slightly among the systems tested, a general pattern in their class distribution could be observed, dominated by the same

Table 1
Approximate mass balance data (kg or L/kg of fruits FW)[d] ($n = 2$) and products dry matter characteristics (g/kg)[e] of different extraction systems: traditional press *vs.* continuous two- and three-phase centrifuge.

Extraction system	Mass balance data (per fruits FW)[d]			
	Paste (kg/kg)	Pomace (kg/kg)	Wastewater (L/kg)	Oil (L/kg)
Traditional press[a]	1.35 ± 0.24αβ (230 g/kg)[f]	0.42 ± 0.08β (400 g/kg)	0.74 ± 0.12α (110 g/kg)	0.20 ± 0.01 α
Continuous two-phase centrifuge[b]	1.12 ± 0.19β (250 g/kg)	0.89 ± 0.11α (200 g/kg)	–	0.20 ± 0.02 α
Continuous three-phase centrifuge[c]	1.65 ± 0.21α (180 g/kg)	0.51 ± 0.09β (500 g/kg)	0.95 ± 0.15α (95 g/kg)	0.20 ± 0.01 α
P value[f]	>0.05	<0.01	>0.05	>0.05

[a] Total amount of water used: 0.3 L/kg of processed olives (washing water: 0.1 L/kg + water for paste dilution.: 0.2 L/kg).
[b] Total amount of water used: 0.1 L/kg of processed olives (washing water: 0.1 L/kg).
[c] Total amount of water used: 0.6 L/kg of processed olives (washing water: 0.1 L/kg + water for paste dilution.: 0.5 L/kg).
[d] Expressed as kg or L/kg of fruits fresh weight (FW).
[e] Dry matter content (g/kg)
[f] Values in the same row marked by the same letter (α, β, χ,) are not significantly different; confidential level = 95%; *P* values <0.05 significant; $P < 0.01$ very significant; $P > 0.05$ insignificant.

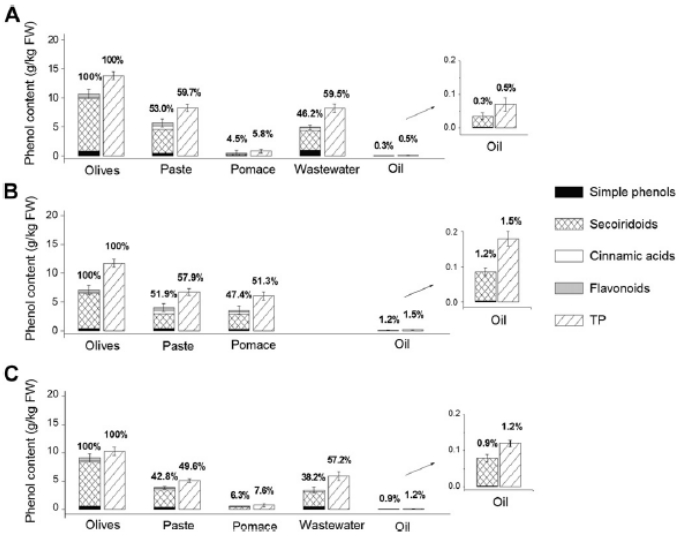

Fig. 3. Phenols transfer rates between individual olive matrices in different extraction systems: continuous three-phase centrifuge (A), continuous two-phase centrifuge (B) and traditional press (C). Results are expressed in % of initial olive fruits phenols content (g/kg of fruits fresh weight (FW)) at two levels; as HPLC sum of individual phenol classes (1st column) and Folin–Ciocalteu determined total phenols (TP, 2nd column).

phenol class of secoiridoids, followed by simple phenols, flavonoids and cinnamic acids, depending on the individual matrix examined. Although the secoiridoidic class was clearly predominant in all matrices, an important difference among its individual representatives was observed. While the fruits were characterized by oleuropein, demethyloleuropein and ligstroside secoiridoidic-type representatives, its process-resulting matrices (paste, wastewater and pomace) were dominated by their hydrolysis products, such as, *p*-HPEA-DEDA, 3,4-DHPEA-DEDA, oleuropein aglycone and ligstroside derivatives (Fig. 2).

Our results further revealed that only 50–60% of total phenols were transferred from fruits to paste (Fig. 3), suggesting that the majority of antioxidants are already lost during crushing and/or malaxation. In fact, both operations were already recognized as the most critical points in overall technology, mainly due to activity of several endogenous enzymes which promote phenols oxidative catabolism to a larger extent (Servili et al., 2004). Moreover, during malaxation, several polysaccharides may link the phenols, and thus limit their available pool for further partition (Vierhuis et al., 2001). Interestingly, the transfer rates of phenols to paste were higher in the case of centrifugal systems (up to 60% *vs*. up to 50% in traditional press), most likely due to different crushing technologies used. In fact, the previous study of Di Giovacchino et al. (2002) has already proven that metallic crushers of centrifugal systems ensure much better breakage of olive flesh *versus* millstones of the press, thus liberating higher quantities of phenols into paste, and hence, to oil as well.

Once phenols are in paste, they are further distributed between the oil and resulting by-products, according to their affinities toward these phases (Rodis et al., 2002). This depends on their solubility as well as on the technology used, producing different types of products at various time/temperature malaxing conditions, with or without water additions and technological co-adjuvants (Di Giovacchino et al., 2002). However, none of the co-adjuvants were added during oil processing, while the temperature and malaxation time remained constant (room temperature, 50 min); thus, any type of differences observed in transfer rates of phenols between systems (Fig. 3) could only be ascribed to different paste dilutions. Apparently, the water addition prior to oil separation has influenced the phenols' partition pattern, as evident from the oils' decreasing TP content trend toward higher water additions (three-phase centrifuge > traditional press). This is in line with previous reports, demonstrating that higher water/paste ratios alter partition equilibrium of phenols and causes reduction in TP yields through the dilution of the aqueous phase (Di Giovacchino et al., 2002; Issaoui et al., 2009). Consequently, most of the phenols are flushed away with the wastewater produced as is clearly evident from the TP yields comparison between the three-phase centrifuge and traditional press (46.2% *vs*. 38.2%), while much lower content remained trapped in pomace (4.5% *vs*. 6.3%). By contrast, a two-phase centrifuge (requiring no process water) has provided the highest TP yields in both resulting matrices (oil and pomace), since the fruit vegetation water containing phenols has remained in a wet pomace, while the rest of available antioxidants have ended up in oil (1.5%).

From a health perspective, a two-phase centrifuge provided the highest TP yields in oil (1.5%), followed by traditional press (1.2%) and a three-phase centrifuge (0.5%), respectively. Moreover, the use of a two-phase centrifuge also provided the highest content of hydroxytyrosol and tyrosol, associated with the greatest health benefits (Servili et al., 2004), although both of them were almost absent in oils of other systems. Indeed, a closer look at matrices phenol class composition has revealed that hydroxytyrosol and tyrosol from the press and three-phase centrifuge were mainly lost

Table 2
Olive fruits phenol composition/content data (g/kg of fruits FW) ($n = 3$) used in different extraction systems: traditional press *vs.* continuous two- and three-phase centrifuge.

Phenolic compounds	Phenol content of olive fruits used in different extraction systems (g/kg of fruits FW)			*P* value[f]
	Traditional press[a]	Continuous two-phase centrifuge[b]	Continuous three-phase centrifuge[c]	
Hydroxytyrosol glucoside	0.515 ± 0.026β	0.413 ± 0.019χ	0.765 ± 0.042α	<0.01
Tyrosol	0.062 ± 0.006α	0.032 ± 0.002β	0.064 ± 0.006α	<0.01
Demethyloleuropein	4.266 ± 0.195α	2.056 ± 0.088β	4.156 ± 0.134α	<0.01
Oleuropein	2.979 ± 0.098χ	3.507 ± 0.109β	4.237 ± 0.112α	<0.01
Ligstroside	0.588 ± 0.031α	0.557 ± 0.021α	0.580 ± 0.028α	>0.05
Verbascoside	0.091 ± 0.009β	0.084 ± 0.009β	0.134 ± 0.010α	<0.01
Comselogoside	0.113 ± 0.010α	0.101 ± 0.007αβ	0.093 ± 0.006β	>0.05
Luteolin-7-*O*-glucoside	0.166 ± 0.010β	0.127 ± 0.009χ	0.299 ± 0.012α	<0.01
Rutin	0.234 ± 0.013β	0.195 ± 0.011χ	0.318 ± 0.015α	<0.01
∑ − HPLC-DAD[d]	9.014 ± 0.398β	7.072 ± 0.275χ	10.646 ± 0.365α	<0.01
TP − FC[e]	10.410 ± 0.785β	11.720 ± 0.750β	13.750 ± 0.896α	<0.01

[a] Istrska belica *cv.* (Slovenian Istria, Slovenia).
[b] Mixture of Oblica *cv.*/Leccino *cv.*/Itrana *cv.* : 80%/15%/5% (Hvar, Croatia).
[c] Istrska belica *cv.* (Goriška Brda, Slovenia).
[d] Sum of HPLC-DAD determined phenols expressed as g/kg of fruits fresh weight (FW).
[e] Total phenols (TP) determined by Folin–Ciocalteu analysis expressed as g/kg of fruits fresh weight (FW).
[f] Values in the same row marked by the same letter (α, β, χ,) are not significantly different; confidential level = 95%; *P* values <0.05 significant; $P < 0.01$ very significant; $P > 0.05$ insignificant.

with their wastewaters produced (simple phenols) owing primarily to their hydrophilic nature, although their content in starting fruit material was significantly higher *vs.* two-phase centrifuge (Table 2). This suggests that both systems are associated with a higher loss of valuable compounds, while on the other hand, they present an opportunity for their waste valorization, in particular for the hydroxytyrosol recovery, already recognized as a target of several food, cosmetic and pharmaceutical industries (De Marco et al., 2007; Jerman Klen & Mozetič Vodopivec, 2011; Obied et al., 2008). By contrast, the phenols of more lipophilic character (secoiridoids) were mainly transferred to oils or have remained trapped in pomace, linked to different cellular tissues of the olive flesh.

Despite the technological differences, none of the systems has provided a sufficient phenol content of oils obtained, especially when compared to the fruits vast pool of available phenolic antioxidants. While some of these losses could be prevented by water addition limitations, the majority of phenol-type antioxidants would still not remain in oil, mainly due to their limited solubility in fatty matrices. Although few improvements in oil phenol yields have been achieved by de-stoning and nitrogen flush (Yorulmaz et al., 2011), as well as by the cell-wall-degrading enzyme additions (Vierhuis et al., 2001), none of these practices proposed could successfully stop a huge loss of phenols during oil processing. Indeed, the latter are amphiphilic in their nature with a higher solubility in water than in oil phase (Rodis et al., 2002), thus, when partitioned, most of them end up in wastes, *i.e.* wastewater and/or pomace. This, however, is not a completely undesired phenomenon, since too high levels, especially of secoiridoids, may negatively influence the oil's sensory characteristics, resulting in bitter taste and/or pungency (Servili et al., 2004). Therefore, the major point of concern in the current olive oil industry is the loss of valuable antioxidants, such as hydroxytyrosol and tyrosol, presenting a challenge for their partition control in future research projects.

3.4. Extracts antioxidant potential (AOP) behavior during oil processing

Antioxidant activity is one of the most studied bioactivities of olive phenols (Servili et al., 2004), thus, the extracts' AOP behavior during oil processing was measured in different extraction systems (Table 3). As seen from the results, all of the systems have resulted in a significant loss of AOP compared to that of fruits, where oils have amounted to only 8.7–18.2% of initial fruits AOP, depending on the system used. In general, all fruit extracts exhibited high AOP which positively correlated with their TP content (Table 2), in particular with hydroxytyrosol glucoside ($R^2 = 0.989$), verbascoside ($R^2 = 0.997$), luteolin-7-*O*-glucoside ($R^2 = 0.999$) and rutin ($R^2 = 0.983$), already associated with high antioxidant activities (Bouaziz, Grayer, Simmonds, Damak, & Sayadi, 2005). However, a comparison of phenols transfer rates (Fig. 3) and AOP results of olive-derived matrices in different systems (Table 3) showed that TP variations were not always consistent with AOP, suggesting that the extract's antioxidant activity is also dependent on individual antiradical activity of phenols, rather than on the total. For example, while the difference in oils TP yields between the traditional press and a two-phase centrifuge was relatively low (20%), their AOPs showed much higher variation (40%), most likely due to a higher content of hydroxytyrosol found in the latter (12.21 μg/g in a two-phase centrifuge *vs.* 4.74 μg/g in a traditional press). Moreover, although the crushing and malaxation have significantly reduced the phenols' content of paste (40–50%), they, on the contrary, had only a minor effect on its corresponding AOP, which was decreased

Table 3
Antioxidant potential (AOP) ($n = 3$) results of individual olive matrices in different extraction systems: traditional press *vs.* continuous two- and three-phase centrifuge.

Extraction system	AOP[a] (mL/μg)				
	Olive fruits	Paste	Pomace	Wastewater	Oil
Traditional press	9.60 ± 0.23β	6.74 ± 0.21β	1.64 ± 0.06β	4.56 ± 0.11β	1.00 ± 0.02β
Continuous two-phase centrifuge	9.20 ± 0.18β	6.80 ± 0.17β	5.46 ± 0.11α	–	1.68 ± 0.04α
Continuous three-phase centrifuge	11.25 ± 0.45α	8.23 ± 0.32α	1.26 ± 0.06χ	6.31 ± 0.13α	0.98 ± 0.02β
P value[b]	<0.01	<0.01	<0.01	<0.01	<0.01

[a] Antioxidant potential (AOP) = $100 \times 1/EC_{50}$.
[b] Values in the same row marked by the same letter (α, β, χ,) are not significantly different; confidential level = 95%; *P* values <0.05 significant; $P < 0.01$ very significant; $P > 0.05$ insignificant.

by less than 30%. This could be explained by the degradation of complex olive fruit phenols *i.e.* oleuropein, demethyloleuropein and ligstroside, generating more active DPPH scavengers of lower molecular weights, such as, *p*-HPEA-DEDA, 3,4-DHPEA-DEDA, oleuropeine aglycone and ligstroside derivatives, hydroxytyrosol and tyrosol, found in olive pastes. The latter had apparently increased the AOP of oils and resulting by-products, as their relative values were always higher than the TP transfer rates obtained. A similar behavior was also observed in a previous study by Bouaziz et al. (2005) demonstrating that olive phenol aglycons obtained after hydrolysis had a higher DPPH scavenging activity *vs.* their respective glycosides.

4. Conclusions

Although olive fruits offer a major pool of available phenol-type antioxidants, unfortunately most of them were lost during oil processing, ending up in wastes instead of oil. While crushing and malaxation resulted in the highest phenols lost (50–60%), the phenols partition rates between wastes and oil was mainly limited by their solubility nature, partially affected by the extraction system used for oil processing. The water addition prior to oil separation has influenced the phenols partition rates, decreasing oil's TP content toward higher water additions. Consequently, only 0.3–1.5% of available phenols were found in olive oil, mainly those of lipophilic nature, newly formed through complex biotransformation-reactions, while the rest ended up in wastes (>30% in wastewater, <10% in pomace), depending on the system used. A two-phase centrifuge has provided the highest oil phenols transfer rate (1.5%) with highest AOP (18.2%), followed by a traditional press (TP; 1.2%, AOP; 10.5%) and a three-phase centrifuge (TP; 0.5%, AOP; 8.7%), where the decrease in TP yields was not paralleled by the AOPs decrease in any of the matrices tested. However, none of the available oil extraction systems examined has obtained a satisfactory phenols transfer to oils, in particular those phenols of highest antioxidant activities such as hydroxytyrosol, offering the challenge for future partition control in further research studies.

Acknowledgments

We thank the Slovenian Research Agency (ARRS) and World Federation of Scientists (WFS) for their financial support.

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Food Anal. Methods (2012) 5:781–783
DOI 10.1007/s12161-011-9310-5

DPPH Solution (In)stability during Kinetic UV/Vis Spectrometry Measurements of Phenols Antioxidant Potential

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Received: 22 June 2011 / Accepted: 28 September 2011 / Published online: 12 October 2011

Abstract The instability of 2,2-diphenyl-1-picrylhydrazil (DPPH) methanol solution during kinetic UV/Vis spectrometry measurement of phenols antioxidant potential is reported, where UV/Vis vs. Vis spectrometry comparison revealed the UV-light dependent degradation of DPPH, while only Vis ensured its high stability (>99%) over 24-h kinetic runs.

Keywords DPPH (in)stability · UV/Vis spectrometry · Phenols · Antioxidant potential (AOP)

Introduction

Over the past decade, several clinical and research studies have correlated the regular consumption of plant phenol antioxidants with the prevention of many oxidative-stress related diseases, and consequently their antioxidant potential (AOP) assessments have become one of the most studied parameters of foodstuffs' quality characterization. As a result, numerous analytical methods have been developed to determine plant extracts' antioxidant activities; however, scavenging of 2,2-diphenyl-1-picrylhydrazil (DPPH) free radical has become one of predominant assays for in vitro AOP characterization of different plant materials and/or their bioactive compounds, including phenols (Villaño et al. 2007).

The assay is based on decolouration of DPPH free radical after phenols addition, assessing their ability to transfer H atoms/electrons to radicals—a likely mechanism of antioxidant protection (Goupy et al. 2003). Although some of the assay's principles have been recently modified for an on-line HPLC (Koleva et al. 2000) and EPR spectroscopy analysis (Polovka et al. 2003), the scavenging of DPPH is typically monitored by a commercially available UV/Vis spectrophotometers measuring absorbance decrease at 515 nm.

However, one of the basic assay requirements is the stability of initial DPPH solution during UV/Vis spectrometry, and though DPPH is known as a stable free radical in methanol solution (Arnao 2000), our preliminary results showed its significant absorbance decrease (60–70%) during 1-h kinetic UV/Vis detection. A limited stability of these radicals was already described before (Arnao 2000; Ozcelik et al. 2003), interfered by the wavelength, light, oxygen, type and pH of the solvents tested. Moreover, also other parameters such as reaction time, reagent and sample concentration may interfere the final assay results (Molyneux 2004); however, best to our knowledge, this is the first report concerned with instability of DPPH methanol solution during continuous UV/Vis spectrometry, although used before in several phenols kinetic AOP characterizations (Villaño et al. 2007; Goupy et al. 2003).

The present communication reports observations of DPPH absorbance decrease (A_{515}) during kinetic UV/Vis spectrometry detection due to UV-lamp interferences, aiming to avoid the potential pitfalls in sample kinetic AOP characterizations.

Materials and Methods

Sample

Olive fruits *Istrska belica* cv. were harvested in Vipava Valley (Slovenia), immediately frozen, freeze-dried, de-stoned and ground into homogeneous powder, further stored at −25 °C prior to analysis.

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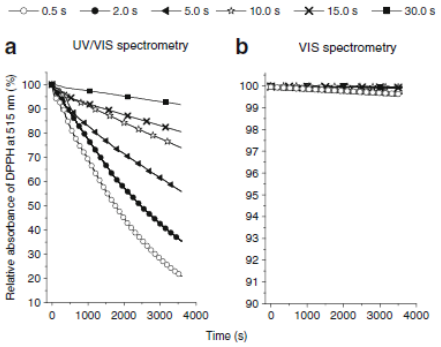

Fig. 1 DPPH methanol solution stability during 1-h kinetic detection using **a** UV/Vis and **b** Vis spectrometry with data acquisition at different time intervals: 0.5, 1.0, 2.0, 5.0, 10.0, 15.0 and 30.0 s

Reagents

2,2-Diphenyl-1-picrylhydrazil (DPPH), methanol and quercetin were purchased from Sigma-Aldrich Co. (Gillingham, GB), while Folin–Ciocalteu (Merck) was from a local supplier.

Phenols Solutions Preparation

Quercetin was dissolved in methanol (1.0 mg mL^{-1}). Olive fruit phenol solution was prepared from freeze-dried olive sample (1.5 g) by extraction (3×20 min) with methanol (25 mL) using high-intensity probe ultrasonication, providing 98% recovery of olive fruit phenols (Jerman et al. 2010). Merged extracts were further diluted to 100 mL with methanol and subjected as such (without purification) to Folin–Ciocalteu analysis of total phenols expressed as μg of gallic acid equivalents (GAE) per mL of olive extract.

Spectrophotometric Measurements

All measurements were performed on HP UV/Vis spectrophotometer 8453 (Agilent Technologies, Santa Clara, USA) equipped with two light sources, Deuterium lamp for UV (190–400 nm) and Tungsten lamp for Vis (400–1100 nm) spectra range scans.

The stability of DPPH solution was evaluated in terms of radical absorbance decrease (A_{515}) using two UV-lamp mode regulations: (a) turned ON (UV/Vis spectrometry) and (b) turned OFF (Vis spectrometry) at different data acquisition intervals (0.5–30.0 s) over 1-h kinetic runs.

Phenols AOP Assessment

The phenols AOP was characterized as previously described, (Villaño et al. 2007) where aliquots of tested solutions were adequately diluted (20 μL) prior to addition to DPPH stock solution (1.5 mL, 80 μM), and then A_{515} decrease was continuously recorded (every 2 s) until reaction reached the plateau at which EC_{50} was calculated

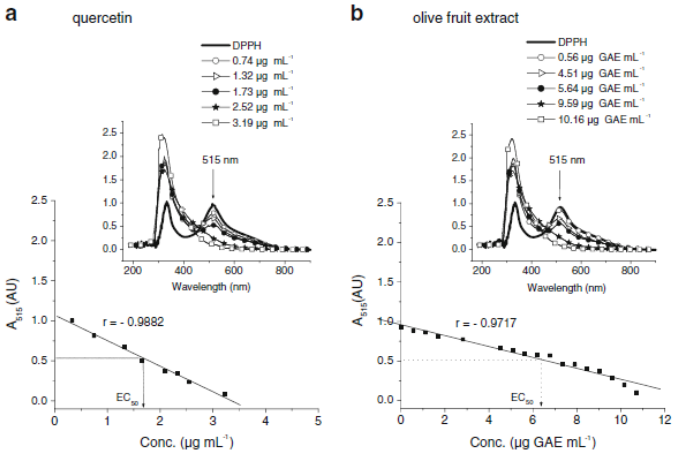

Fig. 2 Determination of EC_{50} for quercetin (**a**) and olive phenol extract (**b**) toward DPPH dissolved in methanol using Vis spectrometry detection

and expressed in μg of phenols per mL of reaction mixture (Fig. 2).

Statistics

All determinations were performed in triplicate with results expressed as means ± SD.

Results and Discussion

Our preliminary results showed a high instability of DPPH methanol solution during continuous UV/Vis spectrometry detection, where up to 70% of DPPH absorbance decrease was observed over 1-h kinetic run. With the aim to obtain its stable absorbance profile needed for correct assay's performance and hence results interpretation, the possibility of UV-lamp interference on DPPH methanol solution stability was tested primarily, as the previous study (Nishizawa et al. 2005) demonstrated the UV-induced degradation of DPPH ethanol solution as a function of radiation time and distance from UV-source (UVGL-25, 4 W).

A comparison of UV/Vis vs. Vis spectrometry kinetic results at different data acquisition intervals (Fig. 1) revealed a UV-light dependent degradation of DPPH methanol solution during UV/Vis detection (turned ON UV-lamp) while not during Vis (turned OFF UV-lamp). In fact, its relative absorbance was hardly changed (<1% decrease) over 1-h Vis detection, providing high stability regardless of the amount of Vis scans emitted from Tungsten lamp. By contrast, a Deuterium UV-lamp has markedly influenced the radicals stability, as their A_{515} decreases have clearly correlated with more (0.5–2.0 s) or less (5.0–30.0 s) continuous UV-scans emissions during UV/Vis detection. Knowing that the latter emit UV-radiation responsible for free radicals production, the results obtained could be most simply explained by the radical–radical interaction, resulting in DPPH solution instability. Up to now, no reports of spectrophotometer's UV-lamp interferences could be found supporting our observations; however, even though cursory, they suggest further investigations in its UV-lamp-induced degradation of DPPH in methanol.

Thus, only Vis spectrometry detection confirmed to provide stable DPPH absorbance was applied for quercetin and olive fruit phenol extract's AOP assessment (Fig. 2). However, as DPPH/extract reaction rates were rather slow (>4 h), additional stability studies of initial DPPH solution were conducted, confirming its stability at even longer Vis detections (>24 h). However, as seen from scavenging profiles of samples tested, a typical gradual DPPH absorbance decrease upon phenols concentration rise was observed in both, allowing undisturbed EC_{50} (quercetin 1.75±0.12 μg mL^{-1}~5.79 μM; extract 6.20±0.92 μg GAE mL^{-1}) and TEC_{50} (quercetin 58±2 min; extract 255± 12 min) evaluation. Noting that EC_{50} presents the quantity of phenols (microgram) needed to decrease initial DPPH concentration to 50%, it could be presumed that 9.4 μg of total olive fruit phenols (corresponding to 0.5 mg of fresh olive flesh) is needed to scavenge 50% of free radicals under experimental conditions applied (1.5 mL, 80 μM DPPH). However, while the final AOP of quercetin (2.98×10^{-3}) was comparable with previous report (Villaño et al. 2007), the extract's AOP was much lower (6.32×10^{-4}), indicating some antagonistic interactions among various constituents of complex olive extract, apparently affecting its antioxidant behavior.

Conclusions

DPPH assay has been and continues to be one of the most used antioxidant assays in food research; however, only Vis spectrometry detection is suggested using kinetic-type of samples AOP spectrometry characterization, since radicals in methanol has shown to be degraded under UV-lamp exposure (up to 70%) while not under Vis (<1%).

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