INSTITUTO SUPERIOR DE AGRONOMIA
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Universidade de Lisboa

Influence of Enzymes and Technology on the Composition of Cobrançosa and Galega Vulgar Virgin Olive Oils

MARIA DE FÁTIMA PRATAS PERES

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TESE ELABORADA PARA OBTENÇÃO DO GRAU DE DOUTOR
EM ENGENHARIA ALIMENTAR

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Resumo

As enzimas endógenas da azeitona, nomeadamente oxidorredutases, têm uma grande importância na composição do azeite virgem (AV), pois estão relacionadas com a presença de compostos fenólicos e voláteis e envolvidas em reações de alteração destes compostos. Por seu turno, a adição de enzimas (pectinases, celulases, hemicelulases) como adjuvantes de extracção pode aumentar o rendimento, sem comprometer a qualidade do AV.

O objectivo da presente tese foi avaliar a influência de enzimas endógenas em frutos colhidos em diferentes estados de maturação e da adição de enzimas no processo de extracção, na composição química e no rendimento de AV. Estes estudos foram realizados em frutos das cultivares 'Cobrançosa' e 'Galega Vulgar', em olivais de sequeiro e regadio, localizados na Beira Baixa.

Assim, estudou-se o efeito da adição de preparações enzimáticas em simultâneo com microtalco natural, o adjuvante utilizado na indústria. A avaliação conjunta da ação destes dois adjuvantes foi realizada pela Metodologia das Superfícies de Resposta. Numa primeira fase, optimizaram-se as condições operacionais do equipamento laboratorial de extração Abencor. Para tal, realizaram-se ensaios segundo uma matriz Placket-Burman, para selecionar as variáveis significativas no processo. Em seguida, procedeu-se à realização de ensaios em função das variáveis significativas - adição de água na centrifugadora e na batedeira - de acordo com uma matriz central compósita rotativa (CCRD) para optimizar os valores dessas variáveis que maximizam a extracção do AV, sem perda da sua qualidade. Todos os ensaios de extracção realizados neste trabalho decorreram nas condições previamente otimizadas. Os efeitos combinados de microtalco natural (*MT*: 0,04-0,46 % (m/m)) e das preparações enzimáticas (*E:* 0,003-0,117 % (v/m)) adicionados no início da batedura foram avaliados, utilizando uma matriz CCRD, relativamente a: critérios químicos de qualidade do azeite (acidez, índice de peróxido e absorvâncias no UV), fenóis totais, pigmentos clorofilinos e rendimento/índice de extratabilidade.

Os resultados da adição simultânea de microtalco e enzimas mostram que pode haver um aumento do rendimento: até 34 % para uma dose de microtalco de 0,4-0,5 % e 0,1 % de enzimas, para a cultivar 'Galega Vulgar', enquanto na cultivar

‘Cobrançosa’, consegue-se um acréscimo máximo de 24 %, para 0,05-0,15 % de microtalco, sem necessidade de adição de enzimas. A adição de adjuvantes não influenciou a concentração de fenóis totais, pigmentos clorofilinos e K_{225}, nem os critérios químicos de qualidade do AV.

Paralelamente ao estudo da adição de enzimas, com o presente trabalho pretendeu-se avaliar o comportamento de oxidorredutases endógenas (polifenoloxidase, peroxidase e lipoxigenase) nas azeitonas ao longo da maturação dos frutos.

A melhoria das características sensoriais do AV é um dos desafios actuais mais importantes para a indústria. Para estudar as características sensoriais do azeite é fundamental conhecer os compostos responsáveis pelo seu aroma. No presente trabalho, selecionaram-se os compostos odorantes mais importantes nos azeites ‘Galega Vulgar’ e ‘Cobrançosa’ por cromotografia gasosa-olfactometria (GC-O). Posteriormente, quantificaram-se estes odorantes-chave, bem como outros compostos voláteis referidos na literatura que influenciam o aroma dos AV. A extracção dos compostos voláteis foi efetuada por microextracção em fase sólida (SPME) e a sua detecção e quantificação realizadas num sistema bidimensional de cromatografia gasosa/espectrometria de massa (GCxGC-ToFMS). Os azeites ‘Galega Vulgar’ e ‘Cobrançosa’ extraídos de azeitonas com índice de maturação (IM) inferior a 4,5 apresentaram perfis semelhantes de aroma por GC-O. Destacam-se os aromas a verde (erva cortada predominante), característicos de compostos como o *trans*-2-hexenal e o hexanal. Após a optimização das condições de extração em SPME, quantificaram-se 22 compostos voláteis em amostras de azeite ‘Galega Vulgar’ e ‘Cobrançosa’ extraídos de azeitonas com distintos índices de maturação. No que se refere à época de colheita (Outubro vs. Novembro) nove compostos voláteis apresentaram poder discriminante entre amostras, nomeadamente o heptanal, o *trans*-2-hexenal, o 1-octeno-3-ol, o nonanal, o 2,3-butanodiona, o etil-2-metilbutirato, o hexanal, o *cis*-3-hexenilacetato e o 3-metilbutilacetato. Para os azeites estudados, a análise sensorial confirmou a predominância dos cheiros orto-nasais e retronasais associados aos atributos verde.

No presente trabalho foram aplicadas várias técnicas de análise multivariada de dados (análise em componentes principais, classificação hierárquica e análise

discriminante) de forma a encontrar relações entre diversas variáveis. Por análise em componentes principais e classificação hierárquica, as amostras de azeite provenientes das duas cultivares foram separadas em dois grupos em função da composição em ácidos gordos. Assim, os azeites 'Cobrançosa' podem ser distinguidos dos azeites 'Galega Vugar' por terem teores mais elevados de ácidos gordos polinsaturados (linoleico (C18:2) e linolénico (C18:3)) e alguns ácidos gordos saturados (margárico (C17:0), esteárico (C18:0), araquídico (C20:0), beénico (C22:0) e lignocérico (C24:0). Os azeites Galega são caracterizados por apresentarem em geral teores mais elevados de ácido oleico (C18:1), palmitoleico (16:1), palmítico (C16:0), mirístico (C14:0), margaroleico (C17:1) e eicosenóico (C20:1).

Os compostos fenólicos do AV apresentam também propriedades sensoriais, nomeadamente pela sua contribuição para o sabor amargo. Além disso, são considerados compostos bioactivos, por apresentarem características antioxidantes e/ou carácter vitamínico. Estes compostos são habitualmente distinguidos pela sua natureza lipofílica e hidrofílica. A partir de amostras obtidas ao longo da maturação, foram quantificados por cromatografia líquida com detector de fluorescência (HPLC-FLD), alfa, beta e gama tocoferóis (fenóis lipofílicos) nos azeites 'Cobrançosa' e 'Galega Vulgar'. Nos azeites de ambas as cultivares, os teores de alfa-tocoferol foram sempre superiores a 200 mg kg^{-1}, apresentando os azeites 'Galega Vulgar' teores mais elevados de gama tocoferol.

Os compostos fenólicos, por terem atividade antioxidante, são também altamente susceptíveis de sofrerem oxidação. As enzimas oxidorredutases endógenas dos frutos, nomeadamente a polifenoloxidase (PPO) e a peroxidase (POD) podem ter um papel muito importante no teor e perfil de compostos fenólicos ao promoverem a sua oxidação e consequentemente a alteração da composição fenólica. Um dos objectivos deste estudo foi avaliar as modificações da actividade da PPO e POD nos frutos das duas cultivares, 'Cobrançosa' e 'Galega Vulgar', ao longo da maturação. Nas análises de actividade enzimática efectuadas ao longo da maturação durante dois anos em olivais de sequeiro e de regadio, não foi detetada atividade da PPO na semente mas verificou-se que é elevada no mesocarpo das azeitonas acompanhando, em geral, a evolução

dos fenóis totais nos azeites. Por seu turno, a actividade da POD é predominante na semente. Em geral, a maior atividade enzimática das oxidorredutases (PPO e/ou POD) para azeitonas com índices de maturação de 3-4, corresponde também a teores mais baixos de fenóis totais nos azeites.

Verificou-se ainda que os azeites 'Cobrançosa' apresentam valores mais elevados de fenóis totais. Por sua vez, os azeites extraídos de azeitonas provenientes de olivais de sequeiro possuem, em geral, teores em fenóis totais mais elevados.

Relativamente ao perfil em compostos fenólicos, avaliados por cromatografia líquida com detetor de díodos (HPLC-DAD), foi possível quantificar os teores de hidroxitirosol, tirosol, ácido vanílico, vanilina, ácido *o*-cumárico, luteolina e apigenina. Por LC/MS identificou-se a presença de 3,4-DHPEA-EDA, p-HPEA-EDA, 3,4-DHPEA-EA e p-HPEA-EA. O composto fenólico maioritário nos azeites foi o 3,4-DHPEA-EDA, representando em alguns azeites mais de 50 % da área total dos picos cromatográficos de fenóis. Foram encontrados baixos teores de hidroxitirosol e tirosol, como é expectável em azeites recentemente extraídos. Os azeites 'Cobrançosa' apresentaram, em geral, teores mais elevados de hidroxitirosol e de flavonóides.

Assim, a decisão acerca da data de colheita vai permitir a produção de azeites virgens com diferentes propriedades sensoriais e valor nutricional. O índice de maturação das azeitonas e a cultivar mostram ser factores chave na quantidade e perfil de compostos fenólicos e voláteis dos azeites 'Galega Vulgar' e 'Cobrançosa'.

Palavras-chave: adjuvantes, fenóis, maturação, odorantes, oxidorredutases

Abstract

The aim of this thesis was to evaluate the effect of endogenous enzymes of olive fruits at early ripening stages and of added enzymes in malaxation, on chemical composition and extraction yield of virgin olive oil (VOO). Fruits from 'Cobrançosa' and 'Galega Vulgar' cultivars grown in rain-fed or irrigated orchards were used.

The optimization of VOO extraction, at laboratory-scale (Abencor system), in the presence of adjuvants (enzymes and natural microtalc), was carried out using Response Surface Methodologies. A Central Composite Rotatable Design was followed as a function of the contents of enzyme preparation (E: 0.003 to 0.1 %, v/w) and of natural microtalc (MT: 0.04 to 0.46 %, w/w). The results showed that an improvement in extractability was always found for both cultivars but the effect of E and/or MT addition depends on the cultivar.

Nowadays, improving sensory characteristics is one of the main goals for olive oil technology. Thus, to know the compounds that have impact on flavour, the major odourants in 'Galega Vulgar' and 'Cobrançosa' olive oils were selected using gas chromatography–olfactometry (GC-O) and analised by SPME-GCxGC-ToFMS. *Trans*-2-hexenal, in higher contents in Galega VOO, was the most abundant compound. Concerning harvesting time and cultivar, nine volatiles showed to discriminate among samples.

Bioactive compounds (eg. phenolic compounds) in VOO affect its nutritional, oxidative and sensory characteristics. Phenolic compounds are highly susceptible to oxidation by oxidorreductases, mainly polyphenol oxidases (PPO) and peroxidases (POD). PPO activity was only detected in fruit mesocarp and POD activity was mainly detected in seeds. Higher contents of alpha-tocopherol were observed in the beginning of harvest and gamma-tocopherol content was higher in 'Galega Vulgar' VOO. Oleacein (3,4-DHPEA-EDA), was the most important phenol compound in VOO. The ripening stage of fruits showed to be a key factor on the amount and profile of bioactive compounds of VOO.

Keywords: adjuvants, phenols, odourants, oxidoreductases, ripening

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Symbols, abbreviations and acronyms

ADH	Alcohol dehydrogenase
AAT	Alcohol acetyl transferase
ACV	Vanillic acid
AT	α-Tocopherol
APG	Apigenin
BT	β-Tocopherol
CA	Cluster Analysis
CAR/PDMS	Carboxen/ Polydimethylsiloxane
CCRD	Central Composite Rotatable Design
CP	Chlorophyll pigments
CW	Water added in centrifugation
DA	Discriminant Analysis
DW	Dry weight
E	Commercial enzyme preparation
EAME	Elenolic acid methyl ester
EI	Extractability index
3,4-DHPEA	3,4-Dihydroxy-Phenyl-Ethanol (hydroxytyrosol)
3,4-DHPEA-AC	4- (acetoxyethyl)-1,2-dihydroxybenzene
3,4-DHPEA-EDA	3,4-Dihydroxy-Phenyl-Ethanol-Elenolic Acid Di-Aldehyde;(oleacein) or dialdehydic form of elenolic acid linked to hydroxytyrosol
3,4-DHPEA-EA	3,4-Dihydroxy-Phenyl-Ethanol-Elenolic Acid; oleuropein aglycone
DVB/CAR/PDMS	Divinylbenzene/Carboxen/Polydimethylsiloxane
EVOO	Extra Virgin Olive Oil
FA	Fatty acid
FAEE	Fatty acids ethyl esters
FID	Flame ionization detector
FW	Fresh weight
GC-O	Gas chromatography - olfactometry
GC × GC-ToF-MS	Comprehensive two-dimensional gas chromatography time of flight mass spectrometry
GT	γ-Tocopherol
HPL	Hydroperoxide lyase
HPLC-DAD	High performance liquid chromatography with diode array detection
HPLC-FLD	High performance liquid chromatography with fluorescence detection
HYT	Hydroxytyrosol
LC-ESI-MS	Liquid chromatography – electrospray ionization mass spectrometry
LOX	Lipoxygenases

LUT	Luteolin
MT	Microtalc
MUFA	Monounsaturated fatty acids
MW	Water added in malaxation
OAVs	Odour activity values
OC	Oil content
OT	Odor threshold
p-HPEA	Hydroxy-Phenyl-Ethanol, (tyrosol)
p-HPEA-EA	p-HPEA-Elenolic Acid mono-Aldehyde; aldehydic form of elenolic acid linked to tyrosol
p-HPEA-EDA	Hydroxy-Phenyl-Ethanol-Elenolic Acid Di-Aldehyde (oleocanthal); dialdehydic form of elenolic acid linked to tyrosol
PB	Plackett-Burman design
PAL	Phenylalanine ammonia lyase
PCA	Principal Component Analysis
PC	Principal components
PCM	*p*-Coumaric acid
PDMS	Polydimethylsiloxane
PDMS/DVB	Polydimethylsiloxane/ Divinylbenzene
PDO	Protected Designations of Origin
POD	Peroxidases
PPO	Polyphenol oxidase
PUFA	Polyunsaturated fatty acids
PV	Peroxide value
RI	Ripening index
RSM	Response Surface Methodology
SAFE	Solvent assisted flavour evaporation
SFA	Saturated fatty acids
SPE	Solid phase extraction
SPME	Solid phase microextraction
TAGs	Triacylglycerols
TP	Total phenols
TYR	Tyrosol
TEOPAC	Time of exposure of olive pastes to air contact
VAN	Vanillin
VOO	Virgin olive oil
Y	Abencor yield (%)
WAF	Weeks after flowering

Chapter 1

Rationale of the thesis and objectives

The main goals of olive oil industry are to improve the yield of the obtained oil and to maximize the production of “extra virgin olive oil”, which is the category that gives a better income. It means that from the research point of view the subjects that can have an immediate technological transfer are the following ones: to study the ways of improving olive oil extraction yield, as well as how to obtain the best quality of the final virgin olive oil (VOO).

The knowledge of the role of endogenous enzymes of olive fruits during ripening, as well as along the extraction process, should be an important information to better control the physical parameters that influence the activity of those enzymes.

Each cultivar, based on its specific compounds will produce different phenolic and volatile compounds, which according to geographical origin will dictate the specificity of each VOO. This is probably responsible for the distinctive character between the 116 protected designations in European Union (POD and IGP) for VOO. Besides cultivar, ripening index can have a great impact on phenol and volatile composition of olive oil.

Nowadays, for olive oil technology, improving sensory characteristics is one of the main goals. To study the sensory characteristics it is important to know the compounds that have impact on flavour of VOO. In what concerns the volatile fraction of virgin olive oils, hundreds of compounds are present, differing by orders of magnitude in their concentration, but only a small fraction contributes to the aroma of olive oil.

Volatile and phenolic compounds are the main compounds that are responsible for the aroma and taste of VOO. Apart from the sensorial characteristics, the presence of different bioactive compounds in virgin olive oil dictates its nutritional and oxidative stability. Moreover, health issues are very important because the scientific evidence is already strong enough for the European Food Safety Authority (EFSA) to enable the legal use of health claims on label. Phenolic compounds are the most important antioxidants present in VOO. Therefore, the study of bioactive compounds in VOO has gained a new insight in the last decade. Bioactive compounds are highly susceptible to degradation and olive endogenous oxidoreductases, mainly polyphenol oxidases (PPO) and

peroxidases (POD), may play an important role on their profile in olive oil by promoting oxidation of phenolic compounds.

The aim of this thesis was to evaluate the effect of endogenous enzymes of olive fruits at early ripening stages and of added enzymes in malaxation, on chemical composition and extraction yield of VOO. These studies were performed with fruits from 'Cobrançosa' and 'Galega Vulgar' cultivars grown in rain-fed or irrigated orchards.

In Chapter 2, a survey of the scientific literature is presented, showing that enzymes can have an important impact on both quality and yield. The application of processing aids, as well as a better understanding of the behaviour of endogenous enzymes, may allow establishing specific technological conditions for each cultivar or blend of cultivars, at different ripening stages.

The Abencor extraction system is the most used laboratory-scale installation in olive oil technology to study yield improvement and/or olive oil characteristics. With this equipment, it is possible to carry out trials that would be very difficult to perform at industrial scale. Thus, in Chapter 3, an optimization of the Abencor extraction process, using a Placket-Burman design to select significant variables followed by a central composite rotatable design (CCRD) is presented. In order to study the effect of adjuvants in yield and in olive oil quality, a CCRD was applied for the optimization of extraction with two adjuvants, namely commercial enzymes and natural microtalc, using fruits from two different cultivars. The influence of adjuvants addition on VOO quality and composition was evaluated.

In the present study, one of our specific goals was to select the main compounds responsible for the major odour notes in 'Galega Vulgar' and 'Cobrançosa' olive oils using gas chromatography – olfactometry (GC-O). Also, the quantification of the detected odourants, as well as other compounds known from the literature to have influence on olive oil flavour, was performed by GC × GC-ToF-MS system. The results obtained are presented in Chapter 4.

The aim of the study presented in Chapter 5 was to evaluate if changes on PPO and POD activities in olive fruits from 'Cobrançosa' and 'Galega Vulgar' cvs were related with the composition of their olive oils, especially phenolic compounds. Pattern recognition techniques (Principal Component Analysis, PCA, Cluster

Analysis, CA, and Discriminant Analysis, DA) were used for multivariate data analysis. The study was performed in two harvests. The first part of the study presents the results obtained in 2010 harvest season and the second part presents the results achieved in 2011 season. Besides the results of phenolic compounds evaluated by HPLC-DAD in the first season, in 2011 it was possible to identify the main compounds found in both oils by LC-MS. Olive oils were also characterized by their fatty acid composition (GC-FID) and tocopherol contents by HPLC-FLD are also presented.

The partial conclusions withdrawn in each experimental chapter are discussed together in an integrated approach, in the Chapter 6 of this thesis.

Chapter 2

Influence of enzymes and technology on virgin olive oil composition

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Abstract

This work aims at presenting the state-of-the-art about the influence of the activity of olive endogenous enzymes, as well as of the application of adjuvants in olive oil technology, discussing their influence on the composition of virgin olive oil, especially in phenols and volatile compounds.

Keywords: adjuvants, oxidoreductases, hydrolases, malaxation, phenols, volatile compounds

1. Introduction

The world production of olive oil accounted for 2.4 million tonnes in 2014, whereas world consumption represents about 3.0 million tonnes per year (IOC, 2014). Olive groves account for close to 5 million hectares in the EU, representing about 1.9 million farms with olive groves. Olive oil production in the EU represents around 73 % of world production. Spain, Italy and Greece account for about 97% of EU olive oil production, with Spain producing approximately 62 % of this amount (EC, 2012). The proportion of groves located in disadvantaged zones (mountainous areas and areas with specific disadvantages) is significant, representing 88 % of total area in Portugal.

In terms of oil quality, in 2009 Spain produced 35 % of extra virgin oil, 32 % of virgin oil and 33 % of lampante oil. The respective figures for Italy in relation to these three categories of oil are 59, 18 and 24%, respectively. Consumption models differ in the EU's three main producer countries. In Italy and Greece, the majority of oil consumed is extra virgin, whereas in Spain this category represents less than half of the consumption (EC, 2012). However, the general trend is towards the consumption of extra virgin olive oils. This trend is based on the general perception of the consumers that extra virgin olive oil category is the best one in terms of health benefits. Also, its sensory properties reflect the sensory character of each monocultivar or blend of cultivars of olives of origin. The presence in olive oil of minor components with antioxidant potential, as well as its high content in monounsaturated fatty acids appear to be essential for the beneficial effect of this food (Trichopoulou and Dilis, 2007). Thus, sensory properties and health characteristics of olive oil are linked to its chemical characteristics, in particular to the presence of several minor components, which are strongly influenced by the operational conditions in the technological extraction process. Therefore, they may be considered as analytical markers of the quality of olive oil processing.

Olive characteristics are probably the key factor that influences the final virgin olive oil (VOO) quality. The chemical and biochemical properties of the fruits rely on some agronomic practices and several studies point out their effect on minor components of VOO. Cultivar, ripening stage, edapho-climatic conditions and irrigation management are some of the factors that can influence the behaviour

of the enzymes present in olive pulp and seeds (Angerosa et al., 2000; Aparicio and Luna, 2002; Servili et al., 2004; Berenguer et al., 2006; Perez et al., 2014). Under optimal extraction conditions, using healthy olives, extra virgin olive oils are always produced, whichever the olive cultivar processed. Only olives attacked by pests and diseases, or fallen to the ground before harvesting, produce olive oils with off-flavours. Other defective sensory notes in VOO are due to inadequate harvesting, post-harvesting, processing or oil storage (Alba et al., 2008).

Moreover, the production of high-quality VOO at the highest yield and minimum cost, as well as using an environmentally friendly olive oil production, is more and more requested (García-González and Aparicio, 2010). Consequently, since 1992, the use of “ecological” technologies (two phase decanter) had the greatest impact in the characteristics of VOO, in the last 25 years.

Although this great progress in olive oil technology the quality of the obtained oil and the extraction yield are still to be optimized, producing a significant economic loss for the oil sector (Chiacchierini et al., 2007; Peres et al., 2014). Considering the eco-sustainability and lower environmental impact of enzymes, the use of biotechnology in olive oil industry, has also been studied for several years (Duarte-Costa and Sameiro, 1978; Alba-Mendoza et al., 1987; Alba et al., 1990; Ranalli and De Mattia, 1997; Ranalli et al., 2005; Aliakbarian et al., 2008; Chih et al., 2012). The use of several inert processing aids, as well, has been tested to increase olive oil extraction yield (Cert et al., 1996; Ranalli and De Mattia, 1997; Moya et al., 2010; Caponio et al., 2014a; Peres et al., 2014).

The enzymatic activities of olives, and consequently the nutritional and the sensory quality of the product, depend on the technological conditions. The changes in VOO characteristics mainly occur in phenol and volatile compounds, which influence decisively VOO nutritional and sensory characteristics (Angerosa, 2000; Clodoveo et al., 2014).

The extraction of olive oil has three main steps: preparation of the paste (crushing and malaxation), solid-liquid and liquid-liquid separation. For every extraction process (solid-liquid separation type), the factors that can be changed are: temperature, time, adjuvants, amount of processing water and oxygen.

The aim of this work is to present the state-of-the-art about the influence of the activity of olive endogenous enzymes, as well as of the application of adjuvants in olive oil industry, discussing their influence on chemical and sensory characteristics of VOO.

2. Main olive enzymes involved in olive oil extraction process

In order to evaluate the need for using processing aids in VOO industry, the knowledge of how endogenous enzymes of olive fruits act during ripening, as well as along the extraction process, should be an important information to better control the physical parameters that influence the activity of those enzymes (Clodoveo et al., 2014).

The olive fruit is a drupe and its components are the exocarp or skin, the mesocarp or pulp, and the endocarp or pit, which consists of a woody shell enclosing one or two seeds. Olives contain several enzyme complexes, distributed differently in several parts of the fruits, like pectinases, lipases, lipoxygenases, glycosidases, peroxidases and polyphenol oxidases, among others, that have been extensively studied (Salas et al., 1999; Mínguez-Mosquera et al., 2002; Panzanaro et al., 2010; Garcia Rodríguez et al., 2011; Sánchez-Ortiz et al., 2011; Gutierrez-Rosales et al., 2012; Romero-Segura et al., 2012; Taticchi et al., 2013).

It is well known the role of enzymes in processing and in the decrease of the quality of post-harvest of fruits. However, in the case of olive fruits this effect has been scarcely studied and very little is known about the complexity of the enzyme composition of the fruits and their influence on the quality of olive oil. However, it is believed that the different types of the existing enzymes, the content and specific activity in the different parts of the fruit may explain some aspects of olive oil technology.

2.1. Oxidoreductases

Oxidoreductases catalyse oxidation-reduction reactions that can play an important role in taste, flavour and nutritional value of VOO.

These enzymes catalyse oxidation-reduction reactions in which hydrogen or oxygen atoms or electrons are transferred between molecules, and are classified

depending on the substrate they act (Ramírez et al., 2003). This class includes lipoxygenases, polyphenol oxidases and peroxidases, among others.

2.1.1. Lipoxygenases

Plant lipoxygenases (LOX) are a class of widespread dioxygenases catalysing the hydroperoxidation of polyunsaturated fatty acids.

LOX (EC 1.13.11.12) catalyses the oxidation of fatty acids containing *cis,cis*-1,4-pentadiene groups to produce the corresponding conjugated unsaturated hydroperoxides, which are the precursors of important volatile compounds of olive oil (Williams and Harwood, 2000). Therefore, the preferred substrates of LOX are linoleic and linolenic acids for the plant enzyme and oleic acid is not oxidized (Belitz et al., 2009). LOX is also capable of use a relatively broad range of other compounds as substrate like carotenoids and polyphenols (Laane et al., 2003).

LOX is a metal-bound protein with a non-heme Fe-atom in its active center; the enzyme is activated by its products and during activation Fe^{2+} is oxidized to Fe^{3+}. According to Belitz et al. (2009), the catalysed oxidation pathway is assumed to have the following reaction steps:

- Abstraction of a methylene H^- atom from the substrate's 1,4-pentadiene system and oxidation of the H^- atom to a proton;

- The pentadienyl radical bound to the enzyme is then rearranged into a conjugated diene system, followed by the uptake of oxygen;

- The peroxy radical formed is then reduced by the enzyme and, after attachment of a proton, the hydroperoxide formed is released.

Lipoxygenases from plants mostly exhibit 9- or 13-regiospecificity. Furthermore regiospecificity studies indicated that the olive lipoxygenase is a 13-LOX, with the preferential production of 13(S)-ZE(Z)-isomers of fatty acid hydroperoxides (80%) (Salas et al., 2000). This specificity data is in good agreement with the composition of olive oil volatiles, in which C6 unsaturated aldehydes and alcohols predominate, these being produced from 13 hydroperoxylinolenic acid (Morales et al., 1995).

Two LOX cDNA clones, Oep1LOX2 and Oep2LOX2, have been isolated from olive (*Olea europaea* cv. Picual). Expression levels of both genes were measured in the mesocarp and seeds during development and ripening of Picual and Arbequina olive fruit and linolenic acid proved to be the preferred substrate; analyses of reaction products showed that both enzymes produce primarily 13-hydroperoxides from linoleic and linolenic acids (Padilla et al., 2009).

Lipoxygenase activity has been detected in olive mesocarp (Salas et al., 1999) and also in oil bodies extracted from olive endosperms (Georgalaki et al., 1998). LOX activity is higher in seeds (Servili et al., 2007) (Table 1).

Table 1. LOX activity found in olives and olive oils (substrate linoleic acid).

Fruit/VOO	Cultivar	LOX activity	Reference
Pulp	FS17	833.4 U mg^{-1} protein	Ridolfi et al., 2002
Pulp	Frantoio	2.26 U mg^{-1} DW	Servili et al., 2007
Seed		6.01 U mg^{-1} DW	
Pulp	Coratina	2.67 U mg^{-1} DW	Servili et al., 2007
Seed		7.16 U mg^{-1} DW	
Olive oil		0.11-1.68 U mg^{-1} protein	Georgalaki et al., 1998

Maximum LOX activity was found in cvs. Kalamata, Ascolana Tenera and FS17 at pH 6.0, using linoleic acid as substrate; the maximum LOX activity was measured at 30 ºC (Ridolfi et al., 2002; Lorenzi et al., 2006). Salas et al. (2000) reported that olive LOX is localized in chloroplasts, thylakoids and microsomes and that the optimum pH is 5.

The high levels of LOX activity detected at early stages of fruit development suggest that in olives, as in other plants, this enzyme is important in the physiological response to stress. A steady decrease of LOX activity was observed at more advanced stages of maturation, from 25 to 35 weeks after anthesis, when the fruits are normally harvested for oil extraction (Salas et al., 1999). In early ripening stages, with ‘Cobrançosa’ olives from irrigated and rain fed orchards, it was not possible to establish any trend in LOX activity measured in fruit mesocarp (Table 2).

Table 2. LOX activity (substrate linoleic acid) in 'Cobrançosa' mesocarp in early ripening stages in two years and in two olive groves (RF-rain fed; IR-irrigated) in Beira Baixa, Portugal

Year	Olive grove	LOX activity (Ug^{-1} FW)		
		RI < 1.0	1.0 < RI <2.0	2.0<RI<3.9
2010	IR	32.45 ± 2.57	45.36 ± 2.29	38.04 ± 4.45
	RF	40.29 ± 3.46	42.18 ± 5.29	36.60 ± 2.17
2011	IR	22.55 ± 1.05	27.92 ±1.96	19.52 ± 2.41
	RF	22.79 ± 4.34	22.51 ± 2.70	35.21 ± 4.64

The widespread presence of the C6- and C9-aldehydes shows that enzymatic-oxidative cleavage of linoleic and linolenic acid by the enzymes lipoxygenase, hydroperoxide-lyase and, if necessary, an aldehyde-isomerase, generally contributes to the formation of aroma in several foods (Belitz et al., 2009). Along the olive oil extraction, the lipoxygenase pathway is very active during the steps of preparation of paste, i.e., crushing and malaxation. Thus, the aroma of the final VOO will be a function of the activity levels and characteristics of the enzymes involved in LOX pathway (Salas et al., 2000; Angerosa et al., 2004). Figure 1 shows a scheme of the LOX pathway with its three different branches for the production of volatiles.

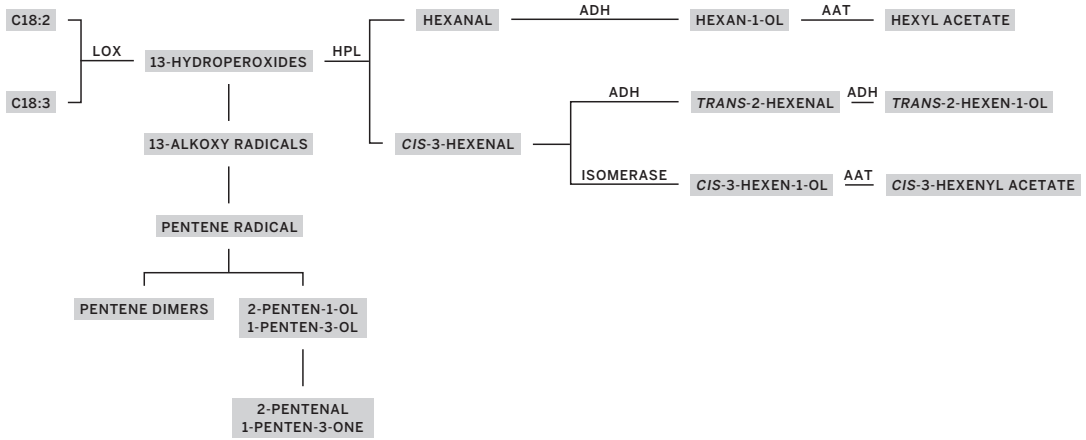


Figure 1. Lipoxygenase pathway involved in the production of C6 and C5 volatile compounds (adapted from Angerosa et al., 2004).

The branch coming from linoleic acid gave rise to hexanal, hexan-1-ol, and hexyl acetate; the former and the latter were responsible for desirable perceptions. This

branch could be seen as the green-sweet aspect of the global green flavour. The second branch could be responsible for the main green notes perception, giving rise to *cis*-3-hexenal, *cis*-3-hexen-1-ol, and *cis*-3-hexenyl acetate. The third branch (*trans*-2-hexenal and *trans*-2-hexen-1-ol) could be considered as the bitter-astringent aspect of the green sensory perceptions (Morales et al., 1999).

Thus, analysis of volatile fractions from virgin olive oils by GCxGC-ToFMS showed that virgin olive oil odourants consists of a complex mixture of more than two hundred volatile compounds, among which saturated and unsaturated aldehydes, alcohols and esters of alcohols of six carbon atoms are especially abundant, accounting for more than 80 % of all of the volatile components in all of the samples analysed (Peres et al., 2013a). Particularly important was the concentration of the aldehyde *trans*-2-hexenal, which was also reported in several other studies to account itself for more than 50 % of those volatile fractions in european olive oils (Angerosa et al., 1999; Angerosa and Basti, 2003; Campeol et al., 2003; Vichi et al., 2003; Cavalli et al., 2004; Berlioz et al., 2006; Tena et al., 2007; Aparicio et al., 2012; Tura et al., 2013).

Virgin olive oils produced from healthy fruits, where the LOX pathway is the predominant source of compounds biogeneration, are usually described by perception of fruity sensations, freshly cut grass, green fruits such as apple, banana, or vegetables like artichoke, celery or tomato, accompanied by more or less intense taste notes of bitterness and pungency (Angerosa, 2002; Aparicio and Luna, 2002; Cerretani et al., 2008c). This confirms that most of the volatile compounds responsible for the ‘green odour’ notes of the aroma of fruits and vegetables are produced by the degradation of polyunsaturated fatty acids through the lipoxygenase pathway (Hatanaka, 1996) (Figure 2).

The profile of volatile compounds present in the aroma of virgin olive oils with sensory defects is quite different (García-González and Aparicio, 2002; Morales et al., 2005; García-González et al., 2007). In those oils the concentrations of C6 and C5 compounds are quite lower than those detected in high quality oils or those compounds are even completely absent (Vichi et al., 2008). Oils from microbiologically contaminated olives showed lower amounts of C5 volatiles and higher levels of C6 volatiles from the lipoxygenase pathway and some fermentation products (Vichi et al., 2011). *Penicillium* (a storage fungi) was

identified as the most potent enzyme producer in olives microbiota, with yields for LOX of 6.8 U mg^{-1} (Fakas et al., 2010).

At the same time, C7-C11 monounsaturated aldehydes or C6, C9 dienals or C5 branched aldehydes or some C8 ketones become important contributors to the oil aroma. Some of these compounds are responsible for virgin olive oil defects, such as rancid, winey-vinegary, fusty, muddy sediment or musty (Morales et al., 1997; Angerosa, 2002; Morales et al., 2005). Branched chain volatiles, supposed to be formed through aminoacid conversion (Figure 2) are more likely derived from keto-acids (Kochevenko et al., 2012).

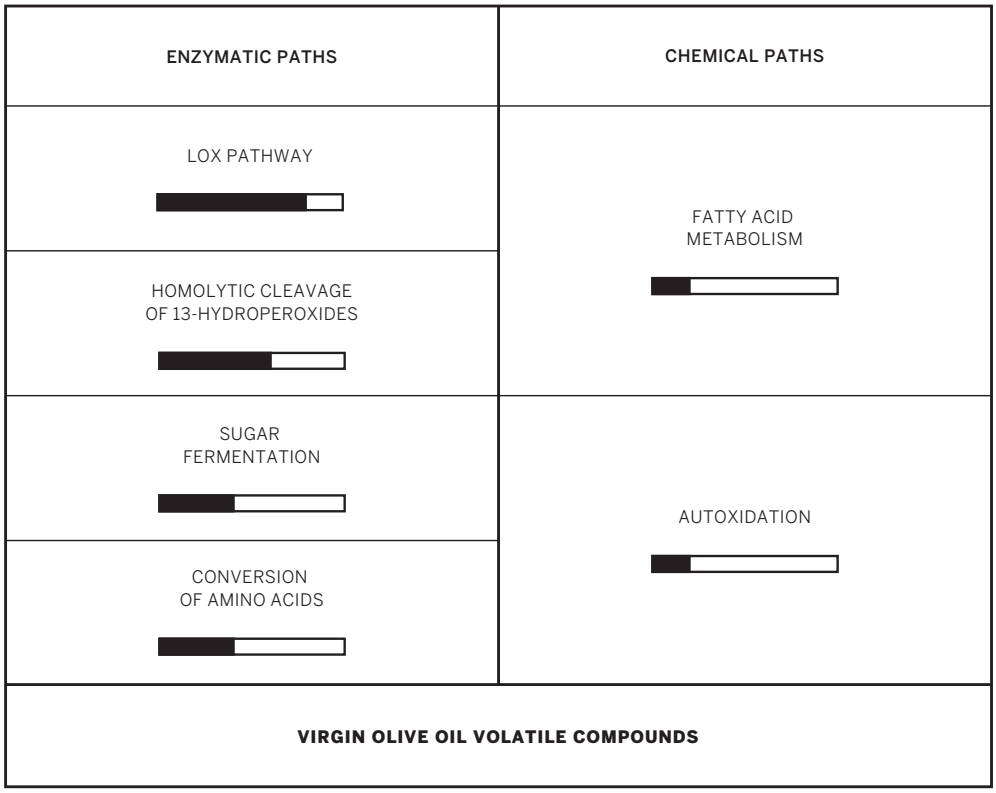


Figure 2. The main pathways involved in the production of volatile compounds of virgin olive oil (adapted from Angerosa, 2002). The size of the black bar gives an idea of the importance of each path.

The most determinant steps of the LOX pathway are the peroxidation of linoleic or linolenic acid by the action of lipoxygenase and then hydroperoxide lyase (HPL) catalyses the cleavage of hydroperoxides from polyunsaturated fatty acids to yield oxoacids and volatile aldehydes (Salas et al., 2005).

The strict specificity of HPL of olive pulp for the n-6 hydroperoxide derivatives from both linoleic and α-linolenic acids, can explain the absence of C9 volatile compounds in the aroma of olive oil (Morales et al., 1995; Salas and Sánchez, 1999).

HPL has been shown to be heat-labile and presented optimal activity under slightly acidic acid conditions (Anthon and Barrett, 2003; Salas et al., 2000). The existence of only one HPL isoform was suggested using data on thermal stabilities of HPL (Luaces et al., 2007b). Maximum activity has been observed at 15 ºC (Anthon and Barrett, 2003), with a clear decline at 35 ºC (Salas and Sánchez, 1999). Thermal stabilities of LOX and HPL enzymatic activities in crude preparations seem to explain the observed decrease of volatile contents as a consequence of heat treatments of olive fruit.

The changes observed in HPL activity during fruit development (from 13 weeks after flowering to 34 weeks after flowering) showed that HPL activity was higher in green olives harvested at the early developmental stages. Thereafter, it decreased slightly to a high constant level along the entire maturation period. Moreover, results proved that only the availability of the HPL substrate could be the limit to volatile aldehyde formation (Salas and Sánchez, 1999; Sánchez-Ortiz et al., 2013).

Other enzymatic systems within the LOX pathway, such as the ADH and AAT enzymatic activities remained apparently unaffected as a consequence of hot-water treatment, so that C6 alcohol and ester contents showed almost no variation (Pérez et al., 2003). Alcohol dehydrogenase (ADH) catalyses the reversible reduction of aliphatic aldehydes to alcohols. Olive stones seem to be a good source of ADH, which is more specific for saturated C6 aldehydes (Luaces et al., 2003). Alcohol acetyl transferase (AAT) catalyses the formation of acetate esters through acetyl-CoA derivatives. In olive oils, ethyl propionate and hexyl acetate are responsible for the sweet and fruity notes (Sánchez and Harwood, 2002; Salas, 2004).

In conclusion, the biosynthesis of VOO aroma compounds depends mainly on the availability of nonesterified polyunsaturated fatty acids, especially linolenic acid, during the process and on the enzymatic activity of the

lipoxygenase/hydroperoxide lyase system. Both availability of substrates and enzymatic activity seem to be cultivar-dependent (Sánchez-Ortiz et al., 2007).

2.1.2. Peroxidases

Peroxidases (EC 1.11.1.7) are mainly heme-iron enzymes that catalyse the reduction of hydrogen peroxide in the presence of a hydrogen donor.

These classes of oxidoreductases are highly specific for hydrogen peroxide, but other organic peroxides, or peroxy acids of the general formula ROOH can also be used as hydrogen acceptors (Laane et al., 2003; Yuan and Jiang, 2003).

Simultaneously they can be very non-specific enzymes for the other substrate that act like the hydrogen donor. In fact peroxidases (PODs) catalyses the oxidation of a variety of organic and inorganic hydrogen donors, such as phenols, aromatic amines, aminophenols, diamines, indophenols, ascorbate, even several amino acids, and can also be able to catalyse other type of reactions such as oxidation and hydroxylation (Laane et al., 2003).

PODs are widely distributed in different plant parts, with the highest activity in roots; POD contents are dependent on plant species, season and growth conditions. In plant cells, PODs are located in soluble form, in the cytoplasm, and also in cell-wall bound form (Vámos Vigyázó and Haard, 1981; Yuan and Jiang, 2003).

Plant PODs play very important roles in physiological processes extensively referred by several authors, such as degrading and synthesizing lignin in cell wall, scavenging of reactive oxidative species in response to oxidative stress (catalysing the removal of excess hydrogen peroxide), participate in defence mechanism against abiotic and biotic stress (like protection against pathogen attack), tolerance, auxin catabolism and other phyto hormones like indole acetic acid (IAA) (Yuan and Jiang, 2003; Mourato et al., 2012; Martins et al., 2013; Vergara-Domínguez et al., 2013).

After olive puncture by olive fly (*Bactrocera olea* (Gmelin)) attack, high levels of peroxidase activity were detected, indicating this key role in the

defence response against insect injuries (Spadafora et al., 2008) and other biotic stress.

The peroxidases catalyse the oxidation of phenolic compounds using either hydrogen peroxide or organic peroxides as the oxidizing agent, thus producing free radicals, highly reactive and easily polymerizable intermediates. While the phenol oxidation by POD activity is limited to the low internal hydrogen peroxide concentration, the autoxidation of phenol compounds in damaged tissues increase the H_2O_2 availability (Takahama and Oniki, 2000). Thus, polyphenol oxidases (PPO) and POD might act synergistically on the oxidative degradation of olive phenolic compounds (Tomás-Barberán and Espín, 2001).

Both PPO and POD activities present in olive fruit at different ripening stages are able to oxidize the main phenol glycosides present in the fruit, as well as those phenol compounds arising from the extraction process, especially secoiridoid compounds derived from oleuropein. Therefore, the activity of endogenous olive PPO and POD enzymes play a key role in the phenolic profile of VOO (Romero-Segura et al., 2012; García-Rodríguez et al., 2015).

Olive seed contains a high level of POD activity (72.4 U g^{-1} FW), accounting for more than 98% of total POD activity in the whole fruit in three main Spanish cultivars (Luaces et al., 2007a). The presence of peroxidase activity, mainly in the seed, was also reported for other cultivars (Servili et al., 2003b; García-Rodríguez et al., 2011; Peres et al., 2011). Moreover, olive seed POD activity was undetectable before 20 weeks after flowering (WAF) in 'Picual' and 'Arbequina' cultivars and has a maximum level at 28 WAF, reaching 20.8 and 15.20 U g^{-1} FW in Picual and Arbequina fruits, respectively, remaining at constant levels thereafter (García-Rodríguez et al., 2011). Table 3 shows some results of maximum POD activity in pulp and seed found for some Spanish and Portuguese cultivars.

The storage of Arbequina olives at 4 ºC and at 20 ºC during four weeks in plastic containers showed that POD in olive seeds during storage and ripening have almost the same behaviour, showing a gradual increase in activity from 9 to 14-15 Ug^{-1} FW (Hbaieb et al., 2015).

Table 3. Maximum guaiacol POD activity levels found in different olive fruit tissues

Cultivar	Plant tissue	POD activity (Ug^{-1} FW)	Reference
Arbequina	seed	15.20 ± 1.6	Garcia-Rodríguez et al., 2011
Picual	seed	20.81 ± 2.2	
Arbequina	mesocarp	0.17 ± 0.05	
Picual	mesocarp	0.05 ± 0.04	
Galega Vulgar	seed	15.7 ± 1.8	Peres et al., 2011
Cobrançosa	seed	11.8 ± 0.8	
Galega Vulgar	mesocarp	2.0 ± 0.11	
Cobrançosa	mesocarp	2.4 ± 0.04	

The purification and characterization of POD of Douro cultivar in the black ripening stage showed that the predominant anionic fraction (PODa4) showed an isoelectric point at 4.4 and optimum pH at 7.0 (optimum temperature of 34.7 ºC) (Saraiva et al., 2007). However, the pH optimum of the peroxidase-catalysed oxidation depended on the substrate used, varying from 4.0 to 6.0 (Tzika et al., 2009).

Concerning the thermal stability, POD loses 60 and 85% of its activity at 40 ºC for 5 and 10 min, respectively. However, no measurable activity could be detected upon heating at 50 and 60 ºC for 5 min (Clodoveo et al., 2014). However, Taticchi et al. (2013) found that olive POD showed the highest activity at 37 °C and high stability in the temperature range tested (0-60 ºC). Olive POD of cultivar Koroneiki showed a very good stability during 45 min of incubation at 30 and 60 °C, while at 40 °C, 34.5% inactivation was noticed after 10 min of incubation (Tzika et al., 2009).

Concerning substrate specificity, POD was unable to oxidize oleuropein, one of the major olive fruit polyphenol, as well as coumaric, ferulic, ascorbic and *p*-hydroxybenzoic acids; although other phenolic compounds, such as gallic acid and protocatechuic acid, were found to be olive POD substrates (Tzika et al., 2009). Plant peroxidases preferencially oxidise phenols at the expense of peroxide species, mainly hydrogen peroxide. So, when the content of H_2O_2 was rised (500 $\mu L\ kg^{-1}$) in the malaxation process, the oxidative degradation of phenols increased as well by 50% in Picual ripened fruits (mainly hydroxytyrosol derived secoiridoids) (García-Rodríguez et al., 2015).

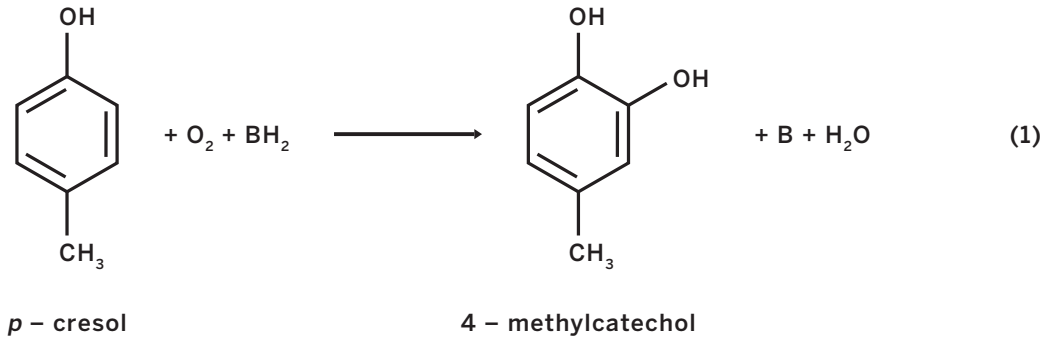
Since the oxidation of endogenous olive oil phenols play significant role for the stability of olive oil, the molecular structure of the phenol as well as its topology inside the olive oil microemulsion, may be crucial for its availability for oxidation by olive fruit POD (Tzika et al., 2009).

2.1.3. Polyphenol oxidases

Polyphenol oxidases (PPO) is a generic name for enzymes that are copper containing metalloproteins, found almost universally in animals, plants, fungi and bacteria. These enzymes are also known as tyrosinase, phenolase, catechol oxidase, catecholase, *o*-diphenol oxidase, monophenol oxidase, and cresolase, due to the diversity and complexity of the reactions that can be involved (Whitaker, 1995).

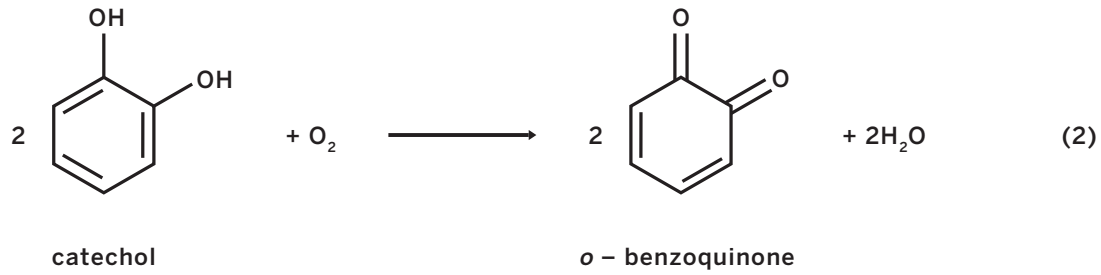
Two main reactions are catalysed by these classes of enzymes in the presence of molecular oxygen (Ramírez et al., 2003; Yoruk and Marshal, 2003) known as:

- monophenol oxidase (EC 1.14.18.1), that catalyses the hydroxylation of monohydroxyphenols (such as *p*-cresol) converting them to *o*-diphenols, and this can be called as monophenolase or cresolase activity; these enzymatic activity cause the hydroxylation in the *o*-position (Equation 1):



- *o*-diphenol oxidase PPO (EC 1.10.3.1), that catalyses the oxidation of *o*-dihydroxyphenols such as catechol, to *o*-quinones, by removal of the hydrogens of the hydroxyl groups converting them on benzoquinones (Equation 2); this can be called as diphenolase or catecholase activity. The resulting benzoquinones formed by *o*-diphenol oxidase are also precursors of many other compounds, as they are very reactive nonenzymatically in the presence of O_2, with sulfhydryl

compounds, amines, amino acids, and proteins. Some coloured compounds can be formed by these reactions, with colors including yellow, red, brown, and black (Ramírez et al., 2003)



These enzymes are located at the inner face of the thylakoids and in the mitochondria (Vaughn and Duke, 1981; Whitaker, 1995). They act on redox reactions that occur during fruit ripening and in response to injury by biotic factors as well as other abiotic stress conditions. PPO has been associated with several cellular processes including resistance to pathogens and herbivores, as well as resistance to stress conditions, and it may be involved in pathogenesis during attack by fungi or other organisms (Mayer, 1986).

PPOs are capable of oxidizing several phenolic compounds reducing the bitter taste associated to these compounds in different food products. The use of laccase to debittering olives by treating stoned, chopped olives was referred as one of the benefic effects of PPOs (Laane et al., 2003). Several authors have studied the time course of fruit PPO activity in several olive cultivars and during fruit ripening (Sciancalepore, 1985; Goupy et al., 1991; Ebrahimzadeh et al., 2003; Ortega-Garcia et al., 2008). The browning rate is well correlated with *o*-diphenols content and polyphenol oxidase activity. The process that leads to browning in the damage areas of olive fruits can be explained by an enzymatic release of hydroxytyrosol from several secoiridoids of the olive fruit due to the action of beta-glycosidase and esterases (Segovia-Bravo et al., 2009). Simultaneously, an additional hydroxytyrosol release, at a less extent than the enzymatic reaction, can also be produced because of the chemical hydrolysis of oleuropein. In a second phase, endogenous fruit PPOs oxidize hydroxytyrosol

together with verbascoside. Due to the pH value of the olive flesh (pH ~5), chemical oxidation of hydroxytyrosol is limited, when compared to the effect caused by the enzymatic action (Brenes-Balbuena et al., 1992).

Results of the kinetics and molecular characterization of PPO in fruits and leaves during ripening of the olive tree cv. Picual showed an increase in PPO-specific activity during fruit ripening for all substrates tested such as catechol and catechin (Ortega-Garcia et al., 2008). Moreover, a new 36-kDa PPO protein was detected in fruits during the last stages of fruit ripening, which may indicate that a new PPO isoform could be present during this stage.

PPO activity was found to be largely present in the fruit mesocarp while no PPO activity was detected in seeds (García-Rodríguez et al., 2011; Peres et al., 2011). Moreover, PPO displays relatively constant values after the onset of fruit ripening (García-Rodríguez et al., 2011). Experiments carried out by us with Cobrançosa and Galega Vulgar Portuguese cultivars showed that PPO activity present in the fruit shows a maximum activity that can be related to ripening index and is dependent on cultivar (Figure 3) (Peres et al., 2013b).

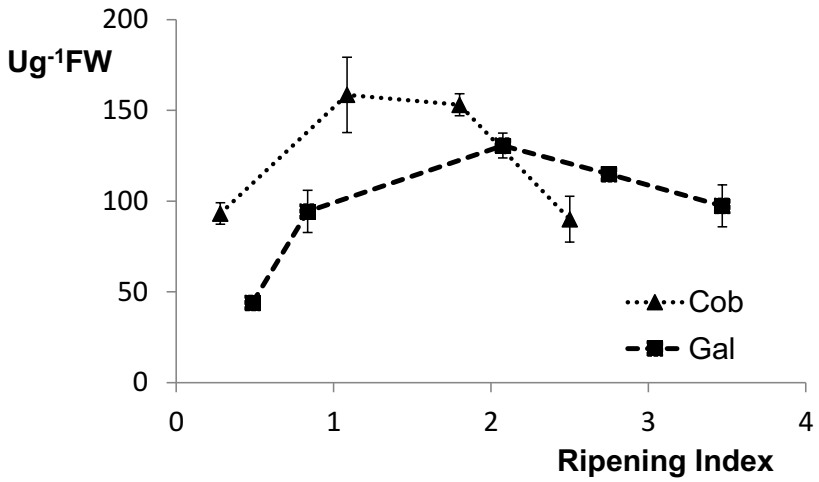


Figure 3. Polyphenol oxidase activity along fruit ripening for two cultivars Cobrançosa (Cob) and Galega (Gal) in 2010 harvest season.

A possible relationship between phenylalanine ammonia lyase (PAL), PPO, and total phenols in Picual, Arbequina, Verdial, and Frantoio cultivars throughout

ripening was performed. The levels of PAL and PPO seem to be coordinated in the different cultivars during ripening (Ortega-García and Peragón, 2010).

Maximum activity of PPO from the Manzanilla cultivar was found to be at pH 6.0. In addition, the enzymatic activity increased with temperature (8 and 25 ºC) and was completely inhibited at pH values below 3.0 regardless of temperature. However, in alkaline conditions, pH inhibition depended on temperature and was observed at values above 9.0 and 11.0 at 8 and 25 ºC, respectively (Segovia-Bravo et al., 2009).

During 7 days of olives storage at 15 ºC, the activity of polyphenol oxidase in the olive pulp decreased drastically in the first 2 days, and then decreased gradually, whereas peroxidase activity showed a gradual reduction throughout the entire storage period. Simultaneously, the PPO activity produced by the spontaneous microbiota in olives, increased along the storage period reaching the maximum levels after the third day of fruit storage (Zullo et al., 2014).

When olives of Arbequina fruits were stored at 4 º C (one month), PPO activity followed the same trend as that in fresh fruit, and was significantly higher than in fruits stored at 20 ºC (Hbaied et al, 2015).

Regarding the thermal stability, PPO has a high stability at 20 ºC, but a high degree of inactivation at 40 ºC, with a large variation in stability according to the olive cultivar (Taticchi et al., 2013).

The oxygen dissolved in the pastes during the malaxation process, activates POD and PPO, which oxidize phenolic compounds and consequently reduce their concentration in VOOs obtained from pastes malaxed at high temperatures. The traditional malaxer, which allows a high amount of oxygen dissolved in the paste during the process due to air contact, represents a classical example of the aforementioned relationship between high temperatures and VOO phenolic loss (Clodoveo et al., 2014).

PPO seems to be the most relevant enzyme involved in phenolic oxidation during crushing. Thus it is important to find suitable inhibitors of this type of phenolic degradation. Citric acid or the addition of citric fruits seems to be a way to control phenol oxidation, by restraining the activity of these enzymes, producing VOO with higher oxidative stability (Cerretani et al., 2008b; Aliakbarian et al., 2009).

2.2. Hydrolases

These enzymes involve hydrolytic reactions and their reversal (degradation of H_2O to OH^- and H^+ products). Hydrolases uses water as a second substrate, and participate in the breakage of the covalent bonds in all biological polymers, such as peptide bonds in proteins, glycosidic bonds in carbohydrates, ester bonds in lipids (Whitaker, 2003).

In the field of olive oil technology the most commonly referred hydrolases are lipases and several other glycosidases.

2.2.1. Lipases

Lipases (glycerol ester hydrolases or triacylglycerol acylhydrolase; EC 3.1.1.3) catalyse the hydrolysis of ester bonds at the interface between an aqueous and nonaqueous phase and are the first enzymes responsible for the degradation path of stored triacylglycerols (TAGs). Hydrolysis of TAGs by lipases can yield di- and mono-acylglycerols, glycerol, and free fatty acids (FFAs). The FFAs released by lipase hydrolytic activity act as a substrate for lipoxygenase activity to produce hydroperoxides.

Olive lipase exhibited a maximum activity at pH 5.0 using triolein as substrate; the presence of calcium increases enzyme activity while the presence of copper reduces this activity by 75% (Panzanaro et al., 2010). However, other results showed that crude olive extracts had an optimal acylhydrolase activity around pH 8.5 (Olias et al., 1993) and pH 7.0 (Fadiloglu and Söylemaz, 1997).

Studies on lipase activity in olives of cv. Ogliarola, at four stages of ripening defined by olives green to purple skin colour, showed that mesocarp lipase activity increases during olive development but declines at purple stage (Panzanaro et al., 2010). The same authors tested the catalytic activity of olive lipase at different temperatures (25 to 45 ºC) and found the maximum activity at 35 ºC.

Moreover, olive TAG can also be hydrolysed by an active lipase present in olive seeds, as well as by exogenous lipases produced by certain fungi, such as *Colletotrichum* spp. (Salas et al., 2000).

Although, fruit endogenous lipase may be responsible for some TAG hydrolyses, the fruit microbiota (lactic and enteric bacteria, fungi and Pseudomonas) has the main influence in the extent of the hydrolytic process (Vichi et al., 2011). *Penicillium* has been identified as the most potent lipase enzyme producer (with yields of 7300 UL^{-1}), which is mostly active at the beginning of its growth (Fakas et al., 2010).

The presence of lipase-positive yeasts in olive oil, immediately after its extraction having an opalescent appearance due to the presence of solid particles and micro-drops of vegetation water, was also demonstrated (Ciafardini et al., 2006b). Laboratory tests highlighted a substantial increase in total diacylglycerols and free fatty acids in the samples of olive oil inoculated with lipase-producing strains of yeasts, isolated from extra virgin olive oil, W. *californica* 1639 and S. *cerevisiae* 1525 (Ciafardini et al., 2006a).

Harvesting time, harvesting methods and the post-harvesting are the main aspects to control the activity of lipase enzymes especially those coming from fruit microbiota. Olive anthracnose caused by *Colletotrichum* spp. is the disease that can cause the main impact in hydrolytic changes of fruits. The lipases produced by this fungus are the most important factor responsible for high acidity of VOO extracted from completely damaged fruit as well as off-flavours (Table 4) (Runcio et al., 2008).

Table 4. Acidity, peroxide value (PV), heptanal, octanal and nonanal from Ottobratica olive oil extracted from healthy and damaged fruits by anthracnose (adapted from Runcio et al., 2008)

Fruits characteristics	Acidity (% oleic acid)	PV (meq O_2 kg^{-1})	Heptanal (mg kg^{-1})	Octanal (mg kg^{-1})	Nonanal (mg kg^{-1})
Healthy	0.3	5	1.5	0.15	1.5
Half damaged	7.7	16	2.79	0.43	5.25
Completely damaged	10.0	23	4.59	1.01	12.90

2.2.2. Glycosidases

Glycosidases, also called glycoside hydrolases, are enzymes that act in the hydrolysis of the β-glycosidic bond between two glycone residues or that between glucose and an aryl or alkyl aglycone (Esen, 2003).

Glycosidases are classified under hydrolases, included in subclass EC 3.2. The subclass 3.1 includes esterases (EC 3.1 acting on ester bonds) that can be divided into several subsubclasses according to specific bond linkage, such as EC 3.1.1 for those acting on carboxylic esters, thioester hydrolases (EC 3.1.2), phosphoric monoester hydrolases, the phosphatases (EC 3.1.3), phosphodiester hydrolases (EC 3.1.4), triphosphoric monoester hydrolases (EC 3.1.5), sulfatases (EC 3.1.6), diphosphoric monoesterases (EC 3.1.7) and phosphoric triester hydrolases (EC 3.1.8) (IUBMB, 2015).

Plant materials contain different glycoside enzymes considering the existing complex mixture of simple and complex glycosides. Within the framework of this study it is important to consider several specific kinds of glycoside hydrolases involved in cell wall polysaccharide degradation, but also other glycoside enzymes that are able to hydrolyse crosslinkages between different polysaccharides, as well as lignin and proteins.

There are numerous enzymes involved in the degradation of cell wall polysaccharides that includes enzymes acting on cellulose and non-cellulosic biopolymers, hemicelluloses (xyloglucans, xylans, arabinoxylan), pectic polysaccharides and proteins.

They can be exo or endo glycoside enzymes dependent upon whether they act at the (usually non-reducing) end or in the middle of an oligo/polysaccharide chain, respectively (Esen, 2003).

The degradation of cell wall polysaccharides is performed by exopolysaccharidases, endopolysaccharidases and other hydrolases that do not belong to these two groups, involved in break of noncarbohydrate groups linked to wall polysaccharides such as *O*-acetyl, *O*-methyl and others. Exopolysaccharidases causes hydrolysis progressively from the nonreducing terminus, and endopolysaccharidases attack the polysaccharide backbone at any position (Minic and Jouanin, 2006).

The major polysaccharides in the cell wall and middle lamella contributing to olive fruit texture were found to be the pectic polysaccharides and the hemicellulosic polysaccharides xyloglucan and xylan (Vierhuis, 2000). During ripening, multiple enzymes contribute to the degradation of complex organization of the cell wall polysaccharides. These include glycoside hydrolases enzymes such as polygalacturonases (PGs), β-D-galactosidases, endo-β-1,4-D-glucanases, and to a lesser extent endo-β-mannanases, β-D-xylosidases, α-D-galactosidase (Minic, 2008).

The progressive loss of firmness of the fruits is the result of a gradual solubilization of protopectin in the cell walls to form pectin and other products. Solubilization followed by depolymerization and deesterification of pectic polysaccharides is the most apparent change occurring during ripening of many fruits like olives (Jimenez et al., 2001). These changes are due to the action of different specific glycosidases that act on cell wall polymers, resulting in their partial or complete degradation. Most of these enzymes are present in low levels and are constitutive throughout fruit development and ripening. However, during ripening, generally all the hydrolases increase in activity, particularly cell wall hydrolases, showing a peak activity at climacteric stage (Prasanna et al., 2007).

The cell wall enzymes, responsible for the changes occurring in the pectic fraction of fruits during ripening and processing, are pectinases, which hydrolyse pectin by different mechanisms.

These enzymes are divided into two broad classes: pectinesterase (PE) and pectin depolymerases (Belitz et al., 2009). PE (EC 3.1.1.11) is a cell-wall-associated enzyme that presents a lot of isoforms facilitating plant cell wall modification and subsequent degradation. In plants, PE plays an important role in cell wall metabolism during fruit ripening. PE catalyses the de-esterification of the methoxyl group of pectin forming pectic acid. The enzyme acts preferentially on a methyl ester group of galacturonate unit next to a nonesterified galacturonate unit (Kashyap et al., 2001). Pectin depolymerases readily split the main chain and they are further classified as polygalacturonase (PG) and pectinlyases (PLs). The depolymerases catalyse the cleavage of glycosidic bonds via hydrolysis (hydrolases) or via β-elimination (lyases) (Hadj-Taieb et al., 2002).

The polysaccharides of olive pulp mainly consist of pectic polysaccharides rich in arabinose, glucuronoxylans, cellulose and minor components as xyloglucans, mannans and glycoproteins (Coimbra et al., 1994; Coimbra et al., 1996; Vierhuis et al., 2003). Seven glycosidases, namely β-glycosidase (EC 3.2.1.21) α-galactosidase (EC 3.2.1.22), β-galactosidase (EC 3.2.1.23), α-arabinosidase (EC 3.2.1.55), α-mannosidase (EC 3.2.1,24), β-xylosidase (EC 3.2.1.37) and β-N-acetylglucosamidase (EC 3.2.1.30), as well as Cx-cellulase (EC 3.2.1.4) and endo-polygalacturonase (EC 3.2.1.15), were identified in olive pulp cell wall preparations, at four ripening stages (Fernández-Bolaños et al., 1995). Many enzymes of this group are involved in a variety of physiological functions such as plant defence, signalling, metabolism of cell wall and lignification (Minic, 2008). Olive fruits can be seriously deteriorated by pre and postharvest damage due to the attack of insects, such as the olive fruit fly, which strongly alters the quality of olives (Rojnić et al., 2015). Olive fruits susceptible to fly infestation could be related to the ability of the oleuropein-degrading-[beta]-glycosidase to produce the highly reactive molecules in the damaged tissues.

The enzyme β-glycosidase (EC 3.2.1.21) is involved in the degradation of oleuropein (Figure 4) and has been shown to play a critical role in shaping the phenolic profile of VOO (García-Rodríguez et al., 2015). Both esterase and β-glycosidase are naturally present in olive pulp (Fernández-Bolaños et al., 1995). Both pathways produce hydroxytyrosol, glucose and elenolic acid as final compounds (Figure 4) (Segovia-Bravo et al., 2009).

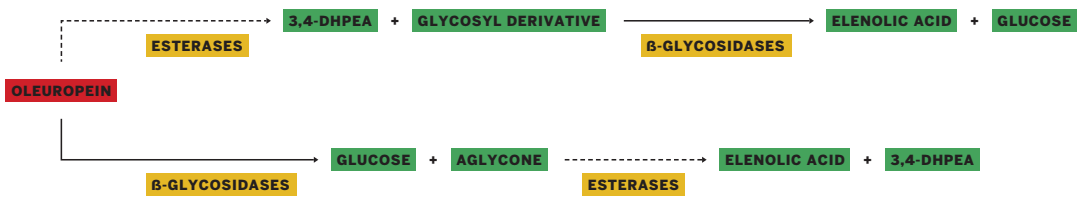


Figure 4. Enzymatic degradation of oleuropein and products formed according to the reaction type (adapted from Segovia Bravo et al., 2009).

Moreover, a clear correlation was established between the contents of oleuropein in olive fruits and the amount in the corresponding VOO of 3,4-DHPEA-EA and OA-isomers (oleuropein derivatives). However, no relationship was found

between the contents of demethyloleuropein in the fruit and the presence of its derived compound 3,4-DHPEA-EDA in VOO (Romero-Segura et al., 2012). Taking into account that the higher selectivity of olive β-glycosidase towards oleuropein and that 3,4-DHPEA-EA is its ultimate product, it is suggested that during VOO production, when no demethyloleuropein is available, 3,4 DHPEA-EDA may be formed by the sequential action of olive β-glycosidase and an endogenous methylesterase acting on the initial products of the oleuropein hydrolysis demethyloleuropein in the fruit and the presence of its derived compound, 3,4-DHPEA-EDA, in the oil (Romero-Segura et al., 2012).

The purified olive β-glycosidase exhibited a broad optimum pH curve with a maximum at pH 5.5 and a rapid decline of activity for higher pH values. This maximum pH is higher than those previously used to assay olive glycosidases (Heredia et al., 1993). Furthermore, the highest activity was observed at 45 ºC, as most plant glycosidases. Thermostability is maintained up to 40 ºC with a dramatic decrease of activity above this temperature (Romero-Segura et al., 2009).

During fruit ripening, β-glycosidases are implicated in the debittering of fruit tissues by oleuropein degradation, and glucose and aglycone molecules release (Brenes-Balbuena et al., 1992; Ryan et al., 1999; Obied et al., 2008). β-glycosidase location during the ripening of olive fruit, by *in situ* activity assay, was detected in mesocarp cell chloroplasts and oleuropein (substrate) is restricted in the vacuoles of olive mesocarp cells (Mazzuca et al., 2006). Moreover, they proposed two different isoforms of beta-glucosidase, which are able to segregate in two different cell compartments. During fruit ripening, significant variations in the number and distribution of reactive cells in mesocarp tissues were observed. In immature fruits the reactive cells are few and located in the inner mesocarp zone; thereafter, in green mature fruits a great number of reactive cells are found distributed in the whole mesocarp. Finally in black fruits, numerous reactive cells are located only in the inner mesocarp close by the woody endocarp, whereas the outer mesocarp cells are devoted to polymeric anthocyanins accumulation. These results clearly showed that the variations of oleuropein-degradative-[beta]-glycosidases activity during ripening are due to changes in the competence of single mesocarp cells to synthesize the enzyme isoforms (Mazzuca et al., 2006).

At 35 WAF Picual fruits display twice as much β-glycosidase activity than Arbequina fruits, which seems to explain the higher phenol content found in Picual VOO (Romero-Segura et al., 2012).

Monitoring the activity of olive endogenous β-glycosidase during fruit ripening and storage showed a similar pattern between one month stored fruits at 4 ºC and fresh harvested ones; moreover, the decrease in this enzyme activity is accompanied by a dramatic decrease in the phenolic content of VOO (Hbaieb et al., 2015).

In conclusion, endogenous enzymes such as β-glycosidase (which hydrolyses phenolic glycosides) and oxidoreductases, like PPO and POD (which oxidase phenolic compounds), may be the main biochemical factors affecting the phenolic content of VOO.

3. Technological factors influencing the activity of the enzymes responsible for some chemical and sensory characteristics of VOO

Sensory characteristics of VOO are strictly related to the contents of volatile and phenol compounds, which are strongly affected by the operative conditions of VOO extraction process. The occurrence of several enzymatic processes (endogenous and exogenous) may affect the amount and the type of phenol and volatile compounds in the final VOO.

3.1. Olive composition: cultivars, ripeness and agro-climatic factors

The composition of VOO depends primarily of olive fruit composition, which depends on the cultivar, the ripening stage and the environmental growth conditions, such as biotic and abiotic stresses. Several studies have already been carried out with the aim of describing the differences found between the phenol profile of different olive cultivars (Esti et al., 1998; Vinha et al., 2005; Cerretani et al., 2006) as well as their evolution throughout the ripening process (Criado et al., 2004; Morelló et al., 2006; Servili et al., 2009; Amiot, 2014). Moreover, phenolic and volatile compounds in VOO have been used to discriminate between fruit ripening stages, geographical origin and cultivar (Aparicio and Morales, 1998; Berlioz et al., 2006; Luna et al., 2006; Vichi et al., 2008; Tura et al., 2013); the influence of environmental and season on flavour

characteristics was also studied (Tura et al., 2009) as well as the site of cultivation on lipophilic and hydrophilic phenols (Tura et al., 2008).

The influence of irrigation on VOO volatile and phenol compounds is an important actual research topic especially for the super high-density orchards (Berenguer et al., 2006; Gómez-Rico et al., 2007; El Riachy et al., 2013).

3.2. Harvest and Post-harvest

The decision of the harvesting date seems to be a key factor to olive oil quality and one of the aspects that most influence the composition of olive oils. From the point of view of the producer the following aspects must be considered: (i) the olives must have the maximum weight of oil, (ii) the quality of the oil must be optimal, (iii) fruit and tree damage must be minimal, (iv) next year's crop must not be affected and (v) harvesting must be as cheap as possible (Prenzler et al., 2007). So, the decision of the harvesting date is usually based on local information, productivity and oil amount or fruit quality. These aspects are certainly crucial for the future development of olive industry but other factors may be analysed as well. Tolerance or sensitiveness to different pests and diseases or climatologic phenomena (frost), studied together with ripening periods are important points to be evaluated in olive growing.

3.3. Storage of the fruits, leaf removal and washing

The effect of olives storage prior to milling is a very important and studied aspect of olive oil technology (Fernández-Bolaños et al., 1997 ; Perez-Camino et al., 2001; Pereira et al., 2002; Kalua et al., 2008). The drastic reduction of total phenols in the oily fraction of the fruit, observed after three days of storage, can probably be attributed to the activity of the oxidoreductases enzymes either from the pulp or from the microbiota grown in the carposphere or in the pulp of the fruits damaged during the harvesting and storage operations (Zullo et al., 2014). Apart from the condition of the fruit at harvest, as the presence of pests and diseases, differences in post-harvest handling of the fruit, produce olive oils with different flavours and off-flavours (Angerosa et al., 2004; Morales et al., 2005; Vichi et al., 2009b). The storage of the fruits after harvest changes the volatile composition of olive oil. 1-octen-3-ol is related with the sensory defect of mustiness-humidity, ethyl butanoate, propanoic and butanoic acids are

responsible for fusty sensory defect; acetic acid, 3-methyl butanol and ethyl acetate for winey-vinegary, while several saturated and unsaturated aldehydes and acids are connected with rancid sensory defect (Morales et al., 2005). The presence of several volatile phenols was also addressed to bad storage conditions of olives (Vichi et al., 2008; Vichi et al., 2009a). So, the storage of fruits before processing is always a non-recommended practice.

Removing leaves and other foreign materials is the first step in olive oil extraction in order to prevent damages in extraction plants caused by stones, as well as to avoid the presence of off-flavours. However, the presence of small amounts of leaves may have benefits in terms of VOO antioxidant characteristics (Malheiro et al., 2013; Sonda et al., 2013).

The washing operation of olives removes soil and pesticides residues but can introduce excessive moisture to the resulting olive paste, leading to a reduction in extractability, because of the formation of water/oil emulsions and to a decrease in hydrophilic phenols due to the extraction of these compounds by the water phase (Uceda et al., 2006). Good practices recommend the frequent renovation of washing water in order to avoid the high microbial concentration in the recycled water and their fermentation activity that might influence sensory VOO quality.

3.4. Milling

Milling is the first unit operation performed in order to prepare the olive paste for olive oil extraction. The crushers, destoner included, as well as time, temperature and oxygen concentration during the malaxation step have shown to influence oxidoreductases activity with consequences on VOO phenolic and volatile composition (Servili et al., 2012). As a matter of fact, the activity of oxidoreductases, mainly POD, can be quite different comparing destoned and the whole fruits (Table 5).

The main hydrophilic phenols of VOO, such as secoiridoid aglycons, are formed during crushing by the hydrolysis of oleuropein, demethyloleuropein and ligstroside, catalysed by endogenous β-glycosidases (Obied et al., 2008). The aglycones are more soluble in the oil phase than the glycoside forms that remain in the water phase. This observation suggests that it is important to know the

adequate conditions to promote β-glycosidase activity, in order to increase phenol contents of VOO. In addition, if the crushing system inhibits β-glycosidases activity, a large proportion of water-soluble phenols will be lost in the waste water (Clodoveo et al., 2014).

Table 5. Oxidoreductases activity (POD and PPO) in two Italian cultivars in milling and malaxation, using destoned and whole fruit (adapted from Servili et al., 2007).

	Cultivar	POD activity (U mg^{-1} DW)	PPO activity (U mg^{-1} DW)
Crushed whole olives	'Frantoio'	38 ± 2.0	17 ± 0.3
Crushed destoned olives		12 ± 1.4	17 ± 1.5
Malaxed whole olives		14 ± 0.3	10 ± 0.7
Malaxed destoned olives		6 ± 0.3	11 ± 1.4
Crushed olives	'Coratina'	16 ± 0.8	5 ± 0.4
Destoned olives		5 ± 0.5	6 ± 0.3
Malaxed whole olives		7 ± 0.2	4 ± 0.4
Malaxed destoned olives		3 ± 0.1	5 ± 0.4

The impact of crushing in VOO phenolic and volatile compounds can be related to the distribution of the endogenous oxidoreductases and phenolic compounds in the different parts of the olive fruit. As previously reported, POD in combination with PPO, are the main endogenous oxidoreductases responsible for phenol oxidation during processing (García-Rodríguez et al., 2011; García-Rodríguez et al., 2015). POD occurs in high amounts in the olive seed; on the contrary, hydrophilic phenol compounds, are largely concentrated in the pulp, whereas the stone and seed contain only small quantities of these substances (Servili et al., 2004; Servili et al., 2007).

In traditional olive mills, the stone crushers, with two or four granite wheels rotating at 12-15 rpm are used. The energy released during breaking is low and the fact that the paste is not heated prevents the LOX pathway from denaturation (Padilla et al., 2009). However, due to the time of exposure to oxygen several compounds produced from the LOX pathway are lost. This type of crusher reduces the phenolic concentration in olive pastes with milling time, due to air contact during processing. Contact with the air stimulates PPO and POD, producing a high oxidation of phenolic compounds. Other drawbacks of the stone crusher are its low working capacity, the high hourly machine footprint, and its low ability to release the chlorophyll found in the olive skin, responsible for the

green colour of VOO, into the oil. This aspect is particularly relevant when the stone crusher is combined with a solid-liquid centrifugal separation (Servili et al., 2012b). The exposition of the olive paste to the atmospheric oxygen promoting the oxidation of phenol compounds causes a reduction of oxidative stability as well as a decrease in bitter and pungent intensity of VOO (Di Giovacchino et al., 2002). Based on this Clodoveo (2012) and Clodoveo et al. (2014) recommend the use of stone mill to process bitter cultivars, like the Italian 'Coratina'.

In VOO extraction by centrifugation the crushing operation is usually performed by hammer crushers. Compared to stone crushers, these machines have some benefits that are attributable to their high working capacity, low footprint and low installation costs. This mills extracts the pigments from the olive skin in a very efficient way, giving VOO with higher concentration in beta-carotene, chlorophyll *a* and pheophytin *a* to produce greener oils (Servili et al., 2002a; Domingues et al., 2009). However, the hammer crushers show some disadvantages, such as the strong emulsifying effect produced on olive paste during crushing, a considerable increase in paste temperature and the high degradation of the seed tissues which, as mentioned earlier, promotes phenolic oxidation by improving peroxidase activity (Servili et al., 2000). Olives treated by the two crushing systems - hammer and stone mills - were observed by scanning electronic microscopy. Micrographs of olive fruits milled in hammer crushing evidence better cell cuts, in contrast to stone mill where olive cell layers have been broken and damaged. The olive oil extracted from pastes obtained by the hammer crusher shows the highest concentration of phenolic compounds and consequently the strongest antioxidant activity (Veillet et al., 2009). Temperature of the olive paste increase by 13-15 ºC with respect to ambient temperature when a fixed-hammer metallic crusher is used (Di Giovacchino et al., 2002). The diameter of grid in hammer crushers as well as the relative speed of fixed hammers may also have a great impact in yield, phenols and pigments extraction (Di Giovacchino et al., 2002). The more violent grinding causes an increase in temperature and the reduction of HPL activity (Amirante et al., 2006).

Disk crushers crush the olives between two-toothed disks, one stationary and one that rotates. The use of the disk-crusher avoids paste overheating, minimizing the risk of oxidation (Amirante et al., 2010). However, this crusher

may promote the formation of emulsions, which inhibit oil–water separation. The use of the disk crusher may also affect the sensory characteristics of the olive oil, which can have a stronger, spicy taste, but less bitter than the one obtained by the hammer-crusher. According to the olive mills manufacturers (e.g. Alfa Laval) this is a technique to obtain “mild taste” or “sweet” olive oils.

A new approach to olive milling is based on separated crushing of the constituent parts of the fruit, such as the skin, pulp and seed. The destoner, also called “de-pitting” machine, crushes only the pulp tissues (Mulinacci et al., 2005; Amirante et al., 2006). In this type of milling, the degradation process of the olive tissues should be strong for the skin and pulp, in order to facilitate the release of oil and pigments, whereas impact on the seed should be limited. The destoning process, excluding the olive seed with high POD activity, reduces the enzymatic degradation of phenols, largely concentrated in the pulp, improving their concentration in VOO and consequently their oxidative stability (Lavelli and Bondesan, 2005; Mulinacci et al., 2005; Amirante et al., 2006; Amirante et al., 2007; Servili et al., 2007; Cerretani et al., 2008a). Simultaneously with the reduction in phenolic degradation an improvement in the concentration of volatile compounds occurs, especially of hexanal, *trans*-2-hexenal and C6 esters, with a consequent positive increase of the intensity of “cut grass” and “floral” sensory notes (Table 6) (Servili et al., 2007).

Table 6. Effect of different crushing methods on total phenols and in the volatile aldehydes hexanal and *trans*-2-hexenal of VOO (mg kg^{-1}).

Types of crushers	Total Phenols	Hexanal	trans-2-hexenal	References
Stone mill	133	28.0	321.0	Di Giovacchino et al., 2002
Disc metallic	247	27.8	121.0	
Hammer mill	260	0.28	43.6	Servili et al., 2012
De-stoner	320	0.58	52.2	
Stone mill	237	8.1	110.8	Amirante et al, 2006
De-stoner	388	13.5	185.4	

Considering that the seeds represent about 25 % of the total paste volume, the use of the destoner can improve the working capacity of the mill plant, excluding about a quarter of the residual solid waste before the extraction process. However, in order to ensure good VOO extraction yields, the de-stoned olive paste requires long mixing times as well as a third-generation decanter to

separate the oil from the olive paste, since the absence of stone fragments causes a change in olive paste rheology (Clodoveo and Hbaieb, 2013).

3.5. Malaxation

From the point of view of yield and VOO quality, malaxation is one of the most important unit operations. The malaxer machine consists of a stainless steel tank containing the olive paste and a malaxing central-screw stirring the paste slowly and continuously, at monitored temperature (Clodoveo, 2012). The oil in olives (about 20-25 %) is found in the mesocarp cells, mainly in the vacuoles and scattered to a lesser extent through the cytoplasm in the form of small lipid inclusions. Mixing and heating (25-35 °C) of the olive pastes during malaxation causes the breakdown of water-oil emulsion, allowing oil droplets to form larger droplets, which separate easily from the aqueous phase during the solid-liquid and liquid-liquid separation processes. Moreover, this operation contributes to activate several natural enzymatic processes after the disruption of olives by milling. However, the knowledge of the critical parameters that control the diffusion and equilibrium phenomena between the aqueous and the oil phases, is crucial, as well as the time needed to trigger enzymatic reactions responsible for the formation of some volatile compounds and for the modification of phenolic compounds (Clodoveo et al., 2014). Many factors, like olive ripeness, olive granulometry in crushing, presence of stone fragments and amount of water may influence these phenomena. Time-temperature relationship is the main critical factor (Peri, 2014).

Operation time, speed of kneading, temperature and atmosphere composition inside the malaxer are the parameters that should be controlled in the malaxation process (Angerosa et al., 2001; Kalua et al., 2006; Parenti et al., 2008; Servili et al., 2008; Inarejos-García et al., 2009; Clodoveo, 2012; Pastore et al., 2014; Reboredo-Rodríguez et al., 2014). The preservation of phenols and volatile compounds in VOO depends on these parameters. Phenol compounds, once released or formed during olive oil extraction, are distributed between the water (approximately 53 % of the available group of antioxidants in the olive fruit) and oil (1-2 %) phases. Approximately 45 % of the phenols are trapped in the solid phase (pomace). This distribution depends on the solubility of phenol compounds between water and oil. Consequently, only a fraction of the phenol compounds is

in the oil phase (Clodoveo, 2012). In turn, volatile compounds are formed by the action of enzymes from the LOX pathway that begin to act as soon as the fruit is crushed, and continue to act during malaxation.

Moreover, risks associated with off-odours or off-flavours, because of “residence time distribution” that can arrive due to incorrect “hygienic design” should be solved by acquiring correctly designed plants (Peri, 2014).

3.5.1. Temperature

During the olive paste malaxing process, increasing temperature levels up to 35 ºC favours the activity of oxidoreductase enzymes present in olive fruit, such as PPO and POD (Taticchi et al., 2013). Also, LOX, that catalyses the formation of hydroperoxides, may be responsible for an indirect oxidation of secoiridoids. Most of these enzymatic reactions occur in the presence of oxygen.

The problems concerning temperature management during malaxation have been widely studied for over twenty years and a negative relationship between processing temperature and the quality of VOO, namely in the amounts of phenols and volatile compounds with their sensory impact in VOO, has been shown (Garcia et al., 2001; Di Giovacchino et al., 2002; Servili et al., 2003b; Kalua et al., 2006; Inarejos-García et al., 2009). In particular, the derivatives of oleuropein, demethyloleuropein and ligstroside are highly affected by the processing temperature, whereas lignans are less affected (Servili et al., 2004).

A significant increase in the secoiridoids of hydroxytyrosol and tyrosol, as the malaxation temperature rises from 20 to 40 °C, is often observed (Ranalli et al., 2001a; Di Giovacchino et al., 2002; Inarejos-García et al., 2009). This is due to the increase in the partition coefficients of these compounds between the oily and water phases in the olive paste and to the increase of the relative solubility in the oily phase (Rodis et al., 2002).

The optimum temperature found to obtain VOO with high total phenol content was 27 ºC (Parenti et al., 2008). In turn, higher total phenol content was found at 40ºC, for 60 min in the experimental oil mill pilot plant and during 45 min for the Abencor laboratory scale system (Inarejos-García et al., 2009). However, VOO obtained in continuous industrial plants at processing temperatures lower than

27 ºC, does not show better chemical and sensory properties than the VOO obtained at 35 ºC in the same plants (Boselli et al., 2009).

PPO is characterized by a lower thermal stability than POD. While olive POD showed the highest activity at 37 °C, PPO exhibited the optimum activity at approximately 50 °C, but showed low stability at 40 °C, with a large variation in stability according to the olive cultivar (Taticchi et al., 2013). This can partly explain the variation in phenol concentrations in the paste during processing depending on the temperature used. In general, an increase in temperature (from 25 to 35 ºC) can reduce the enzymatic oxidative reaction (PPO) causing an increase in both total phenols and in oleocanthal (Taticchi et al., 2014).

At the end of malaxation the residual activity of PPO in the pastes of Moraiolo cultivar was reduced to 30 and 15% of the initial PPO activity in crushed pastes at 20 and 35 ºC, respectively. The lower values for residual activity of PPO at 35 ºC appear to confirm the relationship between the phenolic concentration in the oil and the partial inactivation of PPO at 35 ºC (Taticchi et al., 2013).

The enzymes involved in the LOX pathway such as lipoxygenase, hydroperoxide lyase, alcohol dehydrogenase and alcohol acyltransferase show optimal temperatures between 15 and 25 °C, whereas their activity decreases above 30 °C. Hence, when the malaxing process is carried out at temperatures above 35 °C, a reduction in the generation of volatile compounds during malaxation is observed and less “green” and fruity” VOO are obtained (Taticchi et al., 2014). Oils obtained at 45 ºC were rejected because of “heated or burnt” off-flavour (Boselli et al., 2009). Considering the volatile compounds, the main effect of an increase in the malaxing temperature is a loss of esters and *cis*-3-hexen-1-ol and an accumulation of hexan-1-ol and *trans*-2-hexen-1-ol, responsible for unpleasant odours (Angerosa et al., 2001; Kalua et al., 2006). The activation of amino acid conversion pathway by high temperatures can produce considerable amounts of 2- and 3-methylbutanal, without accumulating any corresponding alcohols, associated with the “fusty” defect (Angerosa et al., 2001).

High malaxation temperature favor the formation of octane, a volatile compound that is produced from the decomposition of 10-hydroxyperoxide of oleic acid and is correlated with “fusty” defect in olive oil (Kalua et al., 2006).

Hexanal formation is promoted at higher temperatures by autoxidation process (Kalua et al., 2006). Based on this, these authors proposed temperatures higher than 35 ºC in malaxation, in order to maximize hexanal formation. However, hexanal odour description, in lower amounts, is green, while in higher amounts (above 900 μg kg^{-1}), is described as unpleasantly sebaceous (Dierkes et al., 2011).

These results are generally obtained by performing malaxation with the pastes in continuous contact with air, as in the case of open-top malaxation machines. The conclusions can be very different in the new generation malaxation equipments that operate in sealed conditions (Servili et al., 2012).

Therefore, the choice of the mixing temperature is a compromise in order to get high quality VOO, rich in volatiles and phenolic compounds. Lower temperatures than 28-30 °C are commonly recommended (Angerosa et al., 2001; Angerosa et al., 2004; Servili et al., 2012). However, even the use of temperatures of paste below 22 °C, in the new generation of confined malaxer, lead to a decrease in the solubilisation of phenolic compounds and chlorophylls (Taticchi et al., 2014).

3.5.2. Time

The time of malaxation can also influence VOO composition (Angerosa et al., 2001; Di Giovacchino et al., 2002; Kalua et al., 2006; Inarejos-García et al., 2009). Therefore, the simultaneous changes in malaxation time-temperature is a feasible systematic approach to promote changes in phenol and volatile composition (Kalua et al., 2006). Table 7 shows the variables (phenol and volatile compounds) in VOO from Frantoio cultivar affected by malaxation time and temperature according to Kalua et al. (2006) and respective impact on flavour.

Regarding the hydrophilic phenols, these compounds are much more affected by the malaxation temperature than the malaxation time (Angerosa et al., 2001; Inarejos-García et al., 2009; Fregapane and Salvador, 2013).

A longer kneading time apparently affects the phenol contents negatively, favouring either their chemical or enzymatic oxidative degradation, and increasing the presence of some undesirable VOO volatiles (Angerosa et al., 2001; Di Giovacchino et al., 2002; Ranalli et al., 2003c; Inarejos-García et al., 2009; Stefanoudaki et al., 2011).

Table 7. Discriminant variables for malaxation time (30, 60, 90 and 120 min) and temperature (15, 30, 45 and 60 º C), Frantoio cultivar, Abencor extraction system (Kalua et al., 2006).

Parameter	Discriminant Variables	Impact on flavour
Time	*cis*-2-penten-1-ol	Olive fruitiness (O) [a]
	hexanal	Green apple (O) [b]
	3,4 –DHPEA-EDA	Bitterness, pungency (G) [a]
	Acetoxypinoresinol	
Temperature	1-penten-3-ol	Grassy (R) [a]
	Hexanal	Green apple (O)
	trans-2-hexenal	Green (O) [b]
	Octane	Sweety, alcane (O) [b]
	Tyrosol	Bitterness (G) [a]
	Vanillic acid	
	3,4 –DHPEA-EDA	Bitterness, pungency (G) [a]

O-orthonasal, R-retronasal, G –gustative; [a] (Cerretani et al, 2008) [b] (Morales et al, 2005)

However, a reduction in the oxygen availability may decrease the activity of polyphenol oxidase and peroxidase, thus avoiding or even increasing the total phenol content of the oil (Servili et al., 2003a; Servili et al., 2008). A small decrease in the secoiridoid derivatives of hydroxytyrosol, as the malaxation time increased, is observed (about 5–10% from 30 to 60 min), whereas the content of secoiridoids of tyrosol increased (15–30%) (Angerosa et al., 2001; Di Giovacchino et al., 2002; Inarejos-García et al., 2009).

For the secoiridoid compound 3,4-DHPEA-EDA high concentrations correspond to short malaxation times (Kalua et al., 2006). The main effect of the duration of malaxation is an increment of C6 and C5 carbonyl compounds, especially of hexanal, which represents an important contribution to the olive oil flavour (Amirante et al., 2006). This increase in C6 and C5 volatile compounds was observed, regardless of the temperature adopted. Thus the production of hexanal, seems mainly to be promoted by extending the malaxation operation to more than 45 min (Morales and Aparicio, 1999; Angerosa et al., 2001) .

It is now generally recognized that temperature and time of malaxation should be evaluated for each cultivar and rheological condition of the pastes. Consequently, the conditions that maximize quality, without compromising yield, should be tested and optimized by the producer. In industrial practice, the residence times of the pastes at the indicated temperature should not be neglected (Peri, 2014).

3.5.3. Oxygen

The time of exposure of olive pastes to air contact (TEOPAC) during malaxation was studied as a processing parameter that could be used to control the activity of endogenous oxidoreductases, such as PPO, POD and LOX, which affect virgin olive oil quality. Servili et al. (2003a) analysed phenol and volatile compounds in VOO using progressive TEOPAC at three ripening stages of olives. The phenol concentration in virgin olive oil progressively decreased with increasing TEOPAC. On the contrary, a positive relationship was found with the concentration of several volatile compounds responsible for virgin olive oil aroma. However, the effect of TEOPAC was strictly related to fruit ripening.

By monitoring the main process parameters (oxygen availability in the malaxer head-space, temperature and time), a selective control of enzyme activities as PPO and POD can be performed. So, the introduction of the new generation of malaxation equipments, such as covered malaxer, permits the regulation of O_2 concentration in the malaxer headspace. Under these conditions, it is possible to increase hydrophilic phenol contents in the olive pastes and in the corresponding EVOO, because of the decrease of phenol oxidation catalysed by endogenous oxidoreductases. Moreover, in covered malaxer, the O_2 concentration can be regulated by using inert gases or the CO_2 naturally produced by the olive pastes during malaxation (Parenti et al., 2006). In fact, saturating the head-space of the malaxer with CO_2 allows the reduction of oxidative phenomena avoiding the use of expensive inert gases (Servili, 2014).

Preliminary studies on some Italian cultivars have been performed to define the best malaxation conditions, in terms of temperature and O_2 concentration. The recommendations are: malaxation should be carried out without oxygen for the cvs. with low phenol content, whereas malaxation should be carried out with controlled supplementation of oxygen for those cvs. characterized with higher phenol contents (Servili et al., 2012). Thus, the optimization of temperature and oxygen concentration requires specific research for each individual cultivar (Selvaggini et al., 2014).

'Coratina' cultivar is characterized by a high amount of phenols and at the same time, shows low polyphenol oxidase and peroxidase activities (Servili et al., 2007). The low activity of these enzymes can explain that an increase of O_2 in

the pastes produces a reduction of phenolic content in the oils, lower than that connected with the malaxing temperature. As a consequence, in 'Coratina' cv. low processing temperatures seem to be more important than high O_2 concentrations in the reduction of the hydrophilic phenolic compounds in the oils. This phenomenon could be explained by reduced activity of the depolymerizing enzymes in the pastes that decrease the release of phenols from the cell wall into the olive oil (Selvaggini et al., 2014).

When pastes were malaxed under a nitrogen atmosphere, oxidation reactions are repressed and no statistical differences were observed in the phenolic concentration of oils obtained from only crushed or malaxed pastes (García et al., 2001a). However, the industrial use of nitrogen during olive oil processing may have some drawbacks. First, nitrogen should be employed not only during the malaxation step but also during crushing. If oxygen is not removed from the paste during crushing, orthodiphenols may be oxidized, even if the paste is malaxed under nitrogen. Second, the mixer should be sealed; otherwise a continuous flow of nitrogen in the mixer should be maintained. Third, from a sensory point of view, an increase of total phenols in oils must also enhance the bitter taste of some olive oils (García et al., 2001a). Malaxation under nitrogen atmosphere raised hydrophilic and lipophilic phenol contents in VOO. Therefore extended induction oxidation time due to increased antioxidant activity was observed in these oils (Yorulmaz et al., 2011).

The reduction of O_2 concentration in the malaxing chamber, to values lower than the O_2 concentration in air, significantly reduced the formation of lipoxygenase derived volatiles decreasing odours and flavours of artichoke, fresh fruity, and fresh cut grass in VOO (Pastore et al., 2014).

3.5.4. Adjuvants

In olive oil extraction process, 10-20 % of the oil remains inside the unsheltered cells or is left in the colloidal system of the olive paste – microgels – and some is bound in an emulsion with the vegetable water (Espínola et al., 2009). The difficulty to extract this ‘‘bound” oil lies mainly in the fact that the droplets of dispersed or emulsified oil are surrounded by a lipoprotein membrane (phospholipids and proteins) that keeps them in that state. The smaller the size

of the droplets, the greater their degree of stabilization, which means that they are prevented from fusing into larger drops (Petrakis, 2006). When this phenomenon is more pronounced, the obtained pastes are called 'difficult pastes' and the positive effects of using processing aids are particularly important for these pastes.

During the last decades, several studies have been performed in order to improve the yield and the quality of olive oil by using processing aids, such as natural microtalc, sodium chloride, enzymes or calcium carbonate adjuvants (Alba et al., 1990; Alba-Mendoza et al., 2005; Pérez et al., 2008; Espínola et al., 2009; Moya et al., 2010; Espínola et al., 2011). For inorganic adjuvants the results obtained can be quite different, and very dependent on cultivar, ripening index (Table 8) and extraction conditions (water addition in malaxation, temperature, time).

According to the EU 2568/1991 and 1989/2003 standards, adjuvants can be added, during malaxation to breakdown emulsions in order to promote a high oil extraction yield. The most frequently employed adjuvants are microtalc and in some countries, although not in Europe, enzyme preparations are used.

Table 8. Effect on yield improvement of some inorganic adjuvants (all trials performed in laboratorial Abencor extraction system)

Adjuvant	Cultivar	Ripening Index	Dose (%)	Yield Improvement (%)	References
NaCl	Picual	1	2.5	21	Perez et al, 2008
	Picual	3	1.2	12	Cruz et al., 2007
	Arbequina	2.2	2	14	Canamassas and Raveti, 2001
$Si_8O_{20}Mg_6(OH)_4$	Picual	3	1.2	30	Cruz et al., 2007
(E553b)	Coratina	1.2	1.0	15	Caponio et al., 2014
	Arbequina	2.2	2	17	Canamassas and Raveti, 2001
$Ca(CO_3)_2$	Picual	3	2	8.9	Espinola et al., 2009
(E170)	Arbequina	2.2	2	27	Canamassas and Raveti, 2001
	Arbequina	4.5	2	11.5	Espinola et al., 2009

For some researchers, adjuvants should always be added in olive oil technology, especially in pastes without adequate rheological properties for phase separation in malaxation. With adjuvants higher yield oil, and a great contribution to environment, as less CO_2 is emitted, less water is consumed and all the olive oil process is more profitable, without modification of olive oil characteristics.

The presence of NaCl in the olive pastes increases the density and the ionic strength of the aqueous phase, which may affect the solubility of certain compounds and may even modulate the activity of enzymes during the malaxation process. In addition, physico-chemical quality parameters of the VOOs were not significantly affected by the use of this adjuvant (Cruz et al., 2007). The addition of NaCl during the extraction process was positively correlated with the presence of *o*-diphenol compounds and the stability of the oils obtained. Furthermore, the use of NaCl resulted in a significant increase in the contents of β-carotene, lutein and chlorophylls *a* and *b* in the oils. The intensity of bitterness was slightly increased (Cruz et al., 2007).

Natural microtalc, which consists of hydrated magnesium silicate, $Si_8O_{20}Mg_6(OH)_4$, is the most important adjuvant used by the olive oil industry, due to its hydrophobic surface and a platy particle shape that adsorbs the natural emulsifiers from the surface of the olive oil droplets. An increase in the amount of extracted oil can be observed with microtalc addition, since it promotes the coalescence of the small oil droplets making easier to separate the oil by centrifugation. Microtalc does not react with oils because of its crystalline structure and water affinity and it is easily removed by centrifugation together with olive pomace due to its high density (2.72 g cm^{-3}) and water affinity (Espínola et al., 2009). The doses for talc addition referred in the literature for olive oil extraction are in the range between 0.5-2 % (Uceda et al., 2006; Clodoveo, 2012; Servili et al., 2012). However, excessive doses can have a negative effect on the extraction process, since the higher pomace oil content do not compensate for the oil content of wastewaters (Uceda et al., 2006). The particle size of the adjuvant could affect the oil yield: the extraction yield decreased as the particle size increased. For the same particle size, calcium carbonate was found to extract a greater oil amount than talc (Moya et al., 2010).

Results from the studies on talc addition show that this adjuvant does not exert any alteration on the VOO quality parameters prescribed by EEC Regulations and successive amendments (Cert et al., 1996; Ben-David et al., 2010; Moya et al., 2010; Pita et al., 2011; Carrapiso et al., 2013; Caponio et al., 2014a). VOO obtained with talc addition had a similar storage behaviour, as those extracted without talc, during six months storage (Caponio et al., 2014a). Addition of talc in

two phase decanter increased oxidative stability, but with no effect in total phenols (Cert et al., 1996). Talc addition (“Talcoliva”) in doses of 5.7% produced a reduction in tocopherols of about 12 % (Carrapiso et al., 2013).

Enzyme preparations are used as processing aids in several food industries. In olive oil technology, several promising studies were performed with the addition of blends of hemicellulases, cellulases and pectinases (Alba-Mendoza et al., 1987; Servili et al., 1992; Di Giovacchino, 1993; Ranalli and De Mattia, 1997; Ranalli et al., 1998; Ranalli et al., 2001b; Vierhuis et al., 2001; Servili et al., 2002b; Ranalli et al., 2003a; Ranalli et al., 2005; Aliakbarian et al., 2008; De Faveri et al., 2008; Najafian et al., 2009; Iconomou et al., 2010; Chih et al., 2012; Peres et al., 2014). Table 9 summarizes some studies performed with enzyme addition usually at the beginning of malaxation, main enzymatic activity, source, dose and yield increase in olive oil extraction.

Usually, enzyme formulations increase both yields and quality level of VOO. Higher contents of phenols, volatile compounds and other key unsaponifiable components are present affecting positively the flavour, odour and shelf-life

Table 9. Commercial names, source, type of enzymes, applied doses and yield improvement in olive oil extraction.

Enzyme commercial name	Type of enzymes	Source	Dose	Yield improvement (%)	References
Roehament O	Glycosidases, cellulases and hemicellulases	*Aspergillus niger*	0.01-0.03 % (w/w)	0.3-11.6	Alba et al., 1987
Cytolase 0	Pectinases, cellulases and hemicellulases	*Aspergillus Trichoderma*	0,3 mL (600 U $kg^{-1}$of paste)	3.2-8.3	Ranalli et al., 2001b
Bioliva	Pectinases with small amounts of cellulases and hemicellulases		600 U kg^{-1} olives	0.8 - 1.2	Ranalli et al., 2003b
Rapidase adex D	Pectinases, cellulases and hemicellulases	*Aspergillus* sp.	600 U kg^{-1}	1.1-1.7	Ranalli et al., 2005
Pectinex Ultra SP-L	Polygalacturonase/pectin methyl esterase/ Pectin lyase (ramno/hemicellulases)	*Aspergillus aculeatus*		22.7 (Barnea) 24.2 (Arbequina)	Canamassas and Ravetti, 2011
NZ 33095	Polygalacturonase/pectin methyl esterase/ Pectin lyase (ramno/hemicellulases)		100-200 mL t^{-1} paste	53.4 (Barnea) 28.0 (Arbequina)	
NZ 33095/ Celluclast 1.5	Polygalacturonase/pectin methyl esterase/ Pectin lyase (ramno/hemicellulases)/Cellulases			24.7 (Barnea) 17.0 (Arbequina)	
Viscozym L	Polygalacturonase/pectin methyl esterase/ Pectin lyase (ramno/hemicellulases)/ β-glucanases	*Aspergillus niger*		62.4 (Barnea) 16.0 (Arbequina)	
Pectinex Ultra SP-L	Pectinases and hemicellulases	*Aspergillus aculeatus*	0.02% v/w – 0.04% v/w Activities >26,000 ^{PG}/mL (pH 3.5)	0.9-2.4	Najafian et al., 2009
Pectinase 1.06021		Aspergillus niger			
Olivex ***Glucanex***	Polygalacturonase β-glucanase	*Aspergillus aculeatus* *Trichoderma* sp.	0.25 and 0.5 mL kg^{-1} (26 000 U mL^{-1}) 0.03 and 0.06 g kg^{-1} (300 U mL^{-1})	15	Iconoumou et al., 2010
Viscozymes	arabanase, cellulase, β-glucanase, hemicellulase and xylanase	*Aspergillus niger*	0.015 – 0.030 w-%	49-69	Chih et al., 2012
Endozym Olea AS	Pectinases with complementary cellulases and hemicellulases activities	*Aspergillus niger*	0.003-0.117 w-%	24-34	Peres et al., 2014

(Di Giovacchino, 1993; Ranalli and Ferrante, 1996; Ranalli and De Mattia, 1997; Ranalli et al., 1999; García et al., 2001b; Ranalli et al., 2003b; Ranalli et al., 2004; Aliakbarian et al., 2008; De Faveri et al., 2008). The addition of commercial enzyme preparations during malaxation can reduce the complexation of hydrophilic phenols with polysaccharides, increasing the concentration of free phenols in the olive paste (Vierhuis et al., 2001). However, due to their hydrophilicity, considerable amounts of these phenols may be lost in wastewaters. The variations in total phenols are quite dependent on the cultivar. Canamasas and Ravetti (2011) did not found any increase in phenol contents for 'Barnea' cultivar when four different enzyme preparations were added at processing plant level, and lower phenol contents were achieved when Pectinex Ultra SP-L was added to 'Arbequina' cultivar (Abencor trials).

The studies of evaluation the authenticity/genuinity of the VOO with enzymes addition shows that no relevant changes occur in compounds like triacylglycerols, waxes, sterols and triterpene dialcohols (Ranalli et al., 2003b). The addition of the enzyme NZ 33095 promoted lower pyropheophytins values and higher 1,2-diacylglycerols contents in VOO (Canamasas and Ravetti, 2011).

The effects of the simultaneous addition of microtalc and enzymes on extraction yield and on olive oil quality and composition was studied by us at lab scale in an Abencor system. Results for Galega Vulgar (black fruits, ripening index (RI) of 6.5) showed that the best extraction conditions are for enzyme (Endozym olea) higher than 0.1% (v/w) and microtalc (FC 8- AW) 0.4-0.5% (w/w) (Figure 5).

Since the first of March 2014, the European Union established the limit of 40 mg kg^{-1} of fatty acids ethyl esters (FAEE) for the EVOO category. Following the recommendations of IOC the limits for FAEE will be 30 mg kg^{-1} in 2015/16 and thereafter (Reg (UE) 1348/2013). So, it is important to evaluate the effect of talc or enzyme addition on the presence of these compounds that result from inadequate practices during olive oil extraction and from using poor quality olives (Giuffrè, 2014). Table 10 shows that no effect in sterols and FAEE content (t test, $p > 0.05$) was found after microtalc (FC 8-AW) and enzyme (*Endozym olea*) addition in cv. 'Galega Vulgar' (RI = 6.5).

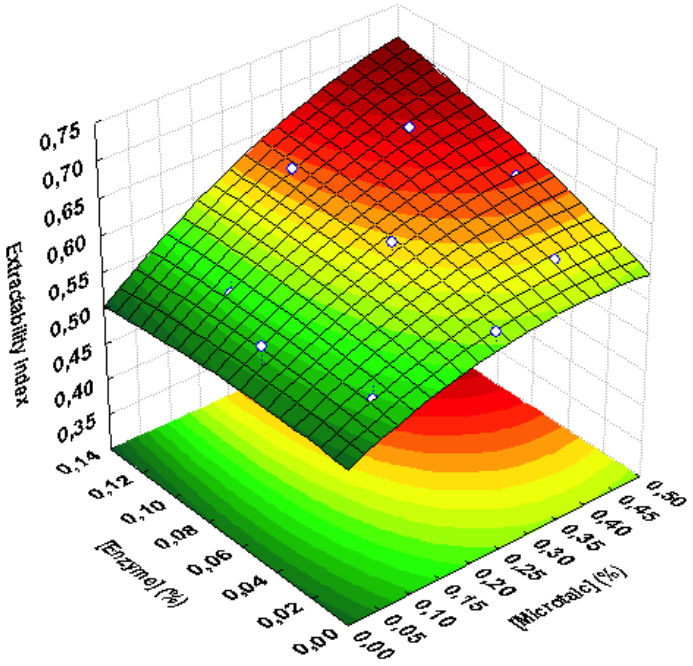


Figure 5. Response surface fitted to Extractability Index for Galega (RI 6.5) as a function of enzyme (E) and microtalc (MT).

Table 10. Effect of microtalc (0.4 % (w/w) and enzyme (0.1% (v/w)) simultaneous addition on sterol composition and FAEE for Galega Vulgar VOO.

Experiment	Control	Microtalc + Enzyme
Campesterol (%)	2.6	2.6
Stigmasterol (%)	0.32	0.30
β-sitosterol (%)	96.14	96.19
δ-7-stigmastenol (%)	0.14	0.14
Total Sterols (mg kg^{-1})	1332	1308
Erythrodiol+Uvaol (%)	0.88	0.77
FAEE (mg kg^{-1})	5.3	6.2

In two-phase decanters water addition in malaxation is considered to have a great impact in pastes extractability, when olive moisture content is very low. Consequently, in these systems water addition is considered as an adjuvant.

3.6. Solid-Liquid Extraction

The extraction system (pressure or centrifugation) plays an important role in the presence of volatile and phenolic compounds in VOO. All systems may provide extra VOO if olive fruits are of good quality, but the centrifugation system helps to avoid or reduce the risk of sensory defects ascribed to the use of recycled mats

in pressure systems (Di Giovacchino et al., 2002). The solid/liquid pressure method is only a valid form of producing high-quality olive oil, if after each extraction, the mat disks are properly cleaned in order to avoid the development of unpleasant odour notes arising from endogenous or microbial enzymatic activities in pastes (Angerosa et al., 2004). In this extraction method, the addition of water is minimal when compared to the continuous system. Thus, the hydrophilic phenol compounds are usually maintained in VOO. On the contrary, the exposition of olive paste to the action of oxygen and light is high.

Nowadays, the majority of VOO in the market is currently extracted by centrifugation. The first operating patents, including the patent by Corteggiani, date back to 1956, followed by the manufacture of olive oil machines by new companies in the early sixties. In 1965, Alfa –Laval Company constructed the "Centriolive Plant", with a three phase "De-Sludger" centrifuge (Ranalli and Martinelli, 1995). This machine, called decanter, consists of a drum containing a cylindrical and a conical part with a horizontal axis, inside which an additional cylinder worm is placed, which acts as a screw conveyor. The differential speed of the latter is slower than that of the outer drum, in order to discharge the solid part. The three phase decanter was till 1992 the only horizontal centrifugation system to perform solid/liquid separation.

In the last decades, this extraction system was modified in order to reduce the amount of water used during the process. According to Servili et al. (2012) the decanters can nowadays be classified as follows:

(a) traditional three phase decanters with water addition between 0.5 and 1 m^3 t^{-1} ;

(b) new three phase decanters, maximum level of water addition 0.2 and 0.3 m^3 t^{-1} ;

(c) two phase decanters that can work without water addition.

In the traditional three-phase decanters, where the oil is separated both from the vegetation water and from the pomace, the humidity level of the pastes must be fixed between 50 and 55 % in order to reduce their viscosity. In addition to the high amounts of vegetation water produced, this implies a decrease in the oil quality, mainly due to the removing of hydrophilic phenol compounds of VOO (De

Stefano et al., 1999; Di Giovacchino et al., 2002; Servili et al., 2004). The addition of warm water, used to dilute the olive pastes in these three-phases decanters, can explain the decrease in C6 alcohols, hexan-1-ol and *trans*-2-hexen-1-ol when compared to oils obtained in pressing systems (Angerosa et al., 2004).

The evolution of this technology for two and three-phase decanters with low water consumption results in VOO with phenol contents higher than those extracted by the traditional centrifugation process. This is mainly due to a decrease in hydrophilic phenolic compounds in the vegetation water (Aparicio and Luna, 2002; Gimeno et al., 2002; Servili et al., 2009). Moreover, the two-phases centrifugal decanters, operating without adding water (or only a minimal amount of water) to olive paste, save heat, energy and the oils obtained are more fruity and have a higher content of antioxidants (Di Giovacchino et al., 2002; Kalogeropoulos et al., 2014). Table 11 summarizes the main differences in some compounds present in VOO obtained by three or two phase decanters.

Table 11. Differences in chemical compounds of VOO obtained from three and two phase decanters

Three-phase Decanter	Two-phase Decanter	References
+ pigments	+ *trans*-2-hexenal	(Di Giovacchino et al., 2002;
+ aliphatic and triterperpenic alcohols	+ total phenols	Aparicio e Luna, 2002;
+ steroid hydrocarbons	+ orthodiphenols	Kalogeropoulos et al., 2014)
+ waxes	+ hydroxytyrosol	
	+ tocopherols	
	+reducing power (FRAP)	

The type of oil extraction plant (three or two phases) markedly affects the level of the hydrophilic phenols in the oil and consequently the related sensory attributes of bitter, pungency and astringency, as well as the level of autoxidation hydrocarbons, esters from fermentations, keto acids derivatives, and all the LOX derived groups, except for C6 aldehydes (Pastore et al., 2014). Drum speed can also affect phenol and volatile contents of VOO. The oils extracted at lower drum speeds are usually bitterer and show higher peroxide and K_{270} values, as well as higher amounts of volatile compounds related to aldehydes oxidation (Caponio et al., 2014b).

Whatever the centrifugal technology used, pomace has always a high level of oil. Pomace olive oil creates damages to the image of the VOO and competes with

VOO determining a detrimental effect on the prices and on producer incomes (Clodoveo, 2014). This will be overcome by the introduction in 2012 by Pieralisi Company, of a multi-phase decanter, the ”Leopard two-phase decanter”, that changes the by-products, producing a dehydrated husk similar to the one coming from a three-phase decanter; it also separates the pulp from the husk, obtaining an ingredient for composting or animal feeding.

3.7. Liquid/Liquid separation

The oil phases are further clarified in an automated discharge vertical disk centrifuge by the addition of tepid water. Vertical centrifugation removes the residual water and the solid impurities from the oil, reducing the VOO moisture content to a mean value of about 0.18 % (Masella et al., 2009). However, the addition of water reduces the content of hydrophilic phenols. Also, vertical centrifugation causes a strong oxygenation of the VOO resulting in a marked increase of dissolved oxygen concentration (Parenti et al., 2007; Masella et al., 2009). This condition can lead to a noticeable shortening of the oil shelf-life as a consequence of accelerated oxidation (Clodoveo et al., 2014). A decrease of C6 and C5 volatile compounds has been observed during the vertical centrifugation as compared to non-centrifuged VOO (Masella et al., 2009).

Since the vertical centrifugation is the processing step that mainly contributes to the oil oxygenation, blanketing the vertical separator with an inert gas would be a feasible way to reduce the dissolved oxygen concentration. VOO vertical centrifugation under inert gas promotes a strong reduction of the oil oxygenation, in terms of reduced dissolved oxygen concentration and oxidative indexes (peroxide value and K_{232}). Moreover, minor compounds as chlorophyll, total phenols and volatile compounds are not affected by this treatment (Masella et al., 2012).

3.8. Storage of olive oils

The storage of olive oils in stainless steel tanks after extraction is actually a common practice all over the world. The maintenance of a constant temperature inside the tank between 12 and 22 °C, before bottling, is a recommended practice (IOC, 2006).

Important losses of chlorophyll, carotenoids, and total phenols in oils occur during the storage period due to oxidation (Psomiadou and Tsimidou, 2002). A significant decrease in secoiridoid derivatives and 3,4-DHPEA-AC was observed after a 12 months storage period, while lignans were the most stable phenol compounds and α-tocopherol disappeared after the storage period (Morelló et al., 2004). Similarly, the main change found in the phenol composition of virgin olive oils of Arbequina, Hojiblanca, and Picual varieties, during storage in darkness at 30 °C was the hydrolysis of the secoiridoid aglycons. This reaction gave rise to an increase in hydroxytyrosol and tyrosol in VOO (Brenes et al., 2001).

Volatile compounds, responsible for off-flavours in VOO, such as hexanal, octane and other C8 and C9 compounds, are formed through nonenzymatic oxidation during VOO storage, favoured by high temperatures, oxygen, light, and pro-oxidants compounds. When VOO was stored in contact with air the levels of some negative sensory components, such as penten-3-ol and hexanal increased while other compounds with positive notes, like *trans*-2-hexenal were reduced (Stefanoudaki et al., 2010). Recently, the use of stripping nitrogen to remove the dissolved oxygen from the oil, immediately after extraction has also been suggested, in order to increase VOO shelf life (Masella et al., 2010).

Therefore, the main deteriorative reaction that occurs during storage is oxidation but the endogenous enzymatic activities contained in the cloudy phase can modify the olive oil phenolic composition (Taticchi et al., 2014). Cloudy, or veiled VOO contains polyphenols, phospholipids and sugars, but it can also contain hydrolytic and oxidative enzymes, such as lipases, LOX, and polyphenol oxidases. These enzymes may reduce the “pungent” and “bitter” sensory notes, the intensity of which is strictly linked to the content of aglycon secoiridoids, and, at the same time, can produce olfactory and taste defects. Consequently, the olive oil profile changes during its storage, due to the simultaneous drastic reduction in compounds by the LOX pathway and to the formation of new volatile compounds, responsible for some common defects as “rancid”, “cucumber” and “muddy sediment”(Morales et al., 1997; Aparicio et al., 2000; Angerosa et al., 2004). This is accompanied by the increase in saturated aldehydes nonanal and hexanal, coming from the oxidation process (Angerosa et al., 2004).

Furthermore, under suitable temperature conditions, the presence of sediment from the sedimentation of unfiltered VOO during its storage, can result in the production of the typical “muddy sediment” defect, due probably to a butyric type fermentation process (Servili et al., 2004).

The racking operation generally leads to a relative decrease of C6 volatiles vs. C5 components, what gives to the resulting oils a more intense bitter character. Moreover, minor alcohols with odour activity levels (OAVs) higher than 0.2, namely 2-methyl-1-butanol and 3-methyl-1-butanol (with whiskey-like and spicy notes), have a slight trend to increase, while minor aldehydes with OAVs that can be as higher as 150, such as 2-methyl-butanal and 3-methyl-butanal (with malty nuances), can be much higher in the oils after racking (Reboredo-Rodríguez et al., 2013).

3.9. Filtration

The filtration process of VOO is a procedure carried out in two steps: first, the suspended solids are removed, and after water traces are removed to give the oil a brilliant aspect (López-Villalta, 2008). Together with water, enzymes are also removed from VOOs. Thus, VOO phenol content is maintained, which contribute to its stabilization during storage. Generally, organic or inorganic materials are used in conjunction with a variety of filtration equipment to enhance or enable the separation of suspended solids and water from the oil by filtration (Lozano-Sánchez et al., 2010).

Veiled oils were found to have longer induction periods, compared to filtered ones (Lercker et al., 1994). It appears that the material in suspension-dispersion that “veils” the oil plays an important role against oxidation, although there is little evidence concerning the chemical nature of the compounds participating in the stable dispersion system. Higher total phenol content in veiled oils in relation to filtered ones may partly explain the higher stability of these VOO (Tsimidou et al., 2005).

During filtration, a loss of phenols occurs favoring a reduction of oxidative stability. Also, the conditions in veiled VOO favors enzymatic reactions. Moreover, the filtration of VOO can avoid the fermentation of sugars or proteins

producing volatile compounds responsible for the “muddy” defect (Clodoveo et al., 2014).

Other possible explanation might be the presence of emulsifiers (e.g. phospholipids) in higher amounts in cloudy VOO than in filtered ones (Koidis and Boskou, 2006).

It has also been suggested that small quantities of proteins may contribute to the higher oxidative stability of unfiltered olive oils (Zamora et al., 2002). There is a discrepancy in the literature concerning the level of proteins and values varying from 0.1 to 400 mg kg^{-1} have been reported (Georgalaki et al., 1998). Koidis and Boskou (2006) demonstrated that the level of proteins in cloudy olive oil is very low, not exceeding 2.5 mg kg^{-1} oil. This indicates that a great antioxidant activity cannot be expected from proteins in the presence of strong antioxidants (α-tocopherol, *o*-diphenols) at much higher concentrations. A lipoxygenase activity has also been detected in freshly prepared olive oil (Georgalaki et al., 1998).

In spite of the presence of a small quantity of water in non-filtered oils (a favourable condition for enzymatic activity), these oils have higher oxidative stabilities. It can be postulated that the polar phenol compounds present not only act as primary antioxidants, but also as inhibitors of oxidizing enzymes (Boskou, 2006).

Ciafardini and Zullo (2002) identified yeasts as the dominant microbial population in non-filtered VOO, in a trial carried out during the sedimentation period, when the solid particles and microdrops of vegetation water present in the newly produced olive oil separate from the oily phase. The authors suggested filtration as a mean to ensure high quality extra virgin olive oil, despite the serious reduction in polar phenols.

Filtration and especially dehydration could help to prolong the shelf life of high-quality and less stable olive oils like those obtained from the ‘Arbequina’ cultivar (Fregapane et al., 2006). Several results show that the impact of filtration depends on the specific monovariety olive oil cultivar. Thus it could be useful to develop targeted technologies for specific VOO quality improvement (Bubola et al., 2012).

4. Conclusions

In the last years the focus of innovation in olive oil technology has been more and more on promoting the aspects that are related with health issues and sensory quality of VOO. In fact, the consumer demand shows an increasing interest towards a product with hedonistic and healthy value. Olive oil extraction is not just a physical, mechanical process. Several biochemical and chemical reactions can occur, from the ripening of the drupes till the end of oil storage. Therefore, it is very important to have a better knowledge of all the factors that influence VOO extraction system in order to develop useful solutions to increase oil yield, process efficiency and quality.

Application of processing aids, as well as a better understanding of the behavior of endogenous enzymes, may allow establishing specific technological conditions for each cultivar or blend of cultivars. Such investigation needs the background of biochemistry, chemistry, technology and physics in a joint effort to bring VOO with high nutritional value and sensory balance to the consumer.

References

Alba, J., Izquierdo, J. R., Gutiérrez , F., and Vossen, P. (2008). Aceite de Oliva Virgen. Análisis Sensorial., 2ª Edition. Editorial Agrícola Española, S.A., Madrid.

Alba, J., Ruiz-Gomez, M. A., Prieto-Gonzalez, M. C., and Gutiérrez -Rosales , F. (1990). Utilización de enzimas en la extracción del aceite de oliva. *Alimentación, Equipos y Tecnología*: 63-71.

Alba-Mendoza, J., Moyano Pérez, M. J., Martinéz Román, F., and Hidalgo Casado, F. (2005). Influencia de la granulometria y del contenido de carbonatos del talco sobre el rendimiento en aceite de oliva virgen. **In:** XII Simposium Cientifico-Técnico Expoliva 2005, Jaen.

Alba-Mendoza, J., Ruiz-Gomez, M. A., Prieto-Gonzalez, M. C., and Gutierrez-Rosales, F. (1987). Efficacy of the enzyme preparation 'Roehament O' in the technology of olive-oil. Composition and organoleptic evaluation of the oil produced. *Grasas-y-Aceites*. **38**: 271-277.

Aliakbarian, B., De Faveri, D., Converti, A., and Perego, P. (2008). Optimisation of olive oil extraction by means of enzyme processing aids using response surface methodology *Biochemical Engineering Journal*. **42**: 34-40.

Aliakbarian, B., Dehghani, F., and Perego, P. (2009). The effect of citric acid on the phenolic contents of olive oil. *Food Chemistry*. **116**: 617-623.

Amiot, M. J. (2014). Olive oil and health effects: from epidemiological studies to the molecular mechanisms of phenolic fraction. *OCL*. **21**: D512.

Amirante, P., Arena, G., Clodoveo, M. L., Dugo, G., Leone, A., Lo Turco, V., Pollicino, D., and Tamborrino, A. (2007). Virgin olive oil production from de-stoned pastes: a new technology to improve the shelf life of the product. *Italian Journal of Food Science*. **19**: 116-120.

Amirante, P., Clodoveo, M. L., Dugo, G., Leone, A., and Tamborrino, A. (2006). Advance technology in virgin olive oil production from traditional and de-stoned pastes: Influence of the introduction of a heat exchanger on oil quality. *Food Chemistry*. **98**: 797-805.

Amirante, P., Clodoveo, M. L., Tamborrino, A., Leone, A., and Paice, A. G. (2010). Influence of the crushing system: phenol content in virgin olive oil produced from whole and de-stoned pastes. **In:** Olives and Olive Oil in Health and Disease Prevention, pp. 69-76. Victor, R. P., and Watson, R. R. (Eds.), Elsevier Inc.

Angerosa, F. (2000). Sensory Quality of Olive oils. **In:** Handbook of olive oil, pp. 355-392. Harwood, J., and Aparicio, R. (Eds.), Aspen Publishers, Maryland.

Angerosa, F. (2002). Influence of volatile compounds on virgin olive oil quality evaluated by analytical approaches and sensor panels. *European Journal of Lipid Science and Technology*. **104**: 639 - 660.

Angerosa, F., and Basti, C. (2003). The volatile composition of samples from the blend of monovarietal olive oils and from the processing of mixtures of olive fruits. *European Journal of Lipid Science and Technology*. **105**: 327 - 332.

Angerosa, F., Basti, C., and Vito, R. (1999). Virgin Olive Oil Volatile Compounds from Lipoxygenase Pathway and Characterization of Some Italian Cultivars. *J. Agric. Food Chem.* **47**: 836-839.

Angerosa, F., Mostallino, R., Basti, C., and Vito, R. (2000). Virgin olive oil odour notes: their relationships with volatile compounds from the lipoxygenase pathway and secoiridoid compounds. *Food Chemistry*. **68**: 283-287.

Angerosa, F., Mostallino, R., Basti, C., and Vito, R. (2001). Influence of malaxation temperature and time on the quality of virgin olive oils *Food Chemistry*. **72**: 19-28.

Angerosa, F., Servili, M., Selvaggini, R., Taticchi, A., Esposto, S., and Montedoro, G. (2004). Volatile compounds in virgin olive oil: occurrence and their relationship with the quality *Journal of Chromatography A*. **1054**: 17-31.

Anthon, G. E., and Barrett, D. M. (2003). Thermal inactivation of lipoxygenase and hydroperoxytrienoic acid lyase in tomatoes. *Food Chemistry*. **81**: 275-279.

Aparicio, R., and Luna, G. (2002). Characterisation of monovarietal virgin olive oils. *European Journal of Lipid Science and Technology*. **104**: 614-627.

Aparicio, R., and Morales, M. T. (1998). Characterization of Olive Ripeness by Green Aroma Compounds of Virgin Olive Oil. *Journal of Agricultural and Food Chemistry*. **46**: 1116-1122.

Aparicio, R., Morales, M. T., and García-González, D. L. (2012). Towards new analyses of aroma and volatiles to understand sensory perception of olive oil. *European Journal of Lipid Science and Technology*. **114**: 1114-1125.

Aparicio, R., Rocha, S. M., Delgadillo, I., and Morales, M. T. (2000). Detection of Rancid Defect in Virgin Olive Oil by the Electronic Nose. *Journal of Agricultural and Food Chemistry*. **48**: 853-860.

Belitz, H., Grosh, D. W., and Shieberle, P. (2009). Food Chemistry. Springer-Verlag Berlin Heidelberg, Leipzig.

Ben-David, E., Kerem, Z., Zipori, I., Weissbein, S., Basheer, L., Bustan, A., and Dag, A. (2010). Optimization of the Abencor system to extract olive oil from irrigated orchards. *European Journal of Lipid Science and Technology*. **112**: 1158-1165.

Berenguer, M. J., Vossen, P. M., Grattan, S. R., Connell, J. H., and Polito, V. S. (2006). Tree Irrigation Levels for Optimum Chemical and Sensory Properties of Olive Oil. *HortScience*. **41**: 427-432.

Berlioz, B., Cordella, C., Cavalli, J.-F., Lizzani-Cuvelier, L., Loiseau, A.-M., and Fernandez, X. (2006). Comparison of the Amounts of Volatile Compounds in French Protected Designation of Origin Virgin Olive Oils. *Journal of Agricultural and Food Chemistry*. **54**: 10092-10101.

Boselli, E., Di Lecce, G., Strabbioli, R., Pieralisi, G., and Frega, N. G. (2009). Are virgin olive oils obtained below 27 °C better than those produced at higher temperatures? *LWT - Food Science and Technology*. **42**: 748-757.

Boskou, D. (2006). Olive Oil Chemistry and Technology. AOCS Press, Ilinois.

Brenes, M., García, A., García, P., and Garrido, A. (2001). Acid Hydrolysis of Secoiridoid Aglycons during Storage of Virgin Olive Oil. *Journal of Agricultural and Food Chemistry*. **49**: 5609-5614.

Brenes-Balbuena, M., Garcia-Garcia, P., and Garrido-Fernandez, A. (1992). Phenolic compounds related to the black color formed during the processing of ripe olives. *Journal of Agricultural and Food Chemistry*. **40**: 1192-1196.

Bubola, K. B., Koprivnjak, O., and Sladonja, B. (2012). Influence of filtration on volatile compounds and sensory profile of virgin olive oils. *Food Chemistry*. **132**: 98-103.

Campeol, E., Flamini, G., Cioni, P. L., Morelli, I., Cremonini, R., and Ceccarini, L. (2003). Volatile Fractions from Three Cultivars of Olea europaea L. Collected in Two Different Seasons. *Journal of Agricultural and Food Chemistry*. **51**: 1994-1999.

Canamasas, P., and Ravetti, L. (2011). Evaluation of processing aids for olive oil extraction and quality improvement. . Rural Industries Research and Development Corporation., Canberra.

Caponio, F., Monteleone, J. I., Martellini, G., Summo, C., Paradiso, V. M., and Pasqualone, A. (2014a). Effect of Talc Addition on the Extraction Yield and Quality of Extra Virgin Olive Oils from Coratina Cultivar after Production and during Storage. *Journal of Oleo Science*. **63**: 1125-1132.

Caponio, F., Summo, C., Paradiso, V. M., and Pasqualone, A. (2014b). Influence of decanter working parameters on the extra virgin olive oil quality. *European Journal of Lipid Science and Technology*. **116**: 1626-1633.

Carrapiso, A. I., García, A., Petrón, M. J., and Martín, L. (2013). Effect of talc and water addition on olive oil quality and antioxidants. *European Journal of Lipid Science and Technology*. **115**: 583-588.

Cavalli, J.-F., Fernandez, X., Lizzani-Cuvelier, L., and Loiseau, A.-M. (2004). Characterization of volatile compounds of French and Spanish virgin olive oils by HS-SPME: Identification of quality-freshness markers. *Food Chemistry*. **88**: 151-157.

Cerretani, L., Baccouri, O., and Bendini, A. (2008a). Improving of oxidative stability and nutritional properties of virgin olive oils by fruit de-stoning. *Agro Food Industry Hi-Tech*. **19**: 21-23.

Cerretani, L., Bendini, A., Del Caro, A., Piga, A., Vacca, V., Caboni, M. F., and Toschi, T. G. (2006). Preliminary characterisation of virgin olive oils obtained from different cultivars in Sardinia. *European Food Research and Technology*. **222**: 354-361.

Cerretani, L., Bendini, A., Poerio, A., and Toschi, T. G. (2008b). Citric acid as co-adjuvant: Improvement of the antioxidant activity of edible olive oils. *Agro Food Industry Hi-Tech*. **19**: 64-66.

Cerretani, L., Salvador, M. D., Bendini, A., and Fregapane, G. (2008c). Relationship Between Sensory Evaluation Performed by Italian and Spanish Official Panels and Volatile and Phenolic Profiles of Virgin Olive Oils. *Chemosensory Perception*. **1**: 258-267.

Cert, A., Alba, J., Leon Camacho, M., Moreda, W., and PerezCamino, M. C. (1996). Effects of talc addition and operating mode on the quality and oxidative stability of virgin olive oils obtained by centrifugation. *Journal of Agricultural and Food Chemistry*. **44**: 3930-3934.

Chiacchierini, E., Mele, G., Restuccia, D., and Vinci, G. (2007). Impact evaluation of innovative and sustainable extraction technologies on olive oil quality *Trends in Food Science & Technology*. **18**: 299-305.

Chih, H. J., James, A. P., Jayasena, V., and Dhaliwal, S. S. (2012). Addition of enzymes complex during olive oil extraction improves oil recovery and bioactivity of Western Australian Frantoio olive oil. *International Journal of Food Science & Technology*. **47**: 1222-1228.

Ciafardini, G., and Zullo, B. A. (2002). Microbiological activity in stored olive oil. *International Journal of Food Microbiology*. **75**: 111-118.

Ciafardini, G., Zullo, B. A., Cioccia, G., and Iride, A. (2006a). Lipolytic activity of Williopsis californica and Saccharomyces cerevisiae in extra virgin olive oil. *International Journal of Food Microbiology*. **107**: 27-32.

Ciafardini, G., Zullo, B. A., and Iride, A. (2006b). Lipase production by yeasts from extra virgin olive oil. *Food Microbiology*. **23**: 60-67.

Clodoveo, M. L. (2012). Malaxation: Influence on virgin olive oil quality. Past, present and future – An overview. *Trends in Food Science & Technology*. **25**: 13-23.

Clodoveo, M. L., and Hbaieb, R. H. (2013). Beyond the traditional virgin olive oil extraction systems: Searching innovative and sustainable plant engineering solutions. *Food Research International*. **54**: 1926-1933.

Clodoveo, M. L., Hbaieb, R. H., Kotti, F., Mugnozza, G. S., and Gargouri, M. (2014). Mechanical Strategies to Increase Nutritional and Sensory Quality of Virgin Olive Oil by Modulating the Endogenous Enzyme Activities. *Comprehensive Reviews in Food Science and Food Safety*. **13**: 135-154.

Coimbra, M. A., Delgadillo, I., Waldron, K. W., and Selvendran, R. R. (1996). Isolation and Analysis of Cell Wall Polymers from Olive Pulp. **In:** Plant Cell Wall Analysis, pp. 19-44. Linskens, H., and Jackson, J. (Eds.), Springer Berlin Heidelberg.

Coimbra, M. A., Waldron, K. W., and Selvendran, R. R. (1994). Isolation and characterisation of cell wall polymers from olive pulp (Olea europaea L.). *Carbohydrate Research*. **252**: 245-262.

Criado, M., Morelló, J., Motilva, M., and Romero, M. (2004). Effect of growing area on pigment and phenolic fractions of virgin olive oils of the arbequina variety in Spain. *Journal of the American Oil Chemists' Society*. **81**: 633-640.

Cruz, S., Yousfi, K., Pérez, A. G., Mariscal, C., and Garcia, J. M. (2007). Salt improves physical extraction of olive oil *European Food Research and Technology*. **225**: 359-365.

De Faveri, D., Aliakbarian, B., Avogadro, M., Perego, P., and Converti, A. (2008). Improvement of olive oil phenolics content by means of enzyme formulations: Effect of different enzyme activities and levels. *Biochemical Engineering Journal*. **41**: 149-156.

De Stefano, G., Piacquadio, P., Servili, M., Di Giovacchino, L., and Sciancalepore, V. (1999). Effect of extraction systems on the phenolic composition of virgin olive oils. *Lipid/Fett*. **101**: 328-332.

Di Giovacchino, L. (1993). L'impiego dei preparati enzimatici nella estrazione dell'olio dalle olive con i sistemi continui di centrifugazione. I: Risultati di esperienze pluriennali *Rivista Italiana delle Sostanze Grasse*. **70**: 279-287.

Di Giovacchino, L., Sestili, S., and Di Vincenzo, D. (2002). Influence of olive processing on virgin olive oil quality. *European Journal of Lipid Science and Technology*. **104**: 587-601.

Dierkes, G., Bongartz, A., Guth, H., and Hayen, H. (2011). Quality Evaluation of Olive Oil by Statistical Analysis of Multicomponent Stable Isotope Dilution Assay Data of Aroma Active Compounds. *Journal of Agricultural and Food Chemistry*. **60**: 394-401.

Domingues, A., Peres, M. F., Ferreira, A., Vitorino, M. C., Gouveia-Martins, C., and Madeira, C. (2009). A moenda da azeitona e as características fisico-químicas dos azeites virgens *Actas Portuguesas de Horticultura*: 198-202.

Duarte-Costa, H. M. P. d.-L., and Sameiro, M. E. M. d.-M. S. (1978). Quality of olive oil extracted using enzyme preparations. *Boletim Instituto do Azeite e Produtos Oleaginosos*. **6**: 25-38.

Ebrahimzadeh, H., Motamed, N., Rastgar-Jazii, F., Montasser-Kouhsari, S., and Shokraii, E. H. (2003). Oxidative enzyme activities and soluble protein content in leaves and fruits of olives during ripening. *Journal of Food Biochemistry*. **27**: 181-196.

EC (2012). Economic analysis of the olive sector. European Commission, Directorate-General for Agriculture and Rural Development, http://ec.europa.eu/agriculture/olive-oil/economic-analysis_en.pdf.

El Riachy, M., Priego-Capote, F., Rallo, L., Luque-de Castro, M. D., and León, L. (2013). Phenolic composition of virgin olive oils in cultivars for narrow hedgerow olive orchards. *European Journal of Lipid Science and Technology*. **115**: 800-810.

Esen, A. (2003). b-glucosidase. **In:** Handbook of Food Enzymology. J. R. Whitaker, A., G. J. Voragen, D. W.S. Wong (Ed.), Marcel Dekker, Inc., New York.

Espínola, F., Moya, M., Fernández, D. G., and Castro, E. (2009). Improved extraction of virgin olive oil using calcium carbonate as coadjuvant extractant. *Journal of Food Engineering*. **92**: 112-118.

Espínola, F., Moya, M., Fernández, D. G., and Castro, E. (2011). Modelling of virgin olive oil extraction using response surface methodology. *International Journal of Food Science & Technology*. **46**: 2576-2583.

Esti, M., Cinquanta, L., and La Notte, E. (1998). Phenolic Compounds in Different Olive Varieties. *Journal of Agricultural and Food Chemistry*. **46**: 32-35.

Fadiloğlu, S., and Söylemez, Z. (1997). Kinetics of lipase-catalyzed hydrolysis of olive oil. *Food Research International*. **30**: 171-175.

Fakas, S., Kefalogianni, I., Makri, A., Tsoumpeli, G., Rouni, G., Gardeli, C., Papanikolaou, S., and Aggelis, G. (2010). Characterization of olive fruit microflora and its effect on olive oil volatile compounds biogenesis. *European Journal of Lipid Science and Technology*. **112**: 1024-1032.

Fernández-Bolaños, J., Heredia, A., Vioque, B., Castellano, J. M., and Guillén, R. (1997). Changes in cell-wall-degrading enzyme activities in stored olives in relation to respiration and ethylene production *Journal Zeitschrift für Lebensmitteluntersuchung und - Forschung A*. **204**: 1431-4630

Fernández-Bolaños, J., Rodríguez, R., Guillén, R., Jiménez, A., and Heredia, A. (1995). Activity of cell wall-associated enzymes in ripening olive fruit. *Physiologia Plantarum*. **93**: 651-658.

Fregapane, G., Lavelli, V., León, S., Kapuralin, J., and Salvador, M. D. (2006). Effect of filtration on virgin olive oil stability during storage. *European Journal of Lipid Science and Technology*. **108**: 134-142.

Fregapane, G., and Salvador, M. D. (2013). Production of superior quality extra virgin olive oil modulating the content and profile of its minor components. *Food Research International*. **54**: 1907-1914.

García, A., Brenes, M., Martínez, F., Alba, J., García, P., and Garrido, A. (2001a). High-performance liquid chromatography evaluation of phenols in virgin olive oil during extraction at laboratory and industrial scale. *Journal of the American Oil Chemists' Society*. **78**: 625-629.

García, A., Brenes, M., Moyano, M. J., Alba, J., García, P., and Garrido, A. (2001b). Improvement of phenolic compound content in virgin olive oils by using enzymes during malaxation *Journal of Food Engineering*. **48**: 189-194.

Garcia, J. M., Yousfi, K., Mateos, R., Olmo, M., and Cert, A. (2001). Reduction of oil bitterness by heating of olive (Olea europaea) fruits. *Journal of Agricultural and Food Chemistry*. **49**: 4231-4235.

García-González, D. L., and Aparicio, R. (2002). Detection of Vinegary Defect in Virgin Olive Oils by Metal Oxide Sensors. *Journal of Agricultural and Food Chemistry*. **50**: 1809-1814.

García-González, D. L., and Aparicio, R. (2010). Research in Olive Oil: Challenges for the Near Future. *Journal of Agricultural and Food Chemistry*. **58**: 12569-12577.

García-González, D. L., Tena, N., and Aparicio, R. (2007). Characterization of olive paste volatiles to predict the sensory quality of virgin olive oil. *European Journal of Lipid Science and Technology*. **109**: 663-672.

García-Rodríguez, R., Romero-Segura, C., Sanz, C., and Pérez, A. G. (2015). Modulating oxidoreductase activity modifies the phenolic content of virgin olive oil. *Food Chemistry*. **171**: 364-369.

García-Rodríguez, R., Romero-Segura, C., Sanz, C., Sánchez-Ortiz, A., and Pérez, A. G. (2011). Role of polyphenol oxidase and peroxidase in shaping the phenolic profile of virgin olive oil. *Food Research International*. **44**: 629-635.

Georgalaki, M., Sotiroudis, T., and Xenakis, A. (1998). The presence of oxidizing enzyme activities in virgin olive oil. *Journal of the American Oil Chemists' Society*. **75**: 155-159.

Gimeno, E., Castellote, A. I., Lamuela-Raventós, R. M., De la Torre, M. C., and López-Sabater, M. C. (2002). The effects of harvest and extraction methods on the antioxidant content (phenolics, α-tocopherol, and β-carotene) in virgin olive oil. *Food Chemistry*. **78**: 207-211.

Giuffrè, A. M. (2014). Wax Ester Variation in Olive Oils Produced in Calabria (Southern Italy) During Olive Ripening. *Journal of the American Oil Chemists' Society*: 1-12.

Gómez-Rico, A., Salvador, M. D., Moriana, A., Pérez, D., Olmedilla, N., Ribas, F., and Fregapane, G. (2007). Influence of different irrigation strategies in a traditional Cornicabra cv. olive orchard on virgin olive oil composition and quality. *Food Chemistry*. **100**: 568-578.

Goupy, P., Fleuriet, A., Amiot, M. J., and Macheix, J. J. (1991). Enzymic browning, oleuropein content, and diphenol oxidase activity in olive cultivars (Olea europaea L.). *Journal of Agricultural and Food Chemistry*. **39**: 92-95.

Hadj-Taieb, N., Ayadi, M., Trigui, S., Bouabdallah, F., and Gargouri, A. (2002). Hyperproduction of pectinase activities by a fully constitutive mutant (CT1) of Penicillium occitanis. *Enzyme and Microbial Technology*. **30**: 662-666.

Hatanaka, A. (1996). The fresh green odor emmited by plants. *Food Rev. Int.* **12**: 303-350.

Hbaieb, R. H., Kotti, F., García-Rodríguez, R., Gargouri, M., Sanz, C., and Pérez, A. G. (2015). Monitoring endogenous enzymes during olive fruit ripening and storage: Correlation with virgin olive oil phenolic profiles. *Food Chemistry*. **174**: 240-247.

Heredia, A., Guillén, R., Jiménez, A., and Fernández-Bolaños, J. (1993). Activity of glycosidases during development and ripening of olive fruit *Journal Zeitschrift für Lebensmitteluntersuchung und -Forschung A*. **196**: 147-151.

Iconomou, D., Arapoglou, D., and Israilides, C. (2010). Improvement of phenolic antioxidants and quality characteristics of virgin olive oil with the addition of enzymes and nitrogen during olive paste processing. *Grasas Y Aceites*. **61**: 303-311.

Inarejos-García, A. M., Gómez-Rico, A., Salvador, M. D., and Fregapane, G. (2009). Influence of malaxation conditions on virgin olive oil yield, overall quality and composition. *European Food Research and Technology*. **228**: 671-677.

IOC (2006). Quality Management Guide for the Olive Oil Industry: Mills.

IOC (2014). World Olive Oil Figures. http://www.internationaloliveoil.org/estaticos/view/131-world-olive-oil-figures.

IUBMB (2015). Recommendations on Biochemical & Organic Nomenclature, Symbols & Terminology. http://www.chem.qmul.ac.uk/iubmb/.

Jimenez, A., Rodriguez, R., Fernandez-Caro, I., Guillen, R., Fernandez-Bolanos, J., and Heredia, A. (2001). Olive Fruit Cell Wall: Degradation of Pectic Polysaccharides during Ripening. *Journal of Agricultural and Food Chemistry*. **49**: 409-415.

Kalogeropoulos, N., Kaliora, A. C., Artemiou, A., and Giogios, I. (2014). Composition, volatile profiles and functional properties of virgin olive oils produced by two-phase vs three-phase centrifugal decanters. *LWT - Food Science and Technology*. **58**: 272-279.

Kalua, C. M., Bedgood, D. R., Bishop, A. G., and Prenzler, P. D. (2006). Changes in Volatile and Phenolic Compounds with Malaxation Time and Temperature during Virgin Olive Oil Production. *Journal of Agricultural and Food Chemistry*. **54**: 7641-7651.

Kalua, C. M., Bedgood, D. R., Bishop, A. G., and Prenzler, P. D. (2008). Changes in Virgin Olive Oil Quality during Low-Temperature Fruit Storage. *Journal of Agricultural and Food Chemistry*. **56**: 2415-2422.

Kashyap, D. R., Vohra, P. K., Chopra, S., and Tewari, R. (2001). Applications of pectinases in the commercial sector: a review. *Bioresource Technology*. **77**: 215-227.

Kochevenko, A., Araújo, W. L., Maloney, G. S., Tieman, D. M., Do, P. T., Taylor, M. G., Klee, H. J., and Fernie, A. R. (2012). Catabolism of branched chain amino acids supports respiration but not volatile synthesis in tomato fruits. *Molecular plant*. **5**: 366-375.

Koidis, A., and Boskou, D. (2006). The contents of proteins and phospholipids in cloudy (veiled) virgin olive oils. *European Journal of Lipid Science and Technology*. **108**: 323-328.

Laane, C., Bruggeman, Y., and Winkel, C. (2003). Applications of oxireductases in foods. **In:** Handbook of food enzymology. J. R., W., A. G. J., Voragen and D. W. S., Wong (Ed.), Marcel Dekker, Inc., New York.

Lavelli, V., and Bondesan, L. (2005). Secoiridoids, Tocopherols, and Antioxidant Activity of Monovarietal Extra Virgin Olive Oils Extracted from Destoned Fruits. *Journal of Agricultural and Food Chemistry*. **53**: 1102-1107.

Lercker, G., Frega, N., Bocci, F., and Servidio, G. (1994). "Veiled" extra-virgin olive oils: Dispersion response related to oil quality. *Journal of the American Oil Chemists' Society*. **71**: 657-658.

López- Villalta, L. C. (2008). Obtención del aceite de oliva virgen, 3th Edition. Editorial Agricola Española, S.A., Madrid.

Lorenzi, V., Maury, J., Casanova, J., and Berti, L. (2006). Purification, product characterization and kinetic properties of lipoxygenase from olive fruit (Olea europaea L.). *Plant Physiology and Biochemistry*. **44**: 450-454.

Lozano-Sánchez, J., Cerretani, L., Bendini, A., Segura-Carretero, A., and Fernández-Gutiérrez, A. (2010). Filtration process of extra virgin olive oil: effect on minor components, oxidative stability and sensorial and physicochemical characteristics. *Trends in Food Science & Technology*. **21**: 201-211.

Luaces, P., Perez, A. G., and Sanz, C. (2003). Role of Olive Seed in the Biogenesis of Virgin Olive Oil Aroma. *J. Agric. Food Chem.* **51**: 4741-4745.

Luaces, P., Romero, C., Gutierrez, F., Sanz, C., and Perez, A. G. (2007a). Contribution of olive seed to the phenolic profile and related quality parameters of virgin olive oil. *Journal of the science of food and agriculture*. **87**: 2721-2727.

Luaces, P., Sanz, C., and Pérez, A. G. (2007b). Thermal Stability of Lipoxygenase and Hydroperoxide Lyase from Olive Fruit and Repercussion on Olive Oil Aroma Biosynthesis. *Journal of Agricultural and Food Chemistry*. **55**: 6309-6313.

Luna, G., Morales, M. T., and Aparicio, R. (2006). Characterisation of 39 varietal virgin olive oils by their volatile compositions. *Food Chemistry*. **98**: 243-252.

Malheiro, R., Casal, S., Teixeira, H., Bento, A., and Pereira, J. A. (2013). Effect of Olive Leaves Addition during the Extraction Process of Overmature Fruits on Olive Oil Quality. *Food and Bioprocess Technology*. **6**: 509-521.

Martins, L. L., Reis, R., Moreira, I., Pinto, F., Sales, J., and Mourato, M. (2013). Antioxidative response of plants to oxidative stress induced by cadmium. **In:** Cadmium:

Characteristics, Sources of Exposure, Health and Environmental Effects. Fujita, M. H. M. (Ed.), Nova Science Publishers, Inc.
Masella, P., Parenti, A., Spugnoli, P., and Calamai, L. (2009). Influence of Vertical Centrifugation on Extra Virgin Olive Oil Quality. *Journal of the American Oil Chemists' Society*. **86**: 1137-1140.
Masella, P., Parenti, A., Spugnoli, P., and Calamai, L. (2010). Nitrogen stripping to remove dissolved oxygen from extra virgin olive oil. *European Journal of Lipid Science and Technology*. **112**: 1389-1392.
Masella, P., Parenti, A., Spugnoli, P., and Calamai, L. (2012). Vertical centrifugation of virgin olive oil under inert gas. *European Journal of Lipid Science and Technology*. **114**: 1094-1096.
Mayer, A. M. (1986). Polyphenol oxidases in plants-recent progress. *Phytochemistry*. **26**: 11-20.
Mazzuca, S., Spadafora, A., and Innocenti, A. M. (2006). Cell and tissue localization of [beta]-glucosidase during the ripening of olive fruit (Olea europaea) by in situ activity assay. *Plant Science*. **171**: 726-733.
Mínguez-Mosquera, I., Gallardo-Guerrero, L., and Roca, M. (2002). Pectinesterase and polygalacturonase in changes of pectic matter in olives (cv. Hojiblanca) intended for milling. *Journal of the American Oil Chemists' Society*. **79**: 93-99.
Minic, Z. (2008). Physiological roles of plant glycoside hydrolases. *Planta*. **227**: 723-740.
Minic, Z., and Jouanin, L. (2006). Plant glycoside hydrolases involved in cell wall polysaccharide degradation. *Plant Physiology and Biochemistry*. **44**: 435-449.
Morales, M., and Aparicio, R. (1999). Effect of extraction conditions on sensory quality of virgin olive oil. *Journal of the American Oil Chemists' Society*. **76**: 295-300.
Morales, M. T., Alonso, M. V., Rios, J. J., and Aparicio, R. (1995). Virgin Olive Oil Aroma: Relationship between Volatile Compounds and Sensory Attributes by Chemometrics. *Journal of Agricultural and Food Chemistry*. **43**: 2925-2931.
Morales, M. T., Angerosa, F., and Aparicio, R. (1999). Effect of the extraction conditions of virgin olive oil on the lipoxygenase cascade: Chemical and sensory implications. *Grasas Y Aceites*. **50**: 114-121.
Morales, M. T., Luna, G., and Aparicio, R. (2005). Comparative study of virgin olive oil sensory defects. *Food Chemistry*. **91**: 293-301.
Morales, M. T., Rios, J. J., and Aparicio, R. (1997). Changes in the Volatile Composition of Virgin Olive Oil during Oxidation: Flavors and Off-Flavors. *Journal of Agricultural and Food Chemistry*. **45**: 2666-2673.
Morelló, J.-R., Motilva, M.-J., Tovar, M.-J., and Romero, M.-P. (2004). Changes in commercial virgin olive oil (cv Arbequina) during storage, with special emphasis on the phenolic fraction. *Food Chemistry*. **85**: 357-364.
Morelló, J. R., Romero, M. P., and Motilva, M. J. (2006). Influence of seasonal conditions on the composition and quality parameters of monovarietal virgin olive oils. *Journal of the American Oil Chemists' Society*. **83**: 683-690.
Mourato, M., Reis, R., and Martins, L. L. (2012). Characterization of plant antioxidative system in response to abiotic stresses: a focus on heavy metal toxicity. **In:** Advances in Selected Plant Physiology Aspects, p. 388. Dichio, G. M. a. B. (Ed.), InTech.
Moya, M., Espínola, F., Fernández, D. G., Torres, A., Marcos, J., Vilar, J., Josue, J., Sánchez, T., and Castro, E. (2010). Industrial trials on coadjuvants for olive oil extraction. *Journal of Food Engineering*. **97**: 57-63.
Mulinacci, N., Giaccherini, C., Innocenti, M., Romani, A., Vincieri, F. F., Marotta, F., and Mattei, A. (2005). Analysis of extra virgin olive oils from stoned olives. *Journal of the science of food and agriculture*. **85**: 662-670.
Najafian, L., Ghodsvali, A., Haddad Khodaparast, M. H., and Diosady, L. L. (2009). Aqueous extraction of virgin olive oil using industrial enzymes. *Food Research International*. **42**: 171-175.
Obied, H. K., Prenzler, P. D., Ryan, D., Servili, M., Taticchi, A., Esposto, S., and Robards, K. (2008). Biosynthesis and biotransformations of phenol-conjugated oleosidic secoiridoids from Olea europaea L. *Natural Product Reports*. **25**: 1167-1179.
Olias, J. M., Perez, A. G., Rios, J. J., and Sanz, L. C. (1993). Aroma of virgin olive oil: Biogenesis of the "green" odor notes. *Journal of Agricultural and Food Chemistry*. **41**: 2368 - 2373.
Ortega-Garcia, F., Blanco, S., Peinado, M. A., and Peragon, J. (2008). Polyphenol oxidase and its relationship with oleuropein concentration in fruits and leaves of olive (Olea europaea) cv. 'Picual' trees during fruit ripening. *Tree Physiol*. **28**: 45-54.

Ortega-García, F., and Peragón, J. (2010). HPLC analysis of oleuropein, hydroxytyrosol, and tyrosol in stems and roots of Olea europaea L. cv. Picual during ripening. *Journal of the science of food and agriculture*. **90**: 2295-2300.

Padilla, M. N., Hernández, M. L., Sanz, C., and Martínez-Rivas, J. M. (2009). Functional Characterization of Two 13-Lipoxygenase Genes from Olive Fruit in Relation to the Biosynthesis of Volatile Compounds of Virgin Olive Oil. *Journal of Agricultural and Food Chemistry*. **57**: 9097-9107.

Panzanaro, S., Nutricati, E., Miceli, A., and De Bellis, L. (2010). Biochemical characterization of a lipase from olive fruit (Olea europaea L.). *Plant Physiology and Biochemistry*. **48**: 741-745.

Parenti, A., Spugnoli, P., Masella, P., and Calamai, L. (2007). Influence of the extraction process on dissolved oxygen in olive oil. *European Journal of Lipid Science and Technology*. **109**: 1180-1185.

Parenti, A., Spugnoli, P., Masella, P., and Calamai, L. (2008). The effect of malaxation temperature on the virgin olive oil phenolic profile under laboratory-scale conditions. *European Journal of Lipid Science and Technology*. **110**: 735-741.

Parenti, A., Spugnoli, P., Masella, P., Calamai, L., and Pantani, O. L. (2006). Improving olive oil quality using CO2 evolved from olive pastes during processing. *European Journal of Lipid Science and Technology*. **108**: 904-912.

Pastore, G., D'Aloise, A., Lucchetti, S., Maldini, M., Moneta, E., Peparaio, M., Raffo, A., and Sinesio, F. (2014). Effect of oxygen reduction during malaxation on the quality of extra virgin olive oil (Cv. Carboncella) extracted through “two-phase” and “three-phase” centrifugal decanters. *LWT - Food Science and Technology*. **59**: 163-172.

Pereira, J. A., Casal, S., Bento, A., and Oliveira, M. B. P. P. (2002). Influence of Olive Storage Period on Oil Quality of Three Portuguese Cultivars of Olea europea, Cobrançosa, Madural, and Verdeal Transmontana. *Journal of Agricultural and Food Chemistry*. **50**: 6335-6340.

Peres, F., Jeleń, H. H., Majcher, M. M., Arraias, M., Martins, L. L., and Ferreira-Dias, S. (2013a). Characterization of aroma compounds in Portuguese extra virgin olive oils from Galega Vulgar and Cobrançosa cultivars using GC–O and GCxGC–ToFMS. *Food Research International*. **54**: 1979-1986.

Peres, F., Martins, L. L., and Ferreira-Dias, S. (2014). Laboratory-scale optimization of olive oil extraction: Simultaneous addition of enzymes and microtalc improves the yield. *European Journal of Lipid Science and Technology*. **116**: 1054-1062.

Peres, F., Martins, L. L., Mourato, M., and Ferreira-Dias, S. (2011). Changes in virgin olive oil antioxidants, polyphenol oxidase and peroxidase activities during fruit ripening. **In:** 4th International Conference Olivebioteq 2011, Chania, pp. 515-520.

Peres, F., Martins, L. L., Mourato, M., and Ferreira-Dias, S. (2013b). The role of polyphenol oxidases and peroxidases from olive fruits on the composition of virgin oil. **In:** EUROFOODCHEM XVII, Istanbul, p. 722.

Perez, A. G., Leon, L., Pascual, M., Romero-Segura, C., Sanchez-Ortiz, A., de la Rosa, R., and Sanz, C. (2014). Variability of Virgin Olive Oil Phenolic Compounds in a Segregating Progeny from a Single Cross in Olea europaea L. and Sensory and Nutritional Quality Implications. *Plos One*. **9**.

Pérez, A. G., Luaces, P., Ríos, J. J., García, J. M., and Sanz, C. (2003). Modification of Volatile Compound Profile of Virgin Olive Oil Due to Hot-Water Treatment of Olive Fruit. *Journal of Agricultural and Food Chemistry*. **51**: 6544-6549.

Pérez, A. G., Romero, C., Yousfi, K., and García, J. (2008). Modulation of Olive Oil Quality Using NaCl as Extraction Coadjuvant. *Journal of the American Oil Chemists' Society*. **85**: 685-691.

Perez-Camino, M. C., Moreda, W., and Cert, A. (2001). Effects of olive fruit quality and oil storage practices on the diacylglycerol content of virgin olive oils. *Journal of Agricultural and Food Chemistry*. **49**: 699-704.

Peri, C. (2014). Quality Excellence in Extra Virgin Olive Oils. **In:** Olive Oil Sensory Science, pp. 1-32. E. Monteleone and S. Langstaff (Eds.), John Wiley & Sons, Ltd.

Petrakis, C. (2006). Olive Oil Extraction. **In:** Olive Oil Chemistry & Technology. Boskou, D. (Ed.), AOCS Press, Champaign, Illinois.

Pita, D., Vitorino, M. C., Gouveia, C., and Peres, F. (2011). Aplicação de Microtalco Natural na Extracção de Azeites Monovarietais *Actas Portuguesas de Horticultura*: 151-155.

Prasanna, V., Prabha, T. N., and Tharanathan, R. N. (2007). Fruit Ripening Phenomena–An Overview. *Critical Reviews in Food Science and Nutrition*. **47**: 1-19.
Prenzler, P., Robards, K., and Bedgood, D. (2007). Quality Enhancement of Australian Extra Virgin Olive Oils. p. 116. Rural Industries Research and Development Corporation.
Psomiadou, E., and Tsimidou, M. (2002). Stability of Virgin Olive Oil. 2. Photo-oxidation Studies. *Journal of Agricultural and Food Chemistry*. **50**: 722-727.
Ramírez, E. C., Whitaker, J. R., and Virador, V. M. (2003). Polyphenol Oxidase. **In:** Handbook of Food Enzymology J. R., W., A. G. J., Voragen and D. W. S., Wong (Ed.), Marcel Dekker, Inc., New York.
Ranalli, A., Contento, S., Schiavone, C., and Simone, N. (2001a). Malaxing temperature affects volatile and phenol composition as well as other analytical features of virgin olive oil. *European Journal of Lipid Science and Technology*. **103**: 228-238.
Ranalli, A., and De Mattia, G. (1997). Characterization of olive oil produced with a new enzyme processing aid. *Journal of the American Oil Chemists' Society*. **74**: 1105 - 1113.
Ranalli, A., De Mattia, G., and Ferrante, M. L. (1998). The characteristics of percolation olive oils produced with a new processing enzyme aid. *International Journal of Food Science & Technology*. **33**: 247-258.
Ranalli, A., and Ferrante, M. L. (1996). Las características fisicoquímicas y analíticas delos aceites virgenes extraídos mediante un auxiliar enzimático pectolítico. *Olivae*: 27-32.
Ranalli, A., Gomes, T., Delcuratolo, D., Contento, S., and Lucera, L. (2003a). Improving Virgin Olive Oil Quality by Means of Innovative Extracting Biotechnologies *J. Agric. Food Chem.* **51**: 2597 -2602.
Ranalli, A., Lucera, L., Contento, S., Simone, N., and Del Re, P. (2004). Bioactive constituents, flavors and aromas of virgin oils obtained by processing olives with a natural enzyme extract. *European Journal of Lipid Science and Technology*. **106**: 187 - 197.
Ranalli, A., Malfatti, A., and Cabras, P. (2001b). Composition and Quality of Pressed Virgin Olive Oils Extracted with a New Enzyme Processing Aid. *Journal of Food Science*. **66**: 592-603.
Ranalli, A., Malfatti, A., Lucera, L., Contento, S., and Sotiriou, E. (2005). Effects of processing techniques on the natural colourings and the other functional constituents in virgin olive oil. *Food Research International*: 873-878.
Ranalli, A., and Martinelli, N. (1995). Integral centrifuges for olive oil extraction, at the third millenium threshold. Transformation yields. *Grasas Y Aceites*. **46,**: 255-263.
Ranalli, A., Pollastri, L., Contento, S., and Iannucci, E. (2003b). The glyceridic and nonglyceridic constituents of virgin olive oil after use of a novel method of enzyme extraction. *International Journal of Food Science & Technology*. **38**: 17-27.
Ranalli, A., Pollastri, L., Contento, S., Iannucci, E., and Lucera, L. (2003c). Effect of olive paste kneading process time on the overall quality of virgin olive oil. *European Journal of Lipid Science and Technology*. **105**: 57 - 67.
Ranalli, A., Sgaramella, A., and Surricchio, G. (1999). The new "Cytolase 0" enzyme processing aid improves quality and yields of virgin olive oil *Food Chemistry*. **66**: 443-454.
Reboredo-Rodríguez, P., González-Barreiro, C., Cancho-Grande, B., and Simal-Gándara, J. (2013). Effects of Sedimentation Plus Racking Process in the Extra Virgin Olive Oil Aroma Fingerprint Obtained by DHS–TD/GC–MS. *Food and Bioprocess Technology*. **6**: 1290-1301.
Reboredo-Rodríguez, P., González-Barreiro, C., Cancho-Grande, B., and Simal-Gándara, J. (2014). Improvements in the malaxation process to enhance the aroma quality of extra virgin olive oils. *Food Chemistry*. **158**: 534-545.
Ridolfi, M., Terenziani, S., Patumi, M., and Fontanazza, G. (2002). Characterization of the Lipoxygenases in Some Olive Cultivars and Determination of Their Role in Volatile Compounds Formation. *Journal of Agricultural and Food Chemistry*. **50**: 835-839.
Rodis, P. S., Karathanos, V. T., and Mantzavinou, A. (2002). Partitioning of Olive Oil Antioxidants between Oil and Water Phases. *Journal of Agricultural and Food Chemistry*. **50**: 596-601.
Rojnić, I. D., Bažok, R., and Barčić, J. I. (2015). Reduction of olive fruit fly damage by early harvesting and impact on oil quality parameters. *European Journal of Lipid Science and Technology*. **117**: 103-111.
Romero-Segura, C., García-Rodríguez, R., Sánchez-Ortiz, A., Sanz, C., and Pérez, A. G. (2012). The role of olive β-glucosidase in shaping the phenolic profile of virgin olive oil. *Food Research International*. **45**: 191-196.

Romero-Segura, C., Sanz, C., and Perez, A. G. (2009). Purification and Characterization of an Olive Fruit β-Glucosidase Involved in the Biosynthesis of Virgin Olive Oil Phenolics. *Journal of Agricultural and Food Chemistry*. **57**: 7983-7988.

Runcio, A., Sorgonà, L., Mincione, A., Santacaterina, S., and Poiana, M. (2008). Volatile compounds of virgin olive oil obtained from Italian cultivars grown in Calabria. Effect of processing methods, cultivar, stone removal, and antracnose attack *Food Chemistry*. **106**: 735-740.

Ryan, D., Robards, K., and Lavee, S. (1999). Changes in phenolic content of olive during maturation. *International Journal of Food Science & Technology*. **34**: 265-274.

Salas, J., Willams, M., Harwood, J., and Sánchez, J. (1999). Lipoxygenase activity in olive (Olea europaea) fruit. *Journal of the American Oil Chemists' Society*. **76**: 1163-1168.

Salas, J. J. (2004). Characterization of Alcohol Acyltransferase from Olive Fruit. *Journal of Agricultural and Food Chemistry*. **52**: 3155-3158.

Salas, J. J., Sanchez, C., Garcia-Gonzalez, D. L., and Aparicio, R. (2005). Impact of the Suppression of Lipoxygenase and Hydroperoxide Lyase on the Quality of the Green Odor in Green Leaves. *Journal of Agricultural and Food Chemistry*. **53**: 1648-1655.

Salas, J. J., and Sánchez, J. (1999). Hydroperoxide lyase from olive (Olea europaea) fruits. *Plant Science*. **143**: 19-26.

Salas, J. J., Sánchez, J., Ramli, U. S., Manaf, A. M., Williams, M., and Harwood, J. L. (2000). Biochemistry of lipid metabolism in olive and other oil fruits. *Progress in Lipid Research*. **39**: 151-180.

Sánchez, J., and Harwood, J. L. (2002). Biosynthesis of triacylglycerols and volatiles in olives *European Journal of Lipid Science and Technology*. **104**: 564 - 573.

Sánchez-Ortiz, A., Pérez, A. G., and Sanz, C. (2007). Cultivar Differences on Nonesterified Polyunsaturated Fatty Acid as a Limiting Factor for the Biogenesis of Virgin Olive Oil Aroma. *Journal of Agricultural and Food Chemistry*. **55**: 7869-7873.

Sánchez-Ortiz, A., Pérez, A. G., and Sanz, C. (2013). Synthesis of aroma compounds of virgin olive oil: Significance of the cleavage of polyunsaturated fatty acid hydroperoxides during the oil extraction process. *Food Research International*. **54**: 1972-1978.

Sánchez-Ortiz, A., Romero-Segura, C., Sanz, C., and Pérez, A. G. (2011). Synthesis of Volatile Compounds of Virgin Olive Oil Is Limited by the Lipoxygenase Activity Load during the Oil Extraction Process. *Journal of Agricultural and Food Chemistry*. **60**: 812-822.

Saraiva, J. A., Nunes, C. S., and Coimbra, M. A. (2007). Purification and characterization of olive (Olea europaea L.) peroxidase – Evidence for the occurrence of a pectin binding peroxidase *Food Chemistry*. **101**: 1571-1579.

Sciancalepore, V. (1985). Enzymatic Browning in Five Olive Varieties. *Journal of Food Science*. **50**: 1194-1195.

Segovia-Bravo, K. A., Jarén-Galán, M., García-García, P., and Garrido-Fernández, A. (2009). Browning reactions in olives: Mechanism and polyphenols involved. *Food Chemistry*. **114**: 1380-1385.

Selvaggini, R., Esposto, S., Taticchi, A., Urbani, S., Veneziani, G., Di Maio, I., Sordini, B., and Servili, M. (2014). Optimization of the Temperature and Oxygen Concentration Conditions in the Malaxation during the Oil Mechanical Extraction Process of Four Italian Olive Cultivars. *Journal of Agricultural and Food Chemistry*. **62**: 3813-3822.

Servili, M. (2014). The phenolic compounds: a commercial argument in the economic war to come on the quality of olive oil? *OCL*. **21**: D509.

Servili, M., Baldioli, M., Begliomini, A. L., Selvaggini, R., and Montedoro, G. F. (2000). The phenolic and volatile compounds of virgin olive oil:relationships with the endogenous oxidorreductases during the mechanical oil extraction process. **In:** Flavour and Fragrance Chemistry, pp. 163-173. Lanzotti, V., and Taglialatela-Scafati, O. (Eds.), Kluwer Academic Publishers, Dordrecht.

Servili, M., Begliomini, A. L., Montedoro, G. F., Petruccioli, M., and Federici, F. (1992). Utilisation of a yeast pectinase in olive oil extraction and red wine making processes. *Journal of the science of food and agriculture*. **58**: 253-260.

Servili, M., Esposto, S., Fabiani, R., Urbani, S., Taticchi, A., Mariucci, F., Selvaggini, R., and Montedoro, G. (2009). Phenolic compounds in olive oil: antioxidant, health and organoleptic activities according to their chemical structure. *Inflammopharmacology*. **17**: 76-84.

Servili, M., Piacquadio, P., De Stefano, G., Taticchi, A., and Sciancalepore, V. (2002a). Influence of a new crushing technique on the composition of the volatile compounds and related

sensory quality of virgin olive oil. *European Journal of Lipid Science and Technology*. **104**: 483-489.

Servili, M., Selvaggini, R., Esposto, S., Taticchia, A., Montedoro, G. F., and Morozzib, G. (2004). Health and sensory properties of virgin olive oil hydrophilic phenols: agronomic and technological aspects of production that affect their occurrence in the oil *Journal of Chromatography A*. **1054**: 113-127.

Servili, M., Selvaggini, R., Taticchi, A., Baldioli, M., and Montedoro, G. F. (2002b). The use of biotechnology means during oil mechanical extraction process: relationship with sensory and nutritional parameters of virgin olive oil. *Acta Horticulturae*: 557-560.

Servili, M., Selvaggini, R., Taticchi, A., Esposto, S., and Montedoro, G. (2003a). Air exposure time of olive pastes during the extraction process and phenolic and volatile composition of virgin olive oil. *Journal of the American Oil Chemists' Society*. **80**: 685-695.

Servili, M., Selvaggini, R., Taticchi, A., Esposto, S., and Montedoro, G. F. (2003b). Volatile Compounds and Phenolic Composition of Virgin Olive Oil: Optimization of Temperature and Time of Exposure of Olive Pastes to Air Contact during the Mechanical Extraction. *Journal of Agricultural and Food Chemistry*. **51**: 7980-7988.

Servili, M., Taticchi, A., Esposto, S., Sordini, B., and Urbani, S. (2012). Technological Aspects of Olive Oil Production. **In:** Olive Germplasm - The Olive Cultivation, Table Olive and Olive Oil Industry in Italy. http://www.intechopen.com/books/olive-germplasm-the- olive-cultivation-table-olive-and-olive-oil-industry-in-italy/technological- aspects-of-olive-oil-production.

Servili, M., Taticchi, A., Esposto, S., Urbani, S., Selvaggini, R., and Montedoro, G. (2007). Effect of Olive Stoning on the Volatile and Phenolic Composition of Virgin Olive Oil. *Journal of Agricultural and Food Chemistry*. **55**: 7028-7035.

Servili, M., Taticchi, A., Esposto, S., Urbani, S., Selvaggini, R., and Montedoro, G. F. (2008). Influence of the Decrease in Oxygen during Malaxation of Olive Paste on the Composition of Volatiles and Phenolic Compounds in Virgin Olive Oil. *Journal of Agricultural and Food Chemistry*. **56**: 10048-10055.

Sonda, A., Akram, Z., Boutheina, G., Guido, F., and Mohamed, B. (2013). Effect of Addition of Olive Leaves before Fruits Extraction Process to Some Monovarietal Tunisian Extra-Virgin Olive Oils Using Chemometric Analysis. *Journal of Agricultural and Food Chemistry*. **62**: 251-263.

Spadafora, A., Mazzuca, S., Chiappetta, F. F., Parise, A., Perri, E., and Innocenti, A. M. (2008). Oleuropein-Specific-[beta]-Glucosidase Activity Marks the Early Response of Olive Fruits (Olea europaea) to Mimed Insect Attack. *Agricultural Sciences in China*. **7**: 703-712.

Stefanoudaki, E., Koutsaftakis, A., and Harwood, J. L. (2011). Influence of malaxation conditions on characteristic qualities of olive oil. *Food Chemistry*. **127**: 1481-1486.

Stefanoudaki, E., Williams, M., and Harwood, J. (2010). Changes in virgin olive oil characteristics during different storage conditions. *European Journal of Lipid Science and Technology*. **112**: 906-914.

Takahama, U., and Oniki, T. (2000). Flavonoids and Some Other Phenolics as Substrates of Peroxidase: Physiological Significance of the Redox Reactions. *Journal of Plant Research*. **113**: 301-309.

Taticchi, A., Esposto, S., and Servili, M. (2014). The Basis of the Sensory Properties of Virgin Olive Oil. **In:** Olive Oil Sensory Science, pp. 33-54. E. Monteleone and S. Langstaff (Eds.), John Wiley & Sons, Ltd.

Taticchi, A., Esposto, S., Veneziani, G., Urbani, S., Selvaggini, R., and Servili, M. (2013). The influence of the malaxation temperature on the activity of polyphenoloxidase and peroxidase and on the phenolic composition of virgin olive oil. *Food Chemistry*. **136**: 975-983.

Tena, N., Lazzez, A., Aparicio-Ruiz, R., and García-González, D. L. (2007). Volatile Compounds Characterizing Tunisian Chemlali and Chétoui Virgin Olive Oils. *Journal of Agricultural and Food Chemistry*. **55**: 7852-7858.

Tomás-Barberán, F. A., and Espín, J. C. (2001). Phenolic compounds and related enzymes as determinants of quality in fruits and vegetables. *Journal of the science of food and agriculture*. **81**: 853-876.

Trichopoulou, A., and Dilis, V. (2007). Olive oil and longevity. *Molecular Nutrition & Food Research*. **51**: 1275-1278.

Tsimidou, M. Z., Georgiou, A., Koidis, A., and Boskou, D. (2005). Loss of stability of "veiled" (cloudy) virgin olive oils in storage. *Food Chemistry*. **93**: 377-383.

Tura, D., Failla, O., Bassi, D., Attilio, C., and Serraiocco, A. (2013). Regional and cultivar comparison of Italian single cultivar olive oils according to flavor profiling. *European Journal of Lipid Science and Technology*. **115**: 196-210.

Tura, D., Failla, O., Bassi, D., Pedò, S., and Serraiocco, A. (2008). Cultivar influence on virgin olive (Olea europea L.) oil flavor based on aromatic compounds and sensorial profile *Scientia Horticulturae*. **118**: 139-148.

Tura, D., Failla, O., Bassi, D., Pedò, S., and Serraiocco, A. (2009). Environmental and seasonal influence on virgin olive (Olea europaea L.) oil volatiles in northern Italy. *Scientia Horticulturae*. **122**: 385-392.

Tzika, E. D., Sotiroudis, T., Papadimitriou, V., and Xenakis, A. (2009). Partial purification and characterization of peroxidase from olives (Olea europaea cv. Koroneiki). *European Food Research and Technology*. **228**: 487-495.

Uceda, M., Jimenez, A., and Beltran, G. (2006). Olive oil extraction and quality. *Grasas Y Aceites*. **57**: 25-31.

Vámos -Vigyázó, L., and Haard, N. F. (1981). Polyphenol oxidases and peroxidases in fruits and vegetables. *Critical Reviews in Food Science and Nutrition*. **15**: 49-127.

Vaughn, K. C., and Duke, S. O. (1981). Tissue localization of polyphenol oxidase in Sorghum. *Protoplasma*. **108**: 319-327.

Veillet, S., Tomao, V., Bornard, I., Ruiz, K., and Chemat, F. (2009). Chemical changes in virgin olive oils as a function of crushing systems: Stone mill and hammer crusher. *Comptes Rendus Chimie*. **12**: 895-904.

Vergara-Domínguez, H., Roca, M., and Gandul-Rojas, B. (2013). Characterisation of chlorophyll oxidation mediated by peroxidative activity in olives (Olea europaea L.) cv. Hojiblanca. *Food Chemistry*. **139**: 786-795.

Vichi, S., Castellote, A. I., Pizzale, L., Conte, L. S., Buxaderas, S., and López-Tamames, E. (2003). Analysis of virgin olive oil volatile compounds by headspace solid-phase microextraction coupled to gas chromatography with mass spectrometric and flame ionization detection. *Journal of Chromatography A*. **983**: 19-33.

Vichi, S., Romero, A., Gallardo-Chacón, J., Tous, J., López-Tamames, E., and Buxaderas, S. (2009a). Volatile phenols in virgin olive oils: Influence of olive variety on their formation during fruits storage. *Food Chemistry*. **116**: 651-656.

Vichi, S., Romero, A., Gallardo-Chacón, J., Tous, J., López-Tamames, E., and Buxaderas, S. (2009b). Influence of Olives' Storage Conditions on the Formation of Volatile Phenols and Their Role in Off-Odor Formation in the Oil. *Journal of Agricultural and Food Chemistry*. **57**: 1449-1455.

Vichi, S., Romero, A., Tous, J., and Caixach, J. (2011). The Activity of Healthy Olive Microbiota during Virgin Olive Oil Extraction Influences Oil Chemical Composition. *Journal of Agricultural and Food Chemistry*. **59**: 4705-4714.

Vichi, S., Romero, A., Tous, J., Tamames, E. L., and Buxaderas, S. (2008). Determination of volatile phenols in virgin olive oils and their sensory significance. *Journal of Chromatography A*. **1211**: 1-7.

Vierhuis, E., Korver, M., Schols, H. A., and Voragen, A. G. J. (2003). Structural characteristics of pectic polysaccharides from olive fruit (Olea europaea cv moraiolo) in relation to processing for oil extraction. *Carbohydrate Polymers*. **51**: 135-148.

Vierhuis, E., Schols, H. A., Beldman, G., and Voragen, A. G. J. (2000). Isolation and characterisation of cell wall material from olive fruit (Olea europaea cv. koroneiki) at different ripening stages. *Carbohydrate Polymers*. **43**: 11-12.

Vierhuis, E., Servili, M., Baldioli, M., Schols, H. A., Voragen, A. G. J., and Montedoro, G. F. (2001). Effect of Enzyme Treatment during Mechanical Extraction of Olive Oil on Phenolic Compounds and Polysaccharides *J. Agric. Food Chem.* **49** 1218 -1223.

Vinha, A. F., Ferreres, F., Silva, B. M., Valentão, P., Gonçalves, A., Pereira, J. A., Oliveira, M. B. P., Seabra, R. M., and Andrade, P. B. (2005). Phenolic profiles of Portuguese olive fruits (Olea europaea L.): Influences of cultivar and geographical origin. *Food Chemistry*. **89**: 561-568.

Whitaker, J. R. (1995). Polyphenol Oxidase. **In:** Food Enzymes, pp. 271-307. Springer US.

Whitaker, J. R. (2003). What enzymes do and why they are highly specific and efficient catalysts. **In:** Handbook of food enzymology. J. R., W., A. G. J., Voragen and D. W. S., Wong (Ed.), Marcel Dekker, Inc., New York.

Williams, M., and Harwood, J. L. (2000). Characterization of lipoxygenase isoforms in olive callus cultures. *Biochem. Soc. Trans.* **28**: 830-831.

Yoruk, R., and Marshal, R. (2003). Physicochemical properties and function of plant polyphenol oxidase: a review. *J. Food Biochem*. **27**: 361-422.

Yorulmaz, A., Tekin, A., and Turan, S. (2011). Improving olive oil quality with double protection: Destoning and malaxation in nitrogen atmosphere. *European Journal of Lipid Science and Technology*. **113**: 637-643.

Yuan, Z. Y., and Jiang, T. J. (2003). Horseradish Peroxidase. **In:** Handbook of Food Enzymology. J. R., W., A. G. J., Voragen and D. W. S., Wong (Ed.), Marcel Dekker, Inc. , New York

Zamora, R., Alaiz, M., and Hidalgo, F. J. (2002). Influence of cultivar and fruit ripening on olive (*Olea europaea*) fruit protein cotent, composition and antioxidant activity. *Journal of Agricultural and Food Chemistry*. **49**: 4267-4270.

Zullo, B. A., Di Stefano, M. G., Cioccia, G., and Ciafardini, G. (2014). Evaluation of polyphenol decay in the oily fraction of olive fruit during storage using a mild sample handling method. *European Journal of Lipid Science and Technology*. **116**: 160-168.

Chapter 3

Laboratory-scale optimization of olive oil extraction: Simultaneous addition of enzymes and microtalc improves the yield

Peres, F., Martins, L.L. & Ferreira-Dias, S. (2014). European Journal of Lipid Science and Technology, 116, 1054-1062.

Summary

The aim of this work was to optimize olive oil extraction, at laboratory-scale (Abencor system), from fruits of 'Cobrançosa' and 'Galega Vulgar' Portuguese cultivars, either in the absence or in the presence of enzymes and natural microtalc. Using a Placket-Burman design to select significant variables followed by a central composite rotatable design (CCRD), Abencor operation conditions were optimized: no water addition in 30 min malaxation and 14 % (w/w) water addition at 50ºC in centrifugation. The combined effects of the concentrations of natural microtalc (*MT*: 0.04-0.46 w-%) and enzyme preparation (*E:* 0.003-0.117 w-%) added at the beginning of malaxation, on quality criteria, total phenols and chlorophyll pigments concentrations, and on oil extraction yield, were investigated using a CCRD. The results showed that the addition of both an enzyme preparation and natural microtalc could enhance olive oil yield (up to 34 % for 0.4-0.5 % of microtalc and 0.1 % of enzymes for 'Galega Vulgar') without any effect on quality of the obtained virgin olive oil.

Practical applications

Abencor system is the most used laboratory-scale installation in olive oil technology to carry out trials that would be very difficult to perform at industrial scale. This study presents an optimization of the Abencor extraction process, as well as the optimization of extraction with two adjuvants, enzymes and natural microtalc, using two different cultivars. The effect of E and/or MT addition depend on the cultivar. However, an improvement in extractability was always found for both cultivars. This study shows that for 'Cobrançosa' olives, considering both olive oil yield and economical aspects, the chosen conditions are 0.05-0.15 % of microtalc in the absence of added enzymes. For 'Galega Vulgar' olives, the highest extractability is observed under the presence of enzyme preparation amount higher than 0.10 % and microtalc amount of 0.4-0.5 %.

Keywords: Abencor, adjuvants, Cobrançosa cultivar, extraction, Galega cultivar, modeling, optimization

1. Introduction

The world virgin olive oil (VOO) demand has greatly increased during the last decade due to the discovery of olive oil benefits by new consumers, especially in nontraditional markets. The production of high-quality VOO at the highest yield and minimum cost, as well as using an environmentally friendly olive oil production, is more and more demanded [1].

At present, olive oil extraction is carried out mainly using continuous centrifugation systems which have been replacing the traditional pressing systems. However, both quality and yields have not been fully optimized. In fact, both batch and continuous systems are capable of extracting no more than 80-90 % of the olive oil contained in the fruit. The overall content of residual oil in the by-products (olive pomace and vegetable waters) reaches values up to 4 % (w/w) of olives processed, resulting in important economic losses for the olive oil sector [2].

During the last decades, several studies have been done in order to improve the yield and the quality of olive oil by using processing aids, such as natural microtalc, sodium chloride, enzymes or calcium carbonate [3-8]. Natural microtalc, which consists of hydrated magnesium silicate, $Si_8O_{20}Mg_6(OH)_4$, is the most important adjuvant used by the olive oil industry, due to its hydrophobic surface and a platy particle shape that adsorbs the natural emulsifiers from the surface of the olive oil droplets. Also, an increase in the amount of extracted oil can be observed with microtalc addition, as it promotes the coalescence of the small oil droplets making easier to separate the oil by centrifugation. The doses for talc addition referred in literature for olive oil extraction are in the range between 0.5- 2% [9-11]. However, excessive doses can have a negative effect on the extraction process [9]. This adjuvant is almost all present in the solid by-product and does not seem to have influence on the analytical characteristics of olive oil and the overall costs of the process [12,13].

One of the most important steps in the olive oil extraction is malaxation, which contributes to activate several natural enzymatic processes after the disruption of olives by milling. Enzyme preparations are used as processing aids in several food industries. In olive oil technology, several promising studies were performed with the addition of blends of hemicellulases, cellulases and pectinases [4-6,14-

18]. However, at the moment, the addition of adjuvants with biochemical action is not allowed by the EU legislation (Regulation EC Nº 865/2004).

The added enzyme preparations degrade the walls of the oil bearing cells, showing similar effects also on the olive paste colloidal systems which retain the oil droplets. Thus, the oil is released by phase inversion and gradually merges into larger droplets until they form a mass of free oil, which is then mechanically extracted [2].

Several authors reported that the addition of commercial enzyme preparations during malaxation can reduce the complexation of hydrophilic phenols with polysaccharides, increasing the concentration of free phenols in the olive paste, with antioxidant benefits for olive oil [19-21]. However, due to their hydrophilicity, considerable amounts of these phenols may be lost in waste waters during processing.

The positive effects of using processing aids are particularly important for pastes without adequate rheological properties for phase separation in malaxation, usually called difficult pastes [22]. In these pastes, extraction yields can drop to 70 % or less [23]. This is also important when producers want to start earlier to harvest in order to obtain high quality oils. Under these conditions, lower olive oil yields can be observed due to the high moisture content of the fruits. So, knowing olive characteristics is essential to adapt malaxation conditions [24].

The effect on olive extractability and olive oil characteristics resulting from the simultaneous addition of microtalc and enzymes has never been studied. However, several studies on the effect of each one of these adjuvants on olive oil extraction yield have been published [3-23]. Thus, the aim of this study was (i) to optimize the operation conditions of the laboratory-scale Abencor olive oil extractor system, in the absence of processing aids and (ii) to evaluate the combined effect of adding natural microtalc and a commercial enzyme preparation on the extraction yield, quality and composition of the olive oils extracted from two Portuguese olive cultivars (‘Cobrançosa’ and ‘Galega Vulgar’). The use of microtalc and/or enzymes on olive oil extraction from these cultivars is attempted for the first time.

2. Materials and methods

2.1. Materials

Portuguese olive fruits of 'Cobrançosa' and 'Galega Vulgar' cultivars used in all of the studies were produced according to the Integrated Protection rules in a rain fed olive grove situated in Beira Baixa region (39º 49' N, 7º 27'W), Portugal. Only healthy fruits were selected for olive oil extraction from 10-kg batches. 'Cobrançosa' fruits had an average weight of 3.2 ± 0.1 g per fruit; 'Galega Vulgar' fruits had an average weight of 2.5 ± 0.1 g per fruit. Solid-phase extraction (SPE) columns filled with 1g of octadecyl (C_{18}) material were from J.T. Baker, US; microtalc FC8-AW was kindly donated by Mondo Minerals, Helsinki, Finland; the enzyme preparation Endozym Olea, was a gift from AEB Bioquímica Portuguesa SA, Viseu, Portugal. The characteristics of microtalc are: mean particle size 2.2 μm, largest particle size 9.5 μm and specific surface area of 12 m^2 g^{-1}. The enzyme preparation was obtained from *Aspergillus niger* and is a pectolitic standard enzyme (9400 UPL/g) with complementary cellulasic and hemicellulasic activities. All reagents were analytical grade and from different suppliers.

2.2. Methods

2.2.1. Laboratory-scale olive oil extraction optimization

The virgin olive oils were extracted in a laboratory oil-mill (Abencor analyser; MC2 Ingenieria y Sistemas S.L., Seville, Spain). The olives (c.a. 10 kg) were crushed with a hammer mill equipped with a 4 mm sieve at 3000 rpm and each assay was performed with 0.7 kg of olives. Malaxation of the pastes was performed at 28-30 ºC and centrifugation at 3500 rpm (3 min). After centrifugation olive oil was separated by settling in a graduated cylinder; water traces were removed with anhydrous sodium sulfate, filtered through a cellulose filter and stored in amber glass bottles at 4 ºC. Olive oils analyses were performed in one week after extraction.

In order to select the most important factors to consider in subsequent experiments for the optimization of Abencor extraction conditions, a set of 8 experiments based on a Plackett-Burman (PB) design (Table 1) was carried out with 'Cobrançosa' olives [25]. In this design, the effect of the following factors

were tested simultaneously at two different levels (coded as -1 and +1, respectively): time of malaxation in thermo-mixer (30 or 60 min); water addition in thermo-mixer (1 or 20% v/w); temperature of water added in malaxation (50 or 70 ºC); water addition in centrifugation (1 or 20% v/w) and temperature of water added in centrifuge (50 or 70 ºC). For each experiment, the extraction yield, extractability index, total phenols and chlorophyll pigments contents in olive oil were evaluated.

Table 1. Plackett-Burman design for screening the variables in malaxation and centrifugation in Abencor extraction system and corresponding results for extractability index (EI), total phenols (TP) and chlorophyll pigments (CP).

	Malaxation			Centrifugation				
Experiment number	Time (min)	Added Water (%)	Temperature of water (º C)	Added Water (%)	Temperature of water (º C)	EI	TP (mg GAE kg^{-1})	CP (mg kg^{-1})
1	30	1	50	20	70	0.51	497.76	28.21
2	60	1	50	1	50	0.50	422.78	34.43
3	30	20	50	1	70	0.47	955.45	19.80
4	60	20	50	20	50	0.56	777.34	25.41
5	30	1	70	20	50	0.51	614.94	30.61
6	60	1	70	1	70	0.44	353.74	37.15
7	30	20	70	1	50	0.44	839.21	19.41
8	60	20	70	20	70	0.60	642.47	23.46

A second set of experiments was carried out following a central composite rotatable design (CCRD) (Table 2), in order to model and optimize the extraction conditions in Abencor system as a function of the factors with significant effect on extractability, total phenols and chlorophyll pigments, previously selected from the Plackett Burman design results. In this design, five different levels were tested for each factor (coded as -α, -1, 0, +1 and +α): water addition in malaxation changed between 1.7 and 58.3 % (v/w) and water addition in centrifugation changed between 0.9 and 29.1 % (v/w). In all the experiments, malaxation time was fixed at 30 min, and the temperature of water added in malaxation and centrifugation was 50 ºC.

Table 2. Central composite rotatable design (CCRD) followed in the experiments for Abencor extraction conditions optimization as a function of the amounts of water added in malaxation MW (% w/w), and in centrifugation CW (% w/w) and respective results obtained in each experiment and predicted CP and EI values from the models.

Experiment number	MW (%, w/w)	CW (%, w/w)	TP (mg GAE kg^{-1})	CP (mg kg^{-1})	Predicted CP (mg kg^{-1})	EI	Predicted EI
1	10.0	5.0	886.33	24.17	23.5	0.57	0.58
2	10.0	25.0	827.50	23.62	23.6	0.54	0.59
3	50.0	5.0	797.72	16.04	15.8	0.31	0.27
4	50.0	25.0	715.13	16.42	16.8	0.39	0.33
5	1.7	15.0	637.94	27.14	27.5	0.66	0.62
6	58.3	15.0	510.40	17.35	17.2	0.15	0.22
7	30.0	0.9	695.69	16.54	17.1	0.43	0.44
8	30.0	29.1	575.16	18.09	17.8	0.46	0.49
9	30.0	15.0	663.24	19.75	19.0	0.49	0.47
10	30.0	15.0	707.17	18.32	19.0	0.52	0.47
11	30.0	15.0	660.58	18.99	19.0	0.45	0.47

2.2.2. Optimization of extraction using adjuvants

The combined effects of the concentrations of natural microtalc (*MT*) and of the enzyme preparation (*E*) on the oil extraction yield, extractability index, the concentration of total phenols, chlorophyll pigments and bitterness intensity, as well as on chemical quality criteria parameters (acidity, peroxide value and UV absorbances) of the extracted olive oil, were investigated by response surface methodology (RSM). A CCRD was followed, as a function of the contents of enzyme preparation and of natural microtalc, using the Abencor extraction system under the previously optimized conditions. In this design, the five levels tested for microtalc concentration and for the concentration of the enzyme preparation were between 0.04 and 0.46 % (w/w) and between 0.003 and 0.1 % (v/w), respectively. Both adjuvants were added at the beginning of malaxation.

The variation ranges for microtalc and enzyme preparation concentrations were chosen taking into account the recommendations of the respective manufacturers: 0.3 % (w/w) for microtalc FC8-AW and 0.02-0.03 % (v/w), for Endozym Olea.

2.2.3. Analytical methods

2.2.3.1. Characterization of olives

The ripening indices (RI) of olives were determined following the guidelines of Estación de Olivicultura y Elaiotecnia, Jaén, Spain, based on the colour of the skin and pulp [26]. Humidity and fat content of the fruits were also evaluated by NIR (Foodscan, Foss, Denmark). The percentage of olive moisture was also determined by the oven method keeping at 105°C a 60 g sample ground by a laboratory hammer mill until constant weight following the indications of the International Olive Council [27].

2.2.3.2. Yield and extractability index

For each extraction experiment, the residual oil content in pomace (%, DW) was evaluated by NIR (Foodscan, Foss, Denmark).

The Abencor yield (Y, %) was also calculated by the following equation:

$$Y = (\rho V_{oo} / m)\ 100 \quad (1)$$

where, V_{OO} is the olive oil volume (cm^3), ρ is the density of olive oil (0.915 g/cm^3) and *m* is the mass (g) of the paste [28].The extractability index (EI) was calculated in accordance with [29], as follows:

$$EI = Y / OC \quad (2)$$

where OC is the oil content in olives in fresh weight), assayed by NIR (Foodscan, Foss, Denmark). The EI may vary between 0 and 1.

2.2.3.3. Chemical quality criteria parameters

The evaluation of European Union chemical quality criteria parameters for olive oil (acidity value, peroxide value, and UV absorbances) was carried out following the analytical methods described in EEC/2568/91 EU Regulation. Acidity value was expressed as the percentage of oleic acid. The peroxide value (PV) was determined in terms of miliequivalents of peroxide per kilogram of olive oil. For

spectrophotometric analysis (K_{232} and K_{270}), the evaluation of absorbances of 1 % (w/v) of olive oil in iso-octane was performed at 232 and 270 nm.

2.2.3.4. Total phenol content

Extraction of total phenol compounds from VOO was performed with solid phase extraction (SPE). The SPE columns were conditioned with 2 x 5 mL of methanol and 2 mL of hexane. Then, 1g of VOO in 10 mL of hexane was loaded in the column, which was after washed three times with 5 mL of hexane. After, phenols were eluted with 2 x 4 mL of methanol [30]. After extraction, total phenol content in olive oil samples was determined employing Folin-Ciocalteau reagent and quantified by VIS spectroscopy in a Jasco 7800 spectrophotometer, using a calibration curve of gallic acid. The results were expressed as gallic acid equivalents (mg GAE kg^{-1}).

2.2.3.5. Chlorophyll pigments

The determination was performed in accordance to IUPAC method proposed by [31] using a single beam spectrophotometer Biochrom Libra S21, evaluating the absorbances of VOO at 630, 670 and 710 nm.

2.2.3.6. Specific absorbance at 225 nm (K_{225})

The method that was followed is based on extraction of the bitter constituents (e.g. polyphenols) of virgin olive oil by SPE followed by elution with methanol/water (1:1) and measurement of the absorbance of the extract at 225 nm in a Jasco 7800 spectrophotometer [32].

2.2.4. Statistical analysis

The obtained results of Plackett-Burman and central composite rotatable designs were analyzed using the software Statistica, version 10, from *Statsoft*, Tulsa, OK, USA.

The linear of each factor (variable) tested by PB or CCRD, as well as the quadratic effects of each factor tested by CCRD, and of their linear interactions, on each response (extraction yield, extractability index, total phenols and chlorophyll pigments contents in olive oil) were calculated. The significance of each effect was evaluated by analysis of variance.

A response surface, described by a first or a second order polynomial equation, was fitted to each set of experimental results obtained for each CCRD. First and second order coefficients of these equations were generated by regression analysis. The goodness of fit of these models was evaluated by the coefficients of determination (R^2) and adjusted R^2 (R^2_{adj}). In presence of high values of both R^2 and $R^2_{adj,}$ a good fit of the model to the experimental data points is expected. Values of R^2 above 0.75 are considered to be good and above 0.90, very good, since they indicate that the response-surface polynomial model explains more than 75 and 90 % of the variability of the experimental results, respectively [33].

3. RESULTS AND DISCUSSION

3.1. Laboratory-scale olive oil extraction optimization

The olives of 'Cobrançosa' cultivar, used both in screening experiments and in the optimization of Abencor extraction conditions, were characterized by a ripening index of 3.5, a moisture content of 46 % (DW) and a fat content of 44 % (DW). The results obtained in the experiments of the PB design for selection of the most important variables for the optimization of the operation conditions of the Abencor extraction system, are presented in Table 1.

In this variable selection step, the oil content of the pomace significantly decreased with the amount of water added in centrifugation (effect of -3.37; p=0.03) and, therefore, the EI increased (effect of 0.081; p=0.07). Total phenols significantly increased (effect of 331.3; p=0.03) with the quantity of water added in malaxing. Although with a p-value higher than 0.05 (effect of -177.76; p = 0.09), it seems that longer times of malaxing reduce total phenols in olive oils, which is generally referred by other authors [34-36]. Conversely to the observed for phenols, the content of chlorophyll pigments significantly increased with the time of malaxing but significantly decrease (effect of -10.58; p=0.02) with the water added in malaxing. The variation of the water temperature added in centrifugation and malaxation, from 50 to 70 ºC, showed to affect neither the yield nor the content of phenols or chlorophyll pigments in the olive oil. Therefore, in order to improve both the oil yield and the content of phenols, which have been related with antioxidant and functional properties of olive oil, the Abencor extraction system was used under the following fixed conditions in subsequent optimization

studies: 30 min of malaxation time and water temperature of 50 ºC, since lower temperatures can also preserve the aromas of olive oil [34,36].

CCRD experiments were performed with the same blend of 'Cobrançosa' olives, used in the previous Plackett-Burman design, changing the two factors that most influenced the oil content of the pomace and consequently the EI, as well as the content of total phenols: amounts of water added in malaxation (MW) and in centrifugation (CW) (Table 2). The results obtained in the 11 experiments concerning the EI and the contents of phenols and chlorophyll pigments in olive oil, are presented in Table 2. The linear and quadratic effects of MW and CW, as well as the effect of the interaction of MW and CW on these parameters, were calculated. The MW presented significant negative linear effects on both EI (-0.283; $p < 0.001$) and chlorophyll pigments (-7.294; $p < 0.001$) indicating that high amounts of water added during malaxation will promote a reduction in olive oil yield and in the content of chlorophyll pigments in the olive oil. The negative and positive quadratic effects of MW on EI (-0.064; $p = 0.213$) and chlorophyll pigments (3.336; $p < 0.001$) indicate that values of these parameters are fitted to convex and concave response surfaces, as a function of MW, respectively.

The amount of water added in centrifugation (CW) showed to be important only for the extraction of chlorophyll pigments, which can be described by a convex surface as a function of CW (significant negative quadratic effect of -1.563; $p = 0.023$).

No significant linear interactions between MW and CW were observed for the evaluated responses.

With respect to the amounts of total phenols in the extracted olive oils, they showed to depend neither on the amount of water added during malaxation nor during centrifugation.

Figure 1 shows the response surfaces fitted to the extractability index (EI) values (Fig. 1-a) and to the contents of chlorophyll pigments in the olive oil (Fig. 1-b), as a function of the water added in malaxation (MW) and in centrifugation (CW). These response surfaces are well described by the following second-order polynomial models using decoded variables:

$$EI = 0.621 - 0.0037\ MW - 0.00007\ MW^2 + 0.00006\ MWxCW \quad (3)$$

($R^2 = 0.910$; $R^2_{adj} = 0.871$)

$$CP = 26.14 - 0.435\ MW + 0.0042\ MW2 + 0.260\ CW - 0.0078\ CW\ 2 \quad (4)$$

($R^2 = 0.982$; $R^2_{adj} = 0.971$)

where EI is the extractability index and CP is the chlorophyll pigments concentration expressed in mg pheophytin/kg of olive oil.

In these polynomial model equations, only the significant effects ($p < 0.05$) and those having a confidence range smaller than the value of the effect, or smaller than the standard deviation (data not shown), were considered. The high values of R^2 and R^2_{adj} indicate a good fit of these models to the experimental results.

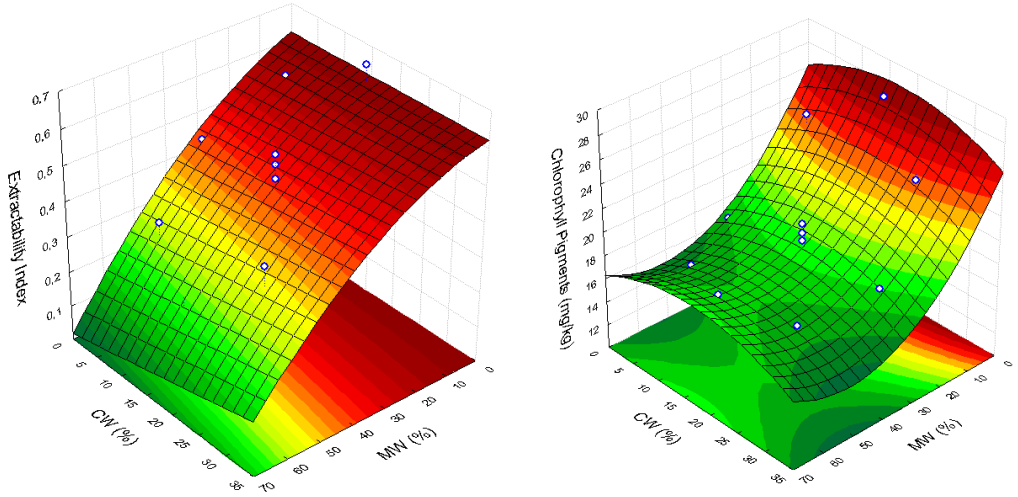


Figure 1. Response surface fitted to the Extractability index and chlorophyll pigments values as a function of water addition in malaxation (MW) and of water addition in centrifugation (CW).

These response surfaces as a function of MW and CW show that the highest EI, and therefore the highest olive oil yields, are obtained when no water is added in

malaxation. These operation conditions correspond to the extraction of olive oils with lower contents in chlorophyll pigments. Thus, in the following experiments to study the effects of adjuvants in olive oil extraction using the Abencor system, no water was added in malaxation step and 14% of water (w/w) was added in centrifugation, maintaining the rest of the conditions previously described. Adding 14 % water in centrifugation will lead to pomace with less oil content (data not shown). Under these moisture conditions, high amounts of chlorophyll pigments in olive oils can be obtained. These optimized extraction conditions were quite different from those originally proposed by [28], when the Abencor lab-scale olive oil extraction system was developed. The modifications of extraction conditions in Abencor system were also proposed when olives from irrigated groves are processed [37].

3.2. Optimization of extraction conditions using adjuvants

For the trials with addition of adjuvants, the characteristics of the olives used were: 'Cobrançosa', ripening index (RI) = 3.5, fat content (DW) = 44 %; Moisture content= 55 %; 'Galega Vulgar', RI= 3.6, fat content (DW) = 43 %; Moisture content= 61 %.

In 'Cobrançosa' and 'Galega Vulgar' cultivars the extractability index (EI) was 0.62 and 0.35 without adjuvants, respectively. In all the experiments of the CCRD, extraction yield was higher than that observed in the trial without processing aids (Tables 3 and 4). Microtalc addition had significant negative linear (-0.022; $p = 0.014$) and quadratic effects (-0.02; $p = 0.036$) on olive oil extraction from 'Cobrançosa' fruits. It means that an increase in microtalc content will promote a decrease in extraction yield, which can be described by a convex surface, as a function of the amount of this adjuvant. For 'Galega Vulgar' cultivar, only the positive effect of the linear interaction of microtalc with enzyme is significant (0.039; $p = 0.024$), meaning that an increase of the concentration of both adjuvants will improve the extraction yield. For 'Cobrançosa' fruits, the positive linear effects of enzyme content and of the interaction microtalc x enzyme are also important.

Table 3. CCRD followed in the experiments for optimization of 'Cobrançosa' olives extraction using adjuvants (enzyme concentration, E, %-v/w, and microtalc, MT, %-w/w), the results obtained in each experiment and predicted EI values from the model: extractability Index (EI), acidity value (%), peroxide value (PV; meq O_2 kg^{-1}), K_{232} and K_{270}, total phenols (TP; mg GAE kg^{-1}), chlorophyll pigments (CP, mg pheophytin kg^{-1}) and K_{225}. Experiment 12 corresponds to the trial without adjuvants.

Experiment	E	MT	Acidity	PV	K_{232}	K_{270}	TP	CP	K_{225}	EI	Predicted EI
1	0.02	0.1	0.37	6.1	1.46	0.20	1103.4	18.1	0.515	0.773	0.777
2	0.1	0.1	0.36	5.2	1.42	0.19	1252.6	19.0	0.503	0.773	0.772
3	0.02	0.4	0.35	4.8	1.42	0.19	1372.5	21.9	0.511	0.750	0.744
4	0.1	0.4	0.34	5.2	1.42	0.19	1403.3	19.8	0.536	0.773	0.761
5	0.06	0.04	0.33	5.6	1.45	0.20	1323.7	16.8	0.499	0.773	0.769
6	0.06	0.46	0.33	6.1	1.41	0.18	1115.0	18.0	0.503	0.727	0.738
7	0.003	0.25	0.34	4.5	1.39	0.18	1334.1	19.4	0.492	0.773	0.770
8	0.117	0.25	0.36	7.4	1.44	0.20	1349.4	11.8	0.525	0.773	0.778
9	0.06	0.25	0.35	7.3	1.25	0.20	1283.1	13.4	0.494	0.773	0.774
10	0.06	0.25	0.34	7.0	1.42	0.19	1406.6	13.0	0.497	0.773	0.774
11	0.06	0.25	0.34	4.9	1.39	0.20	1406.6	18.5	0.520	0.773	0.774
12	0.00	0.00	0.39	7.8	1.43	0.17	1159.9	10.6	0.453	0.62	

Table 4. CCRD followed in the experiments for optimization of 'Galega vulgar' olives extraction using adjuvants (enzyme concentration, E, %-v/w, and microtalc, MT, %-w/w) and results obtained in each experiment: extractability Index (EI), acidity value (%), peroxide value (PV; meq O_2 kg^{-1}), K_{232} and K_{270}, total phenols (TP; mg GAE kg^{-1}), chlorophyll pigments (CP, mg pheophytin kg^{-1}) and K_{225}. Experiment 12 corresponds to the trial without adjuvants.

Experiment	E	MT	Acidity	PV	K_{232}	K_{270}	TP	CP	K_{225}	EI	Predicted EI
1	0.02	0.1	0.29	3.0	1.29	0.11	725.9	11.6	0.359	0.468	0.454
2	0.1	0.1	0.33	2.9	1.32	0.11	724.6	11.3	0.341	0.390	0.389
3	0.02	0.4	0.33	3.0	1.27	0.11	736.2	12.0	0.330	0.429	0.415
4	0.1	0.4	0.30	3.0	1.28	0.11	726.1	11.3	0.358	0.429	0.428
5	0.06	0.04	0.27	2.4	1.27	0.10	680.0	13.3	0.303	0.409	0.417
6	0.06	0.46	0.33	3.2	1.35	0.10	740.6	14.5	0.378	0.409	0.417
7	0.003	0.25	0.32	2.3	1.26	0.09	637.9	14.5	0.334	0.429	0.445
8	0.117	0.25	0.28	3.0	1.26	0.08	743.5	13.8	0.379	0.409	0.408
9	0.06	0.25	0.26	2.2	1.30	0.11	689.5	9.62	0.349	0.429	0.429
10	0.06	0.25	0.32	2.8	1.24	0.11	704.7	10.6	0.331	0.429	0.429
11	0.06	0.25	0.25	2.3	1.30	0.10	678.3	11.0	0.313	0.429	0.429
12	0.00	0.00	0.34	2.7	1.29	0.10	693.2	8.9	0.364	0.35	

Also, no significant effects of the addition of microtalc and/or enzyme were found on quality criteria and total phenols or chlorophyll pigments in the obtained olive oils. Therefore, the presence of these processing aids did not affect these characteristics of the obtained olive oils (Tables 3 and 4).

The use of microtalc did not significantly alter either the phenolic profile or the content of individual phenols of the olive oil obtained from Arbequina cultivar fruits [38]. Conversely, several authors observed an increase in phenol content when enzymes were applied. However, in those studies, higher doses of enzymes and/or different commercial formulations were used, namely 0.5-1.5 % of mixtures of cellulases, pectinases and hemicellulases [18], 0.5-2.5 % Uvazym, Maxoliva [17], 0.05 % of Olivex/Novoferm 12, Olivex/Glucanex [39] and 0.3 % of Pectinex Ultra SP-L [40].

Response surfaces, described by polynomial equations as a function of the significant effects and of those important enough to be neglected, were fitted to the experimental results to visualize the dependence of the responses on the significant variables. For both cultivars, the EI can be described by the following second-order polynomial models, using decoded variables, with a good fit to the experimental data points ($R^2 > 0.8$) representing saddle-like surfaces (Figure 2):

'Cobrançosa' cultivar

$$EI = 0.77+0.10MT – 0.46MT^2 – 0.17E + 0.95\ MT \times E \quad (5)$$

(**$R^2 = 0.835$; $R^2_{adj} = 0.725$**)

'Galega Vulgar' cultivar

$$EI = 0.47 – 0.35MT^2 – 1.08E + 2.99\ MT \times E \quad (6)$$

(**$R^2 = 0.805$; $R^2_{adj} = 0.722$**)

where MT and E are the amounts of microtalc (%, w/w) and enzyme preparation (%, v/w), respectively. In this type of surfaces, it is not possible to identify a single optimal point but we can identify sets of operation conditions that will give the

best results. Therefore, Figure 2 shows that the highest EI for 'Cobrançosa' olives can be attained under completely different conditions: (i) when both microtalc and enzyme preparation are added in small amounts (0.05-0.15 %) of microtalc and enzyme below 0.01 % or when (ii) enzyme amounts higher than 0.13% and microtalc addition between 0.20 and 0.30 % are used. Therefore, for 'Cobrançosa' olives, the chosen conditions, in terms of both olive oil yield and economical aspects, are the use of 0.05-0.15 % of microtalc, in the absence of added enzymes. For 'Galegar Vulgar' olives, the highest extractability is observed under the presence of high enzyme (higher than 0.10 %) and microtalc (0.4-0.5 %) amounts, confirming the significant positive linear effect between enzyme and microtalc (Eq. 6). Under these selected conditions, a 24 % and 34 % increase in extraction yield were observed for 'Cobrançosa' and 'Galega Vulgar' olives, respectively, compared to the values obtained in the absence of adjuvants.

From these results, we can conclude that for each cultivar, the amounts of microtalc and enzymes preparation to be used must be previously optimized. In addition to cultivar effect, moisture content, ripening stage and the content of endogenous enzymes may explain the different behavior observed [39,40].

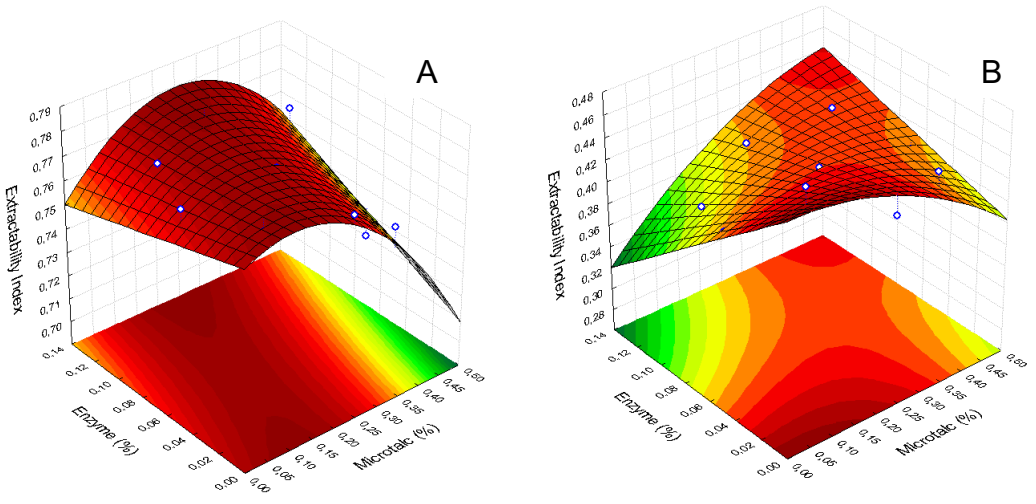


Figure 2. Response surface fitted to the extractability index for 'Cobrançosa' (A) or 'Galega Vulgar' olives (B) as a function of enzyme and microtalc addition.

4. Conclusions

In this study, RSM was successfully applied to model and optimize the conditions used in Abencor extraction as well as the addition of adjuvants. These optimized extraction conditions in Abencor were quite different from those originally proposed, avoiding the addition of water during malaxation and adjusting the quantity and temperature of water in centrifugation.

The addition of blends of microtalc with enzymes during malaxation showed different extraction yields for each cultivar used. For 'Cobrançosa' olives, a maximum of 24 % increase in yield, compared to the results obtained in the absence of adjuvants, was attained when 0.05-0.15 % of microtalc was used, without the need for enzymes addition. For 'Galega Vulgar' olives, the highest extractability was observed under the presence of both high enzyme (higher than 0.10 %) and microtalc (0.4-0.5 %) amounts, corresponding to a 34 % increase in extraction yield. The results showed that the use of adjuvants, as well as their respective amounts, must be adjusted to each cultivar.

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References

[1] García-González, D. L., Aparicio, R.: Research in Olive Oil: Challenges for the Near Future. Journal of Agricultural and Food Chemistry. 2010, **58**, 12569-12577.

[2] Chiacchierini, E., Mele, G., Restuccia, D., Vinci, G.: Impact evaluation of innovative and sustainable extraction technologies on olive oil quality.Trends in Food Science & Technology. 2007, **18**, 299-305.

[3] Ranalli, A., De Mattia, G.: Characterization of olive oil produced with a new enzyme processing aid. Journal of the American Oil Chemists' Society. 1997, **74**, 1105 - 1113.

[4] Ranalli, A., De Mattia, G., Ferrante, M. L.: The characteristics of percolation olive oils produced with a new processing enzyme aid.International Journal of Food Science & Technology. 1998, **33**, 247-258.

[5] Ranalli, A., Sgaramella, A., Surricchio, G.: The new "Cytolase 0" enzyme processing aid improves quality and yields of virgin olive oil. Food Chemistry. 1999, **66**, 443-454.

[6] Ranalli, A., Pollastri, L., Contento, S., Iannucci, E.: The glyceridic and nonglyceridic constituents of virgin olive oil after use of a novel method of enzyme extraction, International Journal of Food Science & Technology. 2003, **38**, 17-27.

[7] Espínola, F., Moya, M., Fernández, D. G., Castro, E.: Improved extraction of virgin olive oil using calcium carbonate as coadjuvant extractant. Journal of Food Engineering. 2009, **92**, 112-118.

[8] Cruz, S., Yousfi, K., Pérez, A. G., Mariscal, C., Garcia, J. M.: Salt improves physical extraction of olive oil European Food Research and Technology. 2007, **225**, 359-365.

[9] Uceda, M., Jimenez, A., Beltran, G.: Olive oil extraction and quality,. Grasas Aceites. 2006, **57**, 25-31.

[10] Valdivia, D. G. F., Lozano, F. E., Vilar, M. I. M.: The influence of different technological co-adjuvants on the quality and yield of virgin olive oil using response surfaces methodology. Grasas Aceites. 2008, **59**, 39-44.

[11] Cert, A., Alba, J., Leon Camacho, M., Moreda, W., Perez Camino, M. C.: Effects of talc addition and operating mode on the quality and oxidative stability of virgin olive oils obtained by centrifugation. Journal of Agricultural and Food Chemistry. 1996, **44**, 3930-3934.

[12] Carrapiso, A. I., García, A., Petrón, M. J., Martín, L.: Effect of talc and water addition on olive oil quality and antioxidants. European Journal of Lipid Science and Technology. 2013, **115**, 583-588.

[13] Moya, M., Espínola, F., Fernández, D. G., Torres, A., Marcos, J., Vilar, J., Josue, J., Sánchez, T., Castro, E.: Industrial trials on coadjuvants for olive oil extraction.Journal of Food Engineering. 2010, **97**, 57-63.

[14] Ranalli, A., Serraiocco, A.: Efectos cuantitativos y cualitativos de un enzima pectolítico en la producción del aceite de oliva. Grasas Aceites. 1996, **47**, 227-236.

[15] Ranalli, A., Malfatti, A., Pollastri, L., Contento, S., Lucera, L.: Analytical quality and genuineness of enzyme-extracted virgin olive oil. Journal of food quality. 2003, **26**, 149-164.

[16] Ranalli, A., Pollastri, L., Contento, S., Iannucci, E.: The glyceridic and nonglyceridic constituents of virgin olive oil after use of a novel method of enzyme extraction, International. Journal of Food Science & Technology. 2003, **38**, 17-27.

[17] Aliakbarian, B., De Faveri, D., Converti, A., Perego, P.: Optimisation of olive oil extraction by means of enzyme processing aids using response surface methodology. Biochemical Engineering Journal. 2008, **42**, 34-40.

[18] De Faveri, D., Aliakbarian, B., Avogadro, M., Perego, P., Converti, A.: Improvement of olive oil phenolics content by means of enzyme formulations: Effect of different enzyme activities and levels. Biochemical Engineering Journal. 2008, **41**, 149-156.

[19] Vierhuis, E., Schols, H. A., Beldman, G., Voragen, A. G. J.: Isolation and characterisation of cell wall material from olive fruit (Olea europaea cv. koroneiki) at different ripening stages. Carbohydrate Polymers. 2000, **43**, 11-12.

[20] Vierhuis, E., Korver, M., Schols, H. A., Voragen, A. G. J.: Structural characteristics of pectic polysaccharides from olive fruit (Olea europaea cv moraiolo) in relation to processing for oil extraction. Carbohydrate Polymers. 2003, **51**, 135-148.

[21] Vierhuis, E., Servili, M., Baldioli, M., Schols, H. A., Voragen, A. G. J., Montedoro, G. F.: Effect of Enzyme Treatment during Mechanical Extraction of Olive Oil on Phenolic Compounds and Polysaccharides. J. Agric. Food Chem. 2001, **49** 1218 -1223.

[22] Di Giovacchino, L., Sestili, S., Di Vincenzo, D.: Influence of olive processing on virgin olive oil quality. European Journal of Lipid Science and Technology. 2002, **104**, 587-601.

[23] Espínola, F., Moya, M., Fernández, D. G., Castro, E.: Modelling of virgin olive oil extraction using response surface methodology. International Journal of Food Science & Technology. 2011, **46**, 2576-2583.

[24] Aguilera, M. P., Beltran, G., Sanchez-Villasclaras, S., Uceda, M., Jimenez, A.: Kneading olive paste from unripe 'Picual' fruits: I. Effect on oil process yield. Journal of Food Engineering. 2010, **97**, 533-538.

[25] Bofinger, V. J.: An introduction to some multivariate techniques with applications in field experiments, in: *Developments in Field Experiment Design and Analysis* Ed. J. L. W. V.J. Bofinger, Commonwealth Agricultural Bureaux, Oxford, UK 1975, pp. 73-83.

[26] Hermoso, M., Uceda, M., Frias, L., Beltran, G.: Maduración, in: *El Cultivo del Olivo* Eds. D. Barranco, D. Fernandez-Escobar, L. Rallo, Ediciones Mundi-Prensa, Madrid 1997, pp. 137- 153.

[27] IOC: Guide for the determination of the characteristics of oil-olives. COI/OH/ 2011.

[28] Martinez, J. M., Muñoz, E., Alba, J., Lanzon, A.: Informe sobre Utilización del Analizador de Rendimientos "Abencor", Grasas Y Aceites. 1975, 379 – 385.

[29] Beltrán, G., Uceda, M., Jiménez, A., Aguilera, M. P.: Olive oil extractability index as a parameter for olive cultivar characterisation. Journal of the science of food and agriculture. 2003, **83**, 503-506.

[30] Favati, F., Caporale, G., Bertuccioli, M.: Rapid determination of phenol content in virgin olive oil. Grasas Aceites. 1994, **45**, 68-70.

[31] Pokorny, J., Kalinová, L., Dysseler, P.: Determination of chlorophyll pigments in crude Vegetable Oils. Pure Appl. Chem. 1995, **67**, 1781-1787.

[32] Gutiérrez -Rosales , F., Perdiguero, S., Gutiérrez, R., Olias, J. M.: Evaluation of the bitter taste in virgin olive oil. Journal of the American Oil Chemists' Society. 1992, **69**, 1558-9331.

[33] Haaland, P. D.: *Experimental Design in Biotechnology* . Marcel Dekker Inc., New York, NY, USA 1989.

[34] Kalua, C. M., Bedgood, D. R., Bishop, A. G., Prenzler, P. D.: Changes in volatile and phenolic compounds with malaxation time and temperature during virgin olive oil Production. Journal of Agricultural and Food Chemistry. 2006, **54**, 7641-7651.

[35] Angerosa, F., Mostallino, R., Basti, C., Vito, R.: Influence of malaxation temperature and time on the quality of virgin olive oils. Food Chemistry. 2001, **72**, 19-28.

[36] Inarejos-García, A., Gómez-Rico, A., Salvador, M. D., Fregapane, G.: Influence of malaxation conditions on virgin olive oil yield, overall quality and composition. European Food Research and Technology. 2009, **228**, 671-677.

[37] Ben-David, E., Kerem, Z., Zipori, I., Weissbein, S., Basheer, L., Bustan, A., Dag, A.: Optimization of the Abencor system to extract olive oil from irrigated orchards. European Journal of Lipid Science and Technology. 2010, **112**, 1158-1165.

[38] Artajo, L.-S., Romero, M.-P., Tovar, M.-J., Motilva, M. J.: Effect of irrigation applied to olive trees (Olea europaea L.) on phenolic compound transfer during olive oil extraction. European Journal of Lipid Science and Technology. 2006, **108**, 19-27.

[39] García, A., Brenes, M., Moyano, M. J., Alba, J., García, P., Garrido, A.: Improvement of phenolic compound content in virgin olive oils by using enzymes during malaxation. Journal of Food Engineering. 2001, **48**, 189-194.

[40] Canamasas, P., Ravetti, L.: Evaluation of processing aids for olive oil extraction and quality improvement. Rural Industries Research and Development Corporation., Canberra 2011.

Chapter 4

Characterization of aroma compounds in Portuguese extra virgin olive oils from Galega Vulgar and Cobrançosa cultivars using GC-O and GCxGC-ToFMS

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Abstract

Aroma compounds of virgin olive oils extracted from two olive cultivars – Galega Vulgar and Cobrançosa - grown in Beira Baixa region in central Portugal were investigated. Gas chromatography – olfactometry (GC-O) was carried out to select the important odorants for subsequent comprehensive gas chromatography/time of flight mass spectrometry (GCxGC-ToFMS) analysis. By GC-O fifteen odorants were identified. For the quantification of volatile compounds, headspace solid phase microextraction (SPME) technique was optimized. Under optimized conditions, 22 volatile compounds were quantified in all samples. *Trans*-2-hexenal was the most abundant compound. A discriminant analysis (DA) was used to discriminate among olive oil samples obtained from olives of the two cultivars with different harvest time/ripening stages. Concerning the harvesting time and cultivar, nine volatiles showed to have discriminant power among samples, namely heptanal, *trans*-2-hexenal, 1-octen-3-ol, nonanal, 2,3-butanedione, ethyl-2-methylbutyrate, hexanal, *cis*-3-hexenylacetate and 3-methylbuthylacetate.

Keywords: Harvest date, olive cultivar, olive oil, volatile compounds

1. Introduction

The research on virgin olive oil (VOO) sensory characteristics is mostly based on trained panels to recognize and evaluate many attributes representing several descriptors. Moreover, the official method to perform organoleptic assessment of VOO is based in well-established methodology, with selected and well trained assessors (EC, 2008). However, this technique is not exempt of risks and sometimes the classification errors may lead to serious economic losses (Aparicio, Morales, & García-González, 2012). More precise and diagnostic chemical information may be expected with gas chromatography/mass spectrometry (GC-MS) analyses of aroma compounds of VOO (Frankel, 2010).

The identification of aroma compounds in VOO is a challenging task, as hundreds of compounds are quite often present in the volatile fraction, differing by orders of magnitude in their concentration (Boskou, 2006). Among these compounds, only a small fraction contributes to the aroma of olive oil (Angerosa et al., 2004). Only those volatiles able to interact with the receptor proteins in the human olfactory bulb are responsible for a certain aroma (Belitz, Grosh, & Shieberle, 2009). The knowledge that not all of the volatiles occurring in a food contribute to its aroma was the reason for changing the methodology of their analysis and the developments of gas chromatography – olfactometry (GC-O) methods. Furthermore, the results led to the conclusion that less than 5 % of the volatiles identified in foods contributed to their aromas (Grosch, 2000).

The aroma of olive oil is mainly attributed to aldehydes, alcohols, esters, hydrocarbons, ketones and furans (Morales & Tsimidou, 2000). Virgin olive oils produced from fruits of good quality, where the lipoxygenase (LOX) pathway is the predominant source of compounds biogeneration, are usually described by perception of fruity sensations, freshly cut grass, green fruits such as apple, banana, or vegetables, such as artichoke or tomato, accompanied by more or less intense taste notes of bitterness and pungency (Angerosa, 2002; Aparicio & Luna, 2002; Cerretani, Salvador, Bendini, & Fregapane, 2008). From a quantitative point of view, C6 linear unsaturated and saturated aldehydes represent the most important fraction of volatile compounds of high quality VOO (Angerosa et al., 2004). An increase on the intensity of fruity vs. green notes was observed in virgin olive oils, after sedimentation and racking, showing that olive

oil flavour profile is affected by the technological operations (Reboredo-Rodríguez, González-Barreiro, Cancho-Grande, & Simal-Gándara, 2013)

Sample preparation is an essential step in the analysis of aroma compounds in VOO, greatly influencing the precision and accuracy of the results and the time and cost of the analysis. Concentration of olive oil aroma compounds using sorptive methods became the most widely used group of methods in the past two decades, and since the last 10 years, solid phase microextraction became the most frequently used of sorbent-based method for the analysis of flavours and off-flavours in virgin olive oil (Cajka et al., 2010; Cavalli, Fernandez, Lizzani-Cuvelier, & Loiseau, 2003; Dierkes, Bongartz, Guth, & Hayen, 2011; Jiménez, Beltrán, & Aguilera, 2004; Kalua, Bedgood, Bishop, & Prenzler, 2006; Kanavouras, Kiritsakis, & Hernandez, 2005; Vaz-Freire, Silva, & Freitas, 2009; Vichi, Castellote et al., 2003). Therefore, gas chromatography – olfactometry (GC-O) is considered a good tool for screening sensory active compounds. The technique consists of isolation of the volatile fraction and concentration of the extract, and then an aliquot is separated by GC and the effluent is split into a flame ionization detector (FID) and a sniffing port (Morales, Rios, & Aparicio, 1997; Reiners & Grosch, 1998).

The identification and the quantification of the compounds causing flavour or off-flavour is considered one of the keys for VOO quality control and a good indicator of olive oil quality changes (Kalua et al., 2007). Moreover, olive cultivar as well as ripening stage, strongly influences the abundance of volatiles compounds and consequently this knowledge may be applied to produce VOO with particular sensory notes from chosen cultivars at certain ripening stages (Prenzler, Robards, & Bedgood, 2007). Also, VOO obtained under irrigation conditions can display higher whole aroma concentration than the rain fed ones (Baccouri et al., 2007). Different water stress levels in olive trees affected the amount of VOO volatile compounds, showing that *trans*-2-hexenal, hexan-1-ol, and *cis*-3- hexen-1-ol concentrations were higher in olive oils extracted from olives from irrigated groves (Gómez-Rico, Salvador, La Greca, & Fregapane, 2006). Hence, different amounts of total volatiles can be related to diverse agronomic conditions.

The aim of the present study was to analyse flavour compounds in two of the most important extra virgin olive oils (EVOO) from Portugal: one produced from

the cultivar Galega Vulgar and the other from cultivar Cobrançosa, in early stages of ripening. Our goal was to select the main compounds responsible for major odour notes in these olive oils using GC-O, and then to quantify the detected odorants, as well as other compounds known from the literature to have influence on olive oil flavour using a dedicated method based on solid phase microextraction and comprehensive gas chromatography/mass spectrometry. Up to now, there is no detailed information available on the identification of the main odorants in Portuguese EVOO by GC-O, although it is known that they are influenced by the time of harvesting (ripening stage) of the fruits, affecting consumers' sensory evaluation of these oils. Concerning this, a correct evaluation of the effects of early stages of ripening on VOO aroma is a very important aspect, especially for Galega cultivar, the most important Portuguese olive cultivar. This cultivar is strongly attacked by pests and diseases, so if the harvesting period could start earlier, this would be very important from a sustainable agriculture point of view, minimizing pesticides use.

2. Material and methods

2.1. Olive samples, chemicals and reagents

Olive samples cvs. Galega Vulgar and Cobrançosa (8 samples of each cultivar), were harvested in 2011 in two olive groves in Beira Baixa region (center of Portugal). Samples were organized into four groups, by cultivar and by two harvest times: first harvest time corresponds to Galega Vulgar and Cobrançosa olives picked in October; second harvest time corresponds to Galega Vulgar and Cobrançosa olives picked in November.

Their ripening indices (RI) were determined following the guidelines of Estación de Olivicultura y Elaiotecnia, Jaén, Spain (Hermoso, Uceda, Frias, & Beltran, 1997). Only healthy fruits were selected for olive oil extraction. Average water content in the crude pastes was 55 and 54% in 'Galega' and 'Cobrançosa', respectively. Olive oils were extracted using an Abencor system (MC2 Ingenieria Sistemas, Seville, Spain), equipped with a hammer mill (3000 rpm), a thermobeater (50 rpm) and a centrifuge (3000 rpm). Oil extraction was performed by thermobeating at 28 - 30 ºC, for 30 min. European Union chemical quality criteria for olive oil (acidity value, peroxide index (IP) and UV light absorption (K_{232}

and K_{270})) was carried out following the analytical methods described in EEC/2568/91 EU Regulation. Fatty acid composition was performed by GC/FID in accordance with EEC/2568/91 EU Regulation. Samples for volatile compound analysis were stored at -20 ºC until analysis. Standards of volatile compounds used for quantitation were purchased at Sigma Aldrich (Poznań, Poland) with the highest available GC standard grade. Dichloromethane was purchased from Sigma Aldrich in a Chromasolv purity (99.9%). SPME fibers – Carboxen/ Polydimethylsiloxane (CAR/PDMS); Polydimethylsiloxane (PDMS); Polydimethylsiloxane/ Divinylbenzene (PDMS/DVB) and 2 cm Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) were purchased from Sigma Aldrich and preconditioned according to manufacturer's recommendation.

2.2. GC-O analysis

Gas chromatography – olfactometry analyses were performed on a HP 5890 II (Hewlett Packard, Santa Clara, CA) gas chromatograph equipped with an olfactory port. To obtain samples for GC-O analyses, 90 g of oil were dissolved in 250 mL of dichlorometane. Volatile compounds were isolated using solvent assisted flavour evaporation (SAFE) apparatus (Engel, Bahr, & Schieberle, 1999). Rotary vane pump was used in SAFE apparatus to reduce the pressure to values lower than 30 kPa to ensure vacuum transfer of volatiles. SAFE apparatus was cooled using liquid nitrogen. Extract containing volatile compounds was concentrated using Kuderna-Danish concentrator to a volume of approximately 0.5 mL. Concentrated extract was analyzed on two columns of different polarities: non polar DB-5 column (30 m × 0.32 mm × 0.5 µm, Agilent Technologies, USA) and a polar Supelcowax-10 (30 m × 0.25 mm × 0.5 µm, Supelco, Bellefonte, PA), both with Y type glass splitters allowing simultaneous detection of compounds by flame ionization detector and sniffing. The oven program for GC-O analysis was the following: 40 ^{o}C for 1 min, then 6 ^{o}C/min to 180 ^{o}C, followed by 20 ^{o}C/min to 260 ^{o}C (3 min), for DB-5 column and 40 ^{o}C (0 min), then 5 ^{o}C/min to 170 ^{o}C, followed by 25 ^{o}C/min to 250 ^{o}C (4 min) for Supelcowax-10 column. A volume of 2.5 µL of extract was injected into the column in a splitless mode with purge valve closed for 2 minutes. Retention

indices were calculated for each compound using homologous series of C_7 – C_{24} n-alkanes.

2.3. Development of SPME extraction method

For the development of the extraction method using solid phase microextraction (SPME), a mixture of 1-pentene-3-one, 2-penten-1-ol, hexanal and 2-hexenal in refined rapeseed oil was used, so the selected volatile compounds represent different chemical classes. In a first step, the fiber providing the highest peak responses was chosen at a temperature of 40 ^{o}C. Then, different extraction temperatures were compared (40 ^{o}C, 50 ^{o}C and 60 ^{o}C), and different extraction times (5, 10, 15, 20, 30 and 60 min). Peak areas for analyzed compounds added to refined rapeseed oil in concentrations of 0.1, 0.5, 1 and 10 mg/L were compared for each fiber to evaluate linearity of compounds adsorption onto the fiber's surface. The evaluation was performed on a HP 6890 gas chromatograph equipped with FID detector and the same polar column as in GC-O experiment. Peak areas were compared in SPME method development procedure.

2.4. SPME-GC × GC-ToF-MS analysis

Quantitative analysis was performed using SPME and gas chromatograph coupled to time of flight mass spectrometer. Volatile compounds were identified and quantified using GC × GC-ToF-MS system (Pegasus 4D LECO, St. Joseph, MI). The GC was equipped with a DB-5 column (25 m x 0.2 mm x 0.33 µm, Agilent Technologies, Santa Clara, CA) and Supelcowax 10 (1.2 m x 0.1 mm x 0.1 µm, Supelco Bellefonte, PA) as a second column. For two dimensional analysis modulation time was optimized and set at 5 s, mass spectra were collected at a rate 150 scans/s. Main oven temperature was 40 ^{o}C (1 min), then increased 5 ^{o}C/min to 180 ^{o}C and 20 ^{o}C/min to 240 ^{o}C. Secondary oven was run at temperatures 5 ^{o}C higher than in the first one. Transfer line was 250 ^{o}C. Injection port temperature was 220 ^{o}C (in case of liquid injections to identify compounds in SAFE extracts) and 260 ^{o}C (in case of samples run using SPME).

2.5. Statistical analysis

A discriminant analysis (DA) was performed using the software Statistica, version 6, from Statsoft, Tulsa, USA. DA was used on a 16 x 12 matrix, containing all the

16 samples characterized by their volatile compounds (12) identified by CG-O, except ethyl isobutyrate, 2,4-heptadienal and *trans*-2-nonenal, as their concentrations were rather low and constant throughout the samples. DA was performed to determine which of these compounds discriminate among the 4 groups of olive oils obtained from olives harvested in the same period (Galega harvested in October and November and Cobraçosa harvested in October and November). These groups were *a priori* defined (Burgard & Kuznicki, 1990). In DA, the basic underlying idea is to see whether groups differ with regard to the mean of a variable and then use that variable to predict group membership. The procedure is identical to the one-way analysis of variance or to the multivariate analysis of variance if several variables are used (Bofinger, 1975; Burgard & Kuznicki, 1990).

The discrimination model was built by forward stepwise analysis using the following options: tolerance of 0.010; F to enter equal to 1.00 and F to remove equal to 0.00. The classification functions obtained to characterize each group of samples can be used in future to determine to which group, each unknown sample most likely belongs. The classification matrix shows the number of samples used in the DA and of known group origin, that were correctly classified and those that were misclassified.

3. Results and discussion

3.1. Olive oils characterization

Fatty acids profiles of analyzed olive oil samples differed between olive cultivars: palmitic (C16:0), palmitoleic (C16:1) and oleic acid (C18:1) contents in Galega oils were higher than in Cobrançosa oils (Table 1). In what concerns polyunsaturated fatty acids (PUFA), mean linoleic acids (C18:2) weight percentage was 4.1 % in Galega and 8.4 % in Cobrançosa olive oils. Together with oleic acid content, they are the main differences between the two oils (> 4%). The amounts of stearic (C18:0) and linolenic acids (C18:3) were higher for Cobrançosa oils. The differences in remaining fatty acids were of a lesser importance. According to the quality criteria defined by the European Union, acidity, peroxide value and UV absorbances, all the samples used in this study can be labeled as “Extra Virgin Olive Oil”. However, Cobrançosa olive oils

presented higher peroxide and UV absorbances values, which may be ascribed to their higher content of PUFA, which are rather prone to oxidation.

Table 1. Fatty acid composition (%) and quality criteria (acidity, peroxide index and UV absorbances) of Galega Vulgar and Cobrançosa virgin olive oils. In each row superscript indexes indicate differences based on Tukey test. No indexes indicate no differences between samples.

	Galega Vulgar		Cobrançosa	
	October	November	October	November
C16:0	16.32 ^{b}	15.97 ^{b}	15.30 ^{b}	14.24 ^{a}
C16:1	2.16 ^{b}	2.29 ^{b}	1.22 ^{a}	1.07 ^{a}
C17:0	0.12	0.11	0.13	0.14
C17:1	0.33 ^{b}	0.31 ^{ab}	0.23 ^{a}	0.24 ^{a}
C18:0	1.74 ^{a}	1.77 ^{a}	2.96 ^{b}	3.27 ^{b}
C18:1	73.81 ^{b}	73.72 ^{b}	70.53 ^{a}	70.63 ^{a}
C18:2	3.85 ^{a}	4.32 ^{a}	7.84 ^{b}	8.90 ^{b}
C18:3	0.78	0.77	0.70	0.64
C20:0	0.38 ^{a}	0.35 ^{a}	0.50 ^{b}	0.49 ^{b}
C20:1	0.28 ^{b}	0.25 ^{b}	0.21 ^{a}	0.20 ^{a}
C22:0	0.10	0.11	0.11	0.12
Acidity (% oleic acid)	0.27 ^{b}	0.19 ^{a}	0.33 ^{b}	0.33 ^{b}
Peroxide Index (meq O_2 kg^{-1})	5.49 ^{a}	4.45 ^{a}	7.41 ^{b}	7.37 ^{b}
K_{270}	0.137 ^{a}	0.120 ^{a}	0.209 ^{b}	0.190 ^{b}
K_{232}	1.221 ^{a}	1.233 ^{a}	1.476 ^{b}	1.379 ^{b}

3.2. GC-O analyses of Galega and Cobrançosa olive oils

Screening volatile compounds by SPME-GCxGC ToF-MS resulted in over 300 chromatographic peaks for each cultivar. When the main peaks were considered (100-120) these compounds could be classified into several classes – aldehydes, ketones, esters and alcohols – to name the dominant ones. Figure 1 shows area percent of peaks of compounds representing these classes in both cultivars. The high amount of data generated by GCxGC ToF-MS (Figure 2) shows that the evaluation of odorants in VOO should be sensory oriented. To select volatile compounds for quantitative analyses, gas chromatography – olfactometry was performed for the olive oils from both varieties. It must be referred that the influence on sensory detection of each volatile compound depends on its concentration and odor threshold, which determine the odor activity value (OAV) (Belitz et al., 2009).

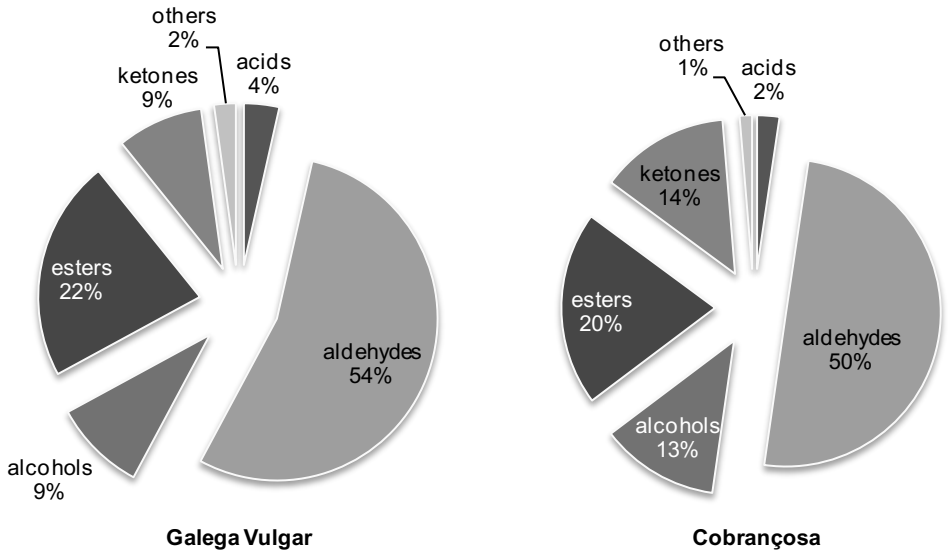

Figure 1. Main classes of volatile compounds in Galega Vulgar and Cobrançosa virgin olive oils evaluated by SPME-GCxGC ToFMS.

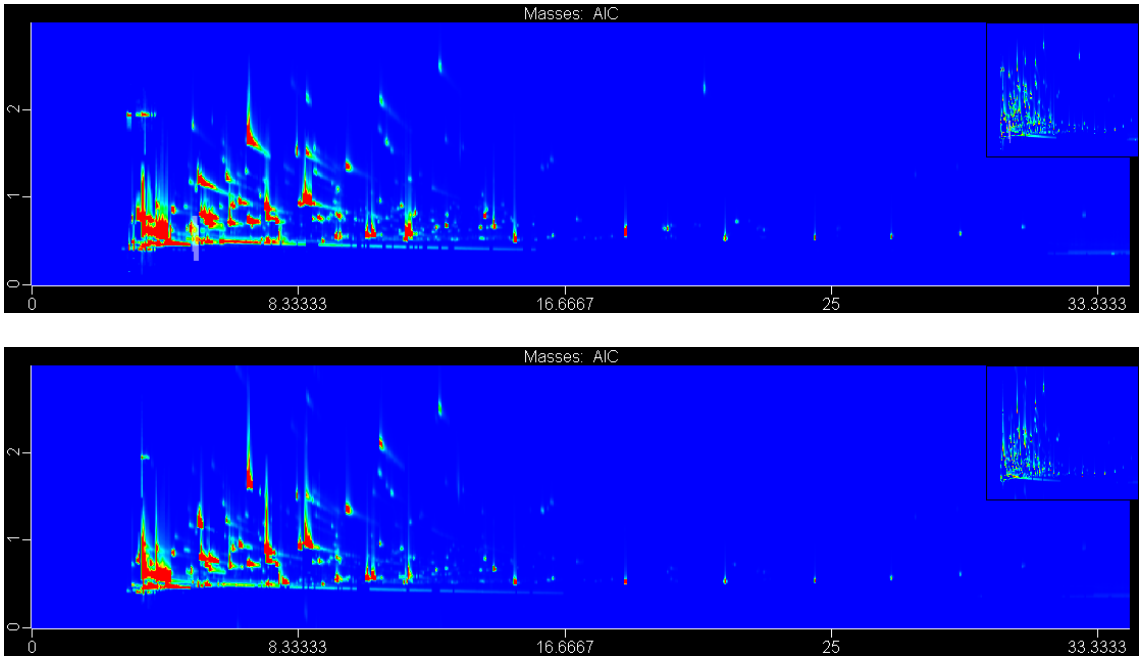

Figure 2. Example of a contour plot for Galega (top) and Cobrançosa (bottom) VOO obtained by CGxGC ToFMS.

Fifteen main odor fractions were detected in analyzed oils. The main odor notes and the respective odor threshold (OT) are listed in Table 2. They ranged from buttery, different fruity notes, fatty and fatty-soapy, as well as several green notes of different character. Compounds responsible for these odor impressions were identified using gas chromatography/mass spectrometry and confirmed by injecting authentic standards of the investigated compounds. The odorants more easily identified were those with descriptors “green”, also prominent when the olive samples were sensory characterized. Consumers dislike high intensities of bitter and pungent, whereas they like almost all aroma descriptors qualified with the adjective “green” (Morales, Angerosa, & Aparicio, 1999). However, "green" is not a single characteristic, but can have several different manifestations (Hongsoongnern & Chambers, 2008). Hexanal, *trans*-3-hexenal, *cis*-3-hexenal, *trans*-2-hexenal, hexyl acetate, *cis*-3-hexenyl acetate, hexan-1-ol, *trans*-3-hexen-1-ol, *cis*-3- hexen-1-ol, and *trans*-2-hexen-1-ol are examples of compounds that give a green-type description covering a wide range from mild green to intense cut grass, in accordance with the results obtained using pure compounds (Hatanaka, 1996). In Spanish olive oils, five compounds (hexanal, *trans*-3-hexenal, *cis*-3-hexenal, *trans*-2-hexenal and hexan-1-ol) had OAVs higher than 1, clearly contributing to the green aroma of olive oil. The other five volatile compounds having green or fruity odors evaluated by GC-O, but with OAVs lower than 1, do not clearly contribute to the green aroma of olive oil by themselves; however, their presence is important for its final overall aroma (Aparicio & Morales, 1998). The odorants with higher OAV are frequently essential for the aroma. However, there are exceptions where odorants with high OAVs are suppressed in the aroma and, conversely, compounds with lower OAVs are important contributors to the final aroma (Grosch, 2001). Another important feature is that the presence of high intensity of green smell enhances the bitterness perception (Caporale, Policastro, & Monteleone, 2004).

The green odorants identified were mainly produced from polyunsaturated fatty acids by the activity of each enzyme from the LOX pathway (Olias, Perez, Rios, & Sanz, 1993). The perception known as “green” odor notes are regarded as freshness and liveliness, which are characteristics of good quality virgin olive oils by consumers (Angerosa, 2002).

Table 2. Main odoriferous fractions of Galega Vulgar and Cobrançosa virgin olive oils detected by GC-O method (RI-retention index; OT-odor threshold, Reiners & Grosh, 1998; Morales et al., 2005)

	Odor	Compound	RI (SPB-5)	OT (µg/kg)
1	Buttery	2,3-butanedione	580	9.2
2	Pungent, green	1-penten-3-one	680	0.73
3	Fruity	Ethyl isobutyrate	757	1.2
4	Fruity	Methyl-2-methylbutyrate	778	0.25
5	Green (fresh cut grass)	Hexanal	803	80
6	Green	*Trans*-2-hexenal	850	420
7	Citrus	Ethyl-2-methylbutyrate	855	0.72
8	Flowery	Cis-3-hexen-1-ol	858	1100
9	Fatty soapy	Heptanal	903	500
10	Fruity	3-methylbutyl acetate	939	5
11	Mushroom	1-octene-3-ol	978	1
12	Banana	*Cis*-3-hexyl acetate	1009	200
13	Fatty	2,4-heptadienal	1011	3620
14	Soapy	Nonanal	1104	150
15	Fatty	*Trans*-2-nonenal	1162	900

Apart from the condition of the fruit at harvest, as the presence of pests and diseases, differences in post-harvest handling of the fruit, produce olive oils with different flavours and off-flavours (Angerosa et al., 2004; Morales, Luna, & Aparicio, 2005; Vichi, Romero, Gallardo-Chacón et al., 2009). Storage of the fruit after harvest and of the olive oil before reaching the consumer changes the volatile composition of olive oil. The highest sensory significance, evaluated by OAV, correspond to 1-octen-3-ol for mustiness-humidity, ethyl butanoate, propanoic and butanoic acids for fusty sensory defect, acetic acid, 3-methyl butanol and ethyl acetate for winey-vinegary and several saturated and unsaturated aldehydes and acids for rancid sensory defect (Morales et al., 2005). The presence of several volatile phenols was also ascribed to bad storage conditions of olives (Vichi, Romero, Gallardo-Chacón et al., 2009; Vichi, Romero, Tous, Tamames, & Buxaderas, 2008). The absence of the C6 aldehydes, alcohols and esters from the LOX pathway and the presence of many aldehydes from chemical oxidation, including hexanal from both chemical and enzymatic reactions, characterise the off-flavour of olive oil (Morales & Przybylski, 2000). In what concerns hexanal, when in lower amounts, it is described as green, while in higher amounts, it is described unpleasantly sebaceous. Dierkes et al. (2011) refer that only concentrations higher than 900 µg/kg have a negative impact on olive oil quality. Nonanal or the ratio hexanal/nonanal was proposed as an

appropriate way to measure olive oil oxidation (Angerosa, Mostallino, Basti, & Vito, 2000; Morales et al., 1997). Hexanal (fatty), 2-heptanal (oxidized, tallow), nonanal (fatty, waxy, painty) and decanal (penetrating, waxy) are examples of volatile compounds present in oxidized olive oils (Morales & Przybylski, 2000). As expected, compounds from final oxidation steps were not detected as olive oil extraction was performed under controlled conditions, as well as storage conditions of olive oils till the moment of analysis.

3.3. Optimization of SPME extraction parameters

Figure 3 shows the summary of the main parameters determined for SPME extraction. In the first stage, efficiency of fibers in extraction of volatiles was determined. Two of the examined fibers – CAR/PDMS and DVB/CAR/PDMS - acted in a similar way extracting vast amounts of volatiles in a 20 minute extraction at 40 ^{o}C. The amounts of extracted compounds for these two fibers were comparable except for 1-penten-3-one. It indicates that the main constituent of the fiber that adsorbs oil volatiles is Carboxen. The fiber containing DVB does not present important higher peak areas. The other fibers extracted minute amounts of volatiles when compared to the CAR/PDMS and DVB/CAR/PDMS fibers. The polymer based fibers are characterized by a high ability to adsorb volatiles when compared to fibers in which absorption takes place. As in polymer based fibers adsorption dominates, the question is whether such fiber provides sufficient linearity to quantify volatile compounds. Therefore, adsorption of tested compounds in a concentration range from 0.5 to 10 mg/L was evaluated. For both most efficient fibers, the linearity in the examined range was very good. The graph presented in Figure 3 presents data for DVB/CAR/PDMS fiber. Similarly, for CAR/DVB fiber the R^2 values were all above 0.98. Therefore, both fibers have almost the same affinity and usability in extraction of oil volatiles. Increasing temperature of extraction favors the migration of compounds into the headspace, which is reflected in the temperatures tested – the highest amounts of extracted compounds were obtained for 60^{o}C. In all cases, samples were preheated for 5 minutes at a given temperature followed by fiber exposure to the sample headspace. Volatiles were extracted for different time from 5 up to 60 minutes. Despite the highest peak areas obtained for 60 minutes sampling, 20 minutes extraction was chosen as a compromise between extraction efficiency and

analysis time. As summarized, SPME was used for the extraction of volatile compounds from oils by various authors (Jeleń, 2006). They noticed the high extraction efficiency of DVB/CAR/PDMS, PDMS/DVB and CAR/DVB fibers. Vichi, Pizzale, Conte, Buxaderas and López-Tamames (2003) found out the highest affinity of DVB/CAR/PDMS fiber for 6-carbon alcohols and that, for many compounds, the polymer based fibers did not attain equilibrium within 40 minutes. The influence of matrix/volatiles composition on the adsorption of particular compounds was investigated by Contini & Esti (2006). They noticed the loss of linearity for compounds present in high concentrations when PDMS/DVB fiber was used, where fiber saturation and compounds displacement can be responsible for this phenomenon. As checked out in the present study for both recommended fibers high linearity was achieved in a range of 0.5 – 10 mg/L. Thus optimized parameters were used for the extraction of volatiles from analyzed olive oil samples.

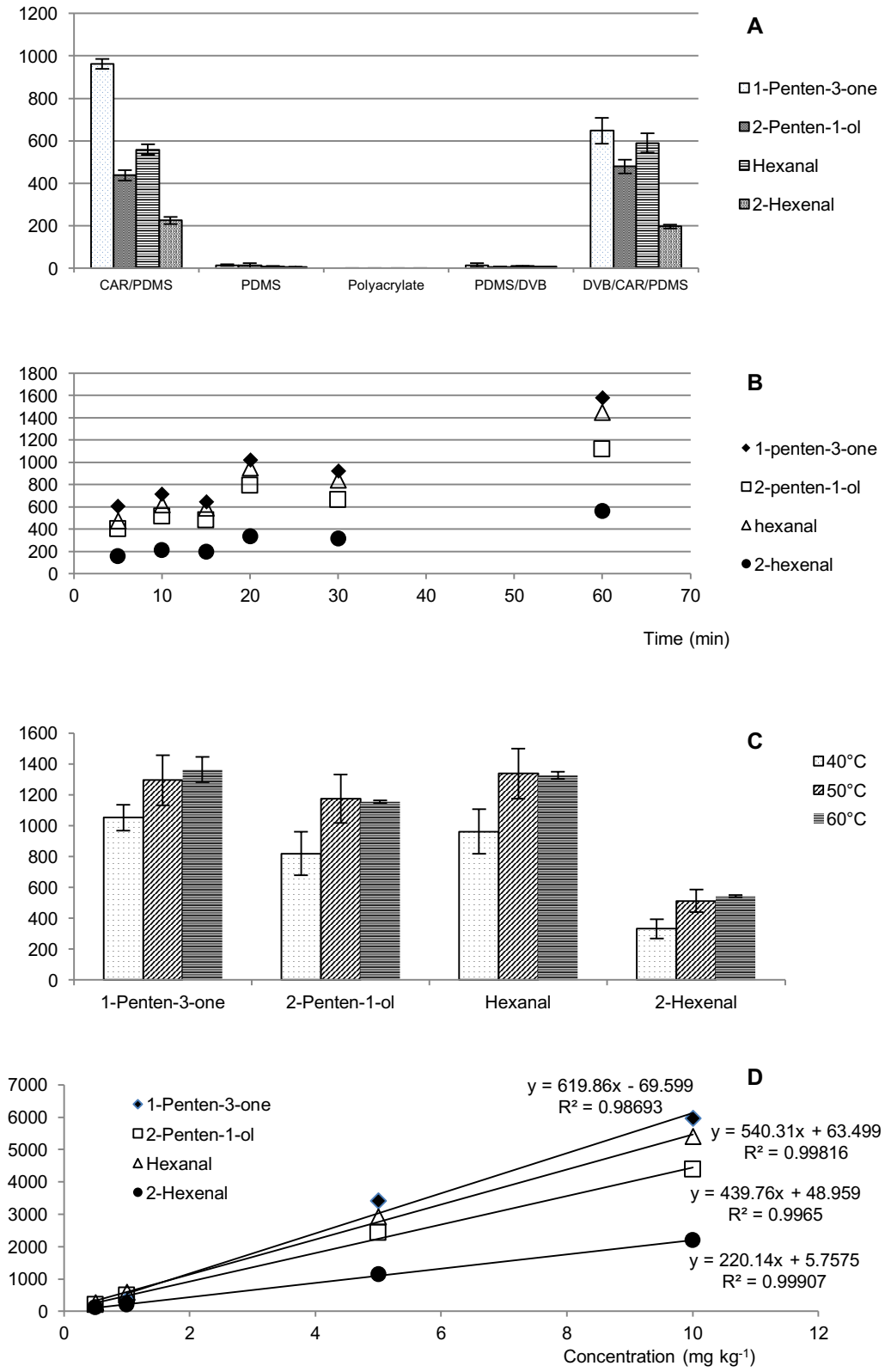

Figure 3. Main parameters determined for SPME extraction (A - efficiency of extraction using different fibers and, for DVB/CAR/PDMS, B - extraction time, C - extraction temperature, C - linearity).

3.4. Comparison of aroma compounds in Galega and Cobrançosa olive oils

For olive oils comparison, 15 aroma compounds were chosen based on GC-O evaluation of main odor fractions in these olive oils. Additionally, seven compounds were selected based on literature data on olive oil aroma compounds: decanal (penetrating, sweet waxy odor; Kalua et al., 2007), *cis*-2-pentene-1-ol (banana; Morales et al., 1997), *trans*-hexen-1-ol (green, grassy; Kalua et al., 2007), hexyl acetate (green fruity, sweet; Kalua et al., 2007) and 2-pentylfurane (butter, green beans; Belitz et al., 2009). Table 3 shows 22 aroma compounds quantified using headspace SPME-GCxGC-ToF-MS for both investigated monovarietal olive oils in the four harvest time/ripening stages. The amounts of compounds varied considerably and the most abundant one was *trans*-2-hexenal. The prevailing ones were also hexanal, nonanal, *cis*-3-hexen-1-ol and 2,3-butanedione. During fruit ripening, *trans*-2-hexenal showed an increase for both cultivars in the early stages of ripening studied. This is not in accordance with the results of Gómez-Rico et al. (2006) for Cornicabra (a Spanish cultivar), as they concluded that, for both rainfed and irrigated groves, *trans*-2-hexenal and hexanal showed a decrease along fruit ripening. However, research in VOO from other cultivars showed the same pattern observed in the VOO of our study in early stages of ripeness (Angerosa, 2002). As expected, the group of C6 aldehydes was the most abundant, which explains the high intensity of orthonasal perception of these oils (Cerretani et al., 2008). Volatile compounds in VOO do not come from the fruit itself, they are formed during processing, namely during crushing and thermobeating, which influence the presence or absence of specific odorant compounds. For instance, 1-penten-3-ol, octane, hexanal and *trans*-2-hexenal significantly discriminate thermobeating temperatures, and 2-penten-1-ol discriminate the time of thermobeating (Kalua et al., 2006). Moreover, Salas (2004) suggest that the conditions that promote hydroperoxide lyase (HPL) and inhibit alcohol dehydrogenases (ADH) and alcohol acyltransferase (AAT) activities can be applied to increase the green aroma. Similarly, the conditions that promote AAT activity can be applied to enhance the fruity aroma.

Table 3. Aroma compounds (mg/kg) in Galega Vulgar and Cobrançosa olive oils determined by SPME-GCxGC-ToF-MS. In each row superscript indexes indicate differences based on Tukey test. No indexes indicate no differences between samples.

	Compounds	Galega		Cobrançosa	
		October	November	October	November
1	hexanal	0.47 ± 0.20	0.89 ± 0.16	0.66 ± 0.32	0.69 ± 0.32
2	heptanal	0.14 ± 0.03 [b]	0.21 ± 0.04 [c]	0.06 ± 0.005 [a]	0.04 ± 0.02 [a]
3	nonanal	0.46 ± 0.10 [ab]	0.72 ± 0.10 [b]	0.37 ± 0.13 [a]	0.22 ± 0.07 [a]
4	decanal	0.02 ± 0.0008 [ab]	0.02 ± 0.001 [b]	0.01 ± 0.002 [a]	0.01 ± 0.003 [a]
5	*trans*-2-hexenal	2.30 ± 0.52 [a]	6.99 ± 1.57 [b]	1.85 ± 0.60 [a]	3.28 ± 1.42 [a]
6	*trans*-2-nonenal	0.03 ± 0.001	0.03 ± 0.001	0.03 ± 0.003	0.03 ±0.002
7	*trans, trans*-2,4-heptadienal	0.009 ± 0.001	0.01 ± 0.0007	0.01 ± 0.007	0.01 ± 0.001
8	*trans, trans*-2,4-decadienal	nd	nd	nd	nd
9	*cis*-2-penten-1-ol	0.32 ± 0.05 [ab]	0.25 ± 0.05 [a]	0.47 ± 0.08 [b]	0.46 ± 0.14 [b]
10	*cis*-3-hexen-1-ol	0.31 ± 0.38	0.54 ± 0.43	0.14 ± 0.03	0.17 ± 0.07
11	*trans*-2-hexen-1-ol	0.02 ± 0.007 [a]	0.05 ± 0.02 [b]	0.006 ± 0.004 [a]	0.009 ± 0.009 [a]
12	1-octene-3-ol	0.10 ± 0.03	0.10 ± 0.04	0.14 ± 0.04	0.13 ± 0.03
13	2,3-butanedione	0.54 ± 0.10	0.51 ± 0.08	0.60 ± 0.17	0.48 ± 0.20
14	6-methyl-5-hepten-2-one	0.35 ± 0.07	0.31 ± 0.06	0.33 ± 0.08	0.27 ± 0.07
15	1-penten-3-one	0.43 ± 0.22	0.20 ± 0.009	0.54 ± 0.19	0.54 ± 0.20
16	*cis*-3-hexenyl acetate	0.02 ± 0.02	0.03 ± 0.03	0.01 ± 0.01	0.02 ± 0.01
17	hexyl acetate	0.001 ± 0.0007	0.002 ± 0.001	0.0008 ± 0.005	0.002 ± 0.001
18	ethyl isobutyrate	0.004 ± 0.0007	0.06 ± 0.09	0.002 ± 0.002	0.002 ± 0.002
19	methyl-2-methylbutyrate	0.06 ± 0.03	0.06 ± 0.02	0.03 ± 0.009	0.05 ± 0.004
20	ethyl-2-methylbutyrate	0.01 ± 0.004	0.07 ± 0.09	0.006 ± 0.0009	0.007 ± 0.0006
21	3-methylbutyl acetate	0.27 ± 0.10	0.29 ± 0.13	0.21 ± 0.06	0.21 ± 0.06
22	2-pentylfuran	0.003 ± 0.002 [a]	0.005 ± 0.0004 [ab]	0.004 ±0.002 [ab]	0.007± 0.003 [b]
	Ripeness Index	2.3 ± 0.5 [a]	3.9 ± 0.5 [b]	1.9 ± 0.7 [a]	3.6 ± 0.6 [b]

Prenzler et al. (2007) refer that aroma volatiles are not present in significant amounts in fresh olive oils and that during the thermobeating step, in presence of very high concentrations of phenolic compounds, flavour development might be hindered. This could be expected in this study, since total phenols from both monovarietal olive oils were very high (data not shown). It should be noted that these conclusions can be quite different if the thermobeating step is performed in the absence of O_2 (Servili, Selvaggini, Taticchi, Esposto, & Montedoro, 2003).

In order to investigate which aroma compounds could discriminate among the two different periods of harvesting/ripening stages for both cultivars, a discriminant analysis was performed.

Concerning the harvesting time, 9 volatiles showed to have discriminant power among samples, namely heptanal, *trans*-2-hexenal, 1-octen-3-ol, nonanal, 2, 3-butanedione, ethyl-2-methylbutyrate, hexanal, *cis*-3-hexenylacetate and 3-

methylbuthylacetate. Table 4 presents the coefficients of the linear classification functions, derived by a stepwise discriminant analysis, describing each harvesting time for each cultivar. These functions can be used to determine to which group each case most likely belongs. In these functions, only the compounds with discriminant power were retained.

Table 4. Coefficients of the linear classification functions, derived by stepwise discriminant analysis, describing each group of olive oils from the same period of harvesting.

	Galega Vulgar		Cobrançosa	
	October (GaOct)	November (GaNov)	October (CobOct)	November (CobNov)
Heptanal	15075,48	24115,7	8931,70	9126,39
Trans-2-hexenal	160,28	291,6	91,43	97,41
1-octen-3-ol	4284,20	6776,2	2854,88	2968,67
Nonanal	-14,58	502,2	-147,04	-165,53
2,3-butadione	-1069,21	-2213,8	-478,34	-502,23
ethyl-2-methylbutyrate	-5663,01	-9238,1	-3090,18	-3075,84
Hexanal	857,07	1427,1	503,92	512,86
Cis-3-hexenylacetate	-8436,52	-14816,9	-4512,18	-4601,62
3-methylbutyl acetate	709,81	1160,2	328,47	316,71
Constant	-1398,34	-3793,0	-532,49	-556,54

When the observed classifications were compared to those predicted by these classification functions, 93.8 % of the samples were correctly classified in terms of harvesting period and cultivar (Table 5).

Table 5. Classification Matrix - The diagonal shows the number of samples correctly classified.

		Predicted classifications			
		Galega		Cobrançosa	
		October	November	October	November
Observed classifications	% Correct	GaOct	GaNov	CobOct	CobNov
GaOct	100	4	0	0	0
GaNov	100	0	4	0	0
CobOct	75	0	0	3	1
CobNov	100	0	0	0	4
Total	93.75	4	4	3	5

For the harvesting period, only the samples from Galega oils were correctly separated by the groups *a priori* defined, while only 87.5 % of the samples from Cobrançosa oils were correctly classified (Table 5). This misclassification may be

due to similar volatile profiles of olive oils from Cobrançosa fruits collected in October and in November. Tura, Failla, Bassi, Attilio and Serraiocco (2013) confirmed that the ripening stage influence both *trans*-2-hexenal and 1-penten-3-one, as well as Garcia, Magalhães, Fregapane, Salvador and Paiva-Martins (2012) for the last compound. Prenzler et al. (2007) without pre-selection of volatile compounds, found that *trans*-2-hexenal, hexanol, 1-penten-3-ol and *cis*-2-penten-1-ol, contribute towards the discrimination of maturity stages.

Figure 4 shows the projections of the olive oil samples on the plane defined by the canonical roots 1 and 2, for the four groups of harvesting periods and cultivar *a priori* defined. In this plot, the clustering of the samples into the groups previously defined is well illustrated.

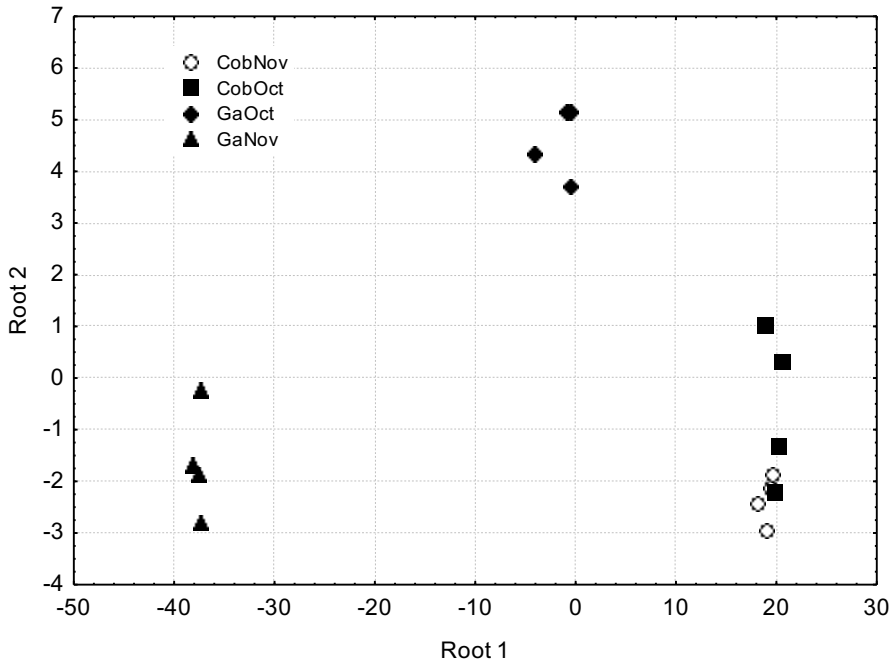

Figure 4. Score plots of EVOO samples on the planes defined by the canonical roots 1 and 2 samples, after performing DA.

4. Conclusion

In Galega and Cobrançosa olive oils, fifteen odorants were identified by GC-O. In fact, the majority of the volatile compounds identified by GC × GC-ToF-MS system could not be sensory detected, probably due to their low concentrations and/or high odor threshold. So, relatively few odorants can explain the aromatic profiles of each monovarietal olive oil.

Galega and Cobrançosa olive oils in early stages of ripening showed a very similar aroma profile by GC-O. For the quantification of volatile compounds, headspace solid phase microextraction (SPME) technique was optimized. Under optimized conditions, volatile compounds were quantified in all samples. *Trans*-2-hexenal was the most abundant compound ranging from 2.3 to 7.0 mg/kg in average, for Galega oils, and from 1.9 to 3.3 mg/kg for Cobrançosa oils.

By discriminant analysis, nine volatiles showed to have discriminant power among samples, from different cultivars and harvest times, namely heptanal, *trans*-2-hexenal, 1-octen-3-ol, nonanal, 2,3-butanedione, ethyl-2-methylbutyrate, hexanal, *cis*-3-hexenylacetate and 3-methylbuthylacetate.

As well as improving the health properties of EVOO, the challenge for the producers should be the enhancement of the sensory quality of fresh olive oils. The present study confirms the importance of the ripening stage in the amount of volatile compounds. Therefore, the decision of the harvesting date will allow the production of high quality VOO with different sensory notes.

Moreover, changes in the crushing and thermobeating steps or the blending of different VOO can be used to improve and promote the generation of other volatile compounds to produce EVOO with more complexity and harmony.

References

Angerosa, F. (2002). Influence of volatile compounds on virgin olive oil quality evaluated by analytical approaches and sensor panels. European Journal of Lipid Science and Technology, 104 (9-10), 639 - 660.

Angerosa, F., Mostallino, R., Basti, C., & Vito, R. (2000). Virgin olive oil odour notes: their relationships with volatile compounds from the lipoxygenase pathway and secoiridoid compounds. Food Chemistry, 68 (3), 283-287.

Angerosa, F., Servili, M., Selvaggini, R., Taticchi, A., Esposto, S., & Montedoro, G. (2004). Volatile compounds in virgin olive oil: occurrence and their relationship with the quality. Journal of Chromatography A, 1054 (1-2), 17-31.

Aparicio, R., & Luna, G. (2002). Characterisation of monovarietal virgin olive oils. European Journal of Lipid Science and Technology, 104 (9-10), 614-627.

Aparicio, R., & Morales, M. T. (1998). Characterization of Olive Ripeness by Green Aroma Compounds of Virgin Olive Oil. Journal of Agricultural and Food Chemistry, 46 (3), 1116-1122.

Aparicio, R., Morales, M. T., & García-González, D. L. (2012). Towards new analyses of aroma and volatiles to understand sensory perception of olive oil. European Journal of Lipid Science and Technology, 114 (10), 1114-1125.

Baccouri, O., Cerretani, L., Bendini, A., Caboni, M. F., Zarrouk, M., Pirrone, L., & Miled, D. D. B. (2007). Preliminary chemical characterization of Tunisian monovarietal virgin olive oils and comparison with Sicilian ones. European Journal of Lipid Science and Technology, 109 (12), 1208-1217.

Belitz, H., Grosh, D. W., & Shieberle, P. (2009). Food Chemistry. Leipzig: Springer-Verlag Berlin Heidelberg.

Bofinger, V. J. (1975). An introduction to some multivariate techniques with applications in field experiments. In J. L. W. V.J. Bofinger (Ed.), Developments in Field Experiment Design and Analysis (pp. 73-83). Oxford, UK: Commonwealth Agricultural Bureaux.

Boskou, D. (2006). Olive Oil Chemistry and Technology. Ilinois: AOCS Press.

Burgard, D. R., & Kuznicki, J. T. (1990). Chemometrics: Chemical and Sensory Data. Boston (USA): CRC Press.

Cajka, T., Riddellova, K., Klimankova, E., Cerna, M., Pudil, F., & Hajslova, J. (2010). Traceability of olive oil based on volatiles pattern and multivariate analysis. Food Chemistry, 121 (1), 282-289.

Caporale, G., Policastro, S., & Monteleone, E. (2004). Bitterness enhancement induced by cut grass odorant (cis-3-hexen-l-ol) in a model olive oil. Food Quality and Preference, 15 (3), 219-227.

Cavalli, J.-F., Fernandez, X., Lizzani-Cuvelier, L., & Loiseau, A.-M. (2003). Comparison of Static Headspace, Headspace Solid Phase Microextraction, Headspace Sorptive Extraction, and Direct Thermal Desorption Techniques on Chemical Composition of French Olive Oils. Journal of Agricultural and Food Chemistry, 51 (26), 7709-7716.

Cerretani, L., Salvador, M. D., Bendini, A., & Fregapane, G. (2008). Relationship Between Sensory Evaluation Performed by Italian and Spanish Official Panels and Volatile and Phenolic Profiles of Virgin Olive Oils. Chemosensory Perception, 1 (4), 258-267.

Contini, M., & Esti, M. (2006). Effect of the matrix volatile composition in the headspace solid-phase microextraction analysis of extra virgin olive oil. Food Chemistry, 94 (1), 143-150.

Dierkes, G., Bongartz, A., Guth, H., & Hayen, H. (2011). Quality Evaluation of Olive Oil by Statistical Analysis of Multicomponent Stable Isotope Dilution Assay Data of Aroma Active Compounds. Journal of Agricultural and Food Chemistry, 60 (1), 394-401.

EC. (2008). Regulation EC Nº 640/2008.

Engel, W., Bahr, W., & Schieberle, P. (1999). Solvent assisted flavour evaporation – a new and versatile technique for the careful and direct isolation of aroma compounds from complex food matrices. European Food Research and Technology, 209 (3), 237-241.

Frankel, E. N. (2010). Chemistry of Extra Virgin Olive Oil: Adulteration, Oxidative Stability, and Antioxidants. Journal of Agricultural and Food Chemistry, 58 (10), 5991-6006.

Garcia, B., Magalhães, J., Fregapane, G., Salvador, M. D., & Paiva-Martins, F. (2012). Potential of selected Portuguese cultivars for the production of high quality monovarietal virgin olive oil. European Journal of Lipid Science and Technology, 114 (9), 1070-1082.

Gómez-Rico, A., Salvador, M. D., La Greca, M., & Fregapane, G. (2006). Phenolic and Volatile Compounds of Extra Virgin Olive Oil (Olea europaea L. Cv. Cornicabra) with Regard to Fruit Ripening and Irrigation Management. Journal of Agricultural and Food Chemistry, 54 (19), 7130-7136.

Grosch, W. (2000). Specificity of the human nose in perceiving food odorants. In P. a. E. Schieberle, K.-H. (eds), (Ed.), Frontiers of Flavour Science. Proceedings of the Ninth Weurman Flavour Research Symposium (pp. 213-219). Garching: Deutsche Forschungsanstalt für Lebensmittelchemie.

Grosch, W. (2000) Specificity of the human nose in perceiving food odorants. In Schieberle, P. and Engel, K.-H. (eds), Frontiers of Flavour Science, Proceedings of the Ninth Weurman Flavour Research Symposium. Deutsche Forschungsanstalt für Lebensmittelchemie, Garching, pp. 213–219

Grosch, W. (2001). Evaluation of the Key Odorants of Foods by Dilution Experiments, Aroma Models and Omission. Chemical Senses, 26 (5), 533-545.

Hatanaka, A. (1996). The fresh green odor emmited by plants. Food Rev. Int., 12 (3), 303-350.

Hermoso, M., Uceda, M., Frias, L., & Beltran, G. (1997). Maduración. In D. Barranco, D. Fernandez-Escobar & L. Rallo (Eds.), El Cultivo del Olivo (pp. 137- 153). Madrid: Ediciones Mundi-Prensa.

Hongsoongnern, P., & Chambers, E. (2008). A lexicon for green odor or flavor and characteristics of chemicals associated with green. Journal of Sensory Studies, 23 (2), 205-221.

Jeleń, H. H. (2006). Solid-Phase Microextraction in the Analysis of Food Taints and Off-Flavors. Journal of Chromatographic Science, 44 (7), 399-415.

Jiménez, A., Beltrán, G., & Aguilera, M. P. (2004). Application of solid-phase microextraction to the analysis of volatile compounds in virgin olive oils. Journal of Chromatography A, 1028 (2), 321-324.

Kalua, C. M., Allen, M. S., Bedgood, J. D. R., Bishop, A. G., Prenzler, P. D., & Robards, K. (2007). Olive oil volatile compounds, flavour development and quality: A critical review. Food Chemistry, 100 (1), 273-286.

Kalua, C. M., Bedgood, D. R., Bishop, A. G., & Prenzler, P. D. (2006). Changes in Volatile and Phenolic Compounds with Malaxation Time and Temperature during Virgin Olive Oil Production. Journal of Agricultural and Food Chemistry, 54 (20), 7641-7651.

Kanavouras, A., Kiritsakis, A., & Hernandez, R. J. (2005). Comparative study on volatile analysis of extra virgin olive oil by dynamic headspace and solid phase micro-extraction. Food Chemistry, 90 (1-2), 69-79.

Morales, M. T., Angerosa, F., & Aparicio, R. (1999). Effect of the extraction conditions of virgin olive oil on the lipoxygenase cascade: Chemical and sensory implications. Grasas Y Aceites, 50 (2), 114-121.

Morales, M. T., Luna, G., & Aparicio, R. (2005). Comparative study of virgin olive oil sensory defects. Food Chemistry, 91 (2), 293-301.

Morales, M. T., & Przybylski, R. (2000). Olive oil oxidation. In J. Harwood & R. Aparicio (Eds.), Handbook of olive oil (pp. 459-490). Maryland: Aspen Publishers, Inc.

Morales, M. T., Rios, J. J., & Aparicio, R. (1997). Changes in the Volatile Composition of Virgin Olive Oil during Oxidation: Flavors and Off-Flavors. Journal of Agricultural and Food Chemistry, 45 (7), 2666-2673.

Morales, M. T., & Tsimidou, M. (2000). The role of volatile compounds and polyphenols in olive oil sensory quality. In J. Harwood & R. Aparicio (Eds.), Handbook of olive oil (pp. 393-458). Maryland: Aspen Publishers, Inc.

Olias, J. M., Perez, A. G., Rios, J. J., & Sanz, L. C. (1993). Aroma of virgin olive oil: Biogenesis of the "green" odor notes. Journal of Agricultural and Food Chemistry, 41 (12), 2368 - 2373.

Prenzler, P., Robards, K., & Bedgood, D. (2007). Quality Enhancement of Australian Extra Virgin Olive Oils. In (pp. 116): Rural Industries Research and Development Corporation.

Reboredo-Rodríguez, P., González-Barreiro, C., Cancho-Grande, B., & Simal-Gándara, J. (2013). Effects of Sedimentation Plus Racking Process in the Extra Virgin Olive Oil Aroma Fingerprint Obtained by DHS–TD/GC–MS. Food and Bioprocess Technology, 6 (5), 1290-1301.

Reiners, J., & Grosch, W. (1998). Odorants of Virgin Olive Oils with Different Flavor Profiles. Journal of Agricultural and Food Chemistry, 46 (7), 2754-2763.Salas, J. J. (2004). Characterization of Alcohol Acyltransferase from Olive Fruit. Journal of Agricultural and Food Chemistry, 52 (10), 3155-3158.

Servili, M., Selvaggini, R., Taticchi, A., Esposto, S., & Montedoro, G. F. (2003). Volatile Compounds and Phenolic Composition of Virgin Olive Oil: Optimization of Temperature and Time of Exposure of Olive Pastes to Air Contact during the Mechanical Extraction. Journal of Agricultural and Food Chemistry, 51, 7980-7988.

Tura, D., Failla, O., Bassi, D., Attilio, C., & Serraiocco, A. (2013). Regional and cultivar comparison of Italian single cultivar olive oils according to flavor profiling. European Journal of Lipid Science and Technology, 115 (2), 196-210.

Vaz-Freire, L. T., Silva, M. D. R. G., & Freitas, A. M. C. (2009). Comprehensive two-dimensional gas chromatography for fingerprint pattern recognition in olive oils produced by two different techniques in Portuguese olive varieties Galega Vulgar, Cobrançosa e Carrasquenha. Analytica Chimica Acta, 633 (2), 263-270.

Vichi, S., Castellote, A. I., Pizzale, L., Conte, L. S., Buxaderas, S., & López-Tamames, E. (2003). Analysis of virgin olive oil volatile compounds by headspace solid-phase microextraction coupled to gas chromatography with mass spectrometric and flame ionization detection. Journal of Chromatography A, 983 (1–2), 19-33.

Vichi, S., Pizzale, L., Conte, L. S., Buxaderas, S., & López-Tamames, E. (2003). Solid-Phase Microextraction in the Analysis of Virgin Olive Oil Volatile Fraction: Modifications Induced by Oxidation and Suitable Markers of Oxidative Status. Journal of Agricultural and Food Chemistry, 51 (22), 6564-6571.

Vichi, S., Romero, A., Gallardo-Chacón, J., Tous, J., López-Tamames, E., & Buxaderas, S. (2009). Volatile phenols in virgin olive oils: Influence of olive variety on their formation during fruits storage. Food Chemistry, 116 (3), 651-656.

Vichi, S., Romero, A., Gallardo-Chacón, J., Tous, J., López-Tamames, E., & Buxaderas, S. (2009). Influence of Olives' Storage Conditions on the Formation of Volatile Phenols and Their Role in Off-Odor Formation in the Oil. Journal of Agricultural and Food Chemistry, 57 (4), 1449-1455.

Vichi, S., Romero, A., Tous, J., Tamames, E. L., & Buxaderas, S. (2008). Determination of volatile phenols in virgin olive oils and their sensory significance. Journal of Chromatography A, 1211 (1-2), 1-7.

Chapter 5

Bioactive compounds of Portuguese virgin olive oils discriminate cultivar and ripening stage

Submitted

Abstract

The presence of different bioactive compounds in virgin olive oil affects its nutritional, oxidative and sensorial properties. Phenolic compounds are olive endogenous bioactive compounds highly susceptible to degradation. Olive endogenous oxidoreductases, mainly polyphenol oxidases (PPO) and peroxidases (POD), may play an important role on the profile of bioactive compounds in olive oil by promoting oxidation of phenolic compounds. The aim of this study was to evaluate if changes on PPO and POD activities in olive fruits from two Portuguese cultivars (*Olea europaea*, cv 'Cobrançosa' and cv 'Galega Vulgar') are related with the composition of their olive oils, especially phenolic compounds. Pattern recognition techniques (Principal Component Analysis, PCA, Cluster Analysis, CA, and Discriminant Analysis, DA) were used for multivariate data analysis. Olive oils characterized by their FA composition were grouped by cultivar. When olive oils were characterized by their phenolic composition, green pigments, and enzymatic activities in fruits, they could be discriminated by olive ripening stage. Along ripening, PPO activity was only detected in the fruit mesocarp of both cultivars and POD activity was mainly detected in the seeds. The POD activity, as well as vanillin and gamma-tocopherol contents in olive oil increased with the ripening index. Conversely, higher PPO activity in fruits at early ripening stages together with higher levels of total phenols, green pigments, beta-tocopherol, hydroxytyrosol and *p*-coumaric acid in olive oils were observed. The ripening stage of fruits showed to be a key factor on the amount and profile of bioactive compounds of olive oil.

Keywords: 'Cobrançosa', 'Galega Vulgar', olive oil, oxidoreductases, phenols, tocopherols

1. Introduction

Extra virgin olive oil (EVOO) phenolic compounds play an important role in organoleptic characteristics namely in attributes related to bitterness and pungency [1, 2]. Moreover, the scientific evidence is already strong enough to enable the legal use of health claims for phenolic compounds on labels of EVOO [3]. The shelf life of EVOO, which is higher than in other vegetable oils, is mainly due to fatty acid composition and to the presence of phenolic molecules having a catechol group, such as hydroxytyrosol and its secoiridoid derivatives [4].

The hydrophilic phenolic compounds of EVOO belong to different classes: phenolic alcohols (hydroxytyrosol and tyrosol), phenolic acids and derivates (vanillic acid and vanillin, respectively), lignans (pinoresinol and acetoxypinoresinol), flavonoids (luteolin and apigenin) and secoiridoid derivates (dialdehydic form of elenolic acid linked to hydroxytyrosol (3,4-DHPEA-EDA, or oleacein), dialdehydic form of elenolic acid linked to tyrosol (p-HPEA-EDA or oleocanthal), aldehydic form of elenolic acid linked to tyrosol (p-HPEA-EA), 4-(acetoxyethyl)-1,2-dihydroxybenzene (3,4-DHPEA-AC), oleuropein aglycone (3,4-DHPEA-EA) and its methylated form (methyl 3,4-DHPEA-EA) [4-7].

The lipophilic phenols present in olive oil are tocopherols and tocotrienols. Alpha-tocopherol is considered the major tocopherol of olive oil, since the rest of the tocopherols represent less than 5% of total tocopherols [8]. When phenolic compounds like 3,4-dihydroxy phenylethanol, 3,4-DHPEA-EDA and 3,4-DHPEA-EA are in association with α-tocopherol, they have a synergistic antioxidant effect [9]. Thus, the initial amount of α-tocopherol is one of the components that influences the susceptibility of virgin olive oil (VOO) to oxidation [10].

The phenolic fraction of EVOO can be modified by the activity of (i) hydrolytic enzymes that catalyze the release of aglycon secoiridoids from their respective glucoside forms and by (ii) oxidative degradation activities catalysed by two enzymes – polyphenol oxidases (PPO) and peroxidases (POD). These enzymes are believed to have a negative effect on olive oil quality, not only in relation to its sensory properties but also by decreasing its level of antioxidants [11, 12]. PPO (EC 1.14.18.1) is the main enzyme involved in the oxidation of phenols, which is performed by two different reactions in the presence of oxygen: the

hydroxylation of monophenols to *o*-diphenols and the oxidation of *o*-diphenols to *o*-quinones, followed by condensation and polymerization reactions [13]. POD (EC 1.11.1.7) performs single-electron oxidation of a wide variety of compounds in the presence of hydrogen peroxide. Although the contribution of POD to the oxidation of phenols is limited by the low internal level of hydrogen peroxide, it has been proposed that PPO could act as promoter of POD activity, which could be due to the generation of hydrogen peroxide during the oxidation of phenolic compounds [14, 15].

Phenolic compounds are enzymatically oxidized by PPO, which results in color changes of olive pastes as soon as the rupture of olive fruit tissues occurs. In plants, the PPO activity occurs in damaged tissues by insect attack. Thus, the usually low phenolic compounds content in olive oils of the Portuguese cultivar 'Galega Vulgar' may be associated with the high susceptibility of this cultivar to pests and diseases [16]. Moreover, PPO seems to be the most relevant enzyme involved in phenolic oxidation in the milling process, while POD enzymes are mainly active in the malaxation process [17]. Therefore, the study of the influence of enzymatic activity on EVOO phenolic compounds profile changes is a current research challenge [18, 19] since EVOO processing should include, as a main objective, the improvement of sensory and nutritional properties [20].

Cultivar, ripening stage of the fruit and cultural practices such as irrigation, are the main agronomic factors affecting olive oil fatty acids, phenols and volatile composition, with a great impact on sensory and nutritional characteristics [21-25]. It is expected that changes of phenolic compounds and other compounds like pigments, as well as in enzymatic activity levels, occur during ripening [26-28].

The aim of this study was to evaluate if changes on oxidoreductase (PPO and POD) activities in olives (fruit and seeds) from two of the main Portuguese olive cultivars (*Olea europaea*, cv 'Cobrançosa' and cv 'Galega Vulgar'), along ripening, are related with the composition of their olive oils. Special attention will be drawn to the phenolic compounds in olive oils extracted from olives at different ripening stages.

2. Materials and methods

2.1. Plant material characterization

A trial was conducted in two olive groves under integrated production, located in Beira Baixa (39º 49' N, 7º 27'W and 39º 50´N, 7º 42W) an inland region in the centre of Portugal. Olive fruits (*Olea europaea* L.) cvs. 'Galega Vulgar' and 'Cobrançosa' were picked from the beginning of October till the second fortnight of November. Each sample of healthy olives (5 kg) was characterized by its ripening index following the guidelines of the "Estación de Olivicultura y Elaiotecnia", Jaén, Spain [29]. This method is based on color changes of epicarp and mesocarp of 100 fruits randomly collected from 2 kg of olives which are divided into eight groups: intense green (group 0), yellowish-green (group 1), green with reddish spots (group 2), reddish or light violet (group 3), black with white mesocarp (group 4), black with less than 50 % purple mesocarp (group 5), black with 50 % or more purple mesocarp (group 6) and black with 100 % purple mesocarp (group 7). The ripening index (RI) is expressed as follows:

$$RI = \Sigma(Ni \times ni)/100 \qquad \text{(eq.1)}$$

where Ni is the group number and ni is the number of fruits in that group (I=0,…7).

The moisture (assayed by oven at 103 ºC) and fat content (assayed by Soxtec) of the fruits were also evaluated. Only healthy fruits were selected for enzyme activity assays and for olive oil extraction. Olive oil samples were grouped into four groups according to their ripening stage: RS1 (Ripening index 0.5 - 1.4); RS2 (Ripening index 1.5 - 2.4); RS3 (Ripening index 2.5 - 3.4) and RS4 (Ripening index 3.5 - 4.6).

2.2. Enzymatic assays

Acetone powders were prepared by homogenizing destoned olive fruits and seeds with cold acetone (-20 °C) in a mechanical stirrer for 2 min, followed by filtration in fiber glass filters, washing the pellet with cold acetone (-20 °C) until total removal of pigments, followed by drying at room temperature with nitrogen [30, 31]. POD and PPO extracts were prepared from 0.4 g of acetone powder suspended in 5 mL of extraction buffer (0.05 M potassium phosphate, pH 6.2 containing 1 M KCl) [12] and 2% (w/w) of polyvinylpyrrolidone (PVP) and stirred

for 30 min, 4 ºC, 400 rpm; the suspension was centrifuged at 12000 rpm for 30 min and filtered (0.45 μm). Enzymatic activity assays were performed using continuous spectrophotometric methodologies. PPO was evaluated using 30 mM catechol as substrate based on the Oktay et al. methodology [32], following the increase in absorbance at 420 nm, during 1 min. One unit of PPO was defined as the quantity of enzyme that caused the absorbance variation of 0.001 min^{-1} mL^{-1} of enzyme extract, at room temperature. Results were expressed as Ug^{-1} of FW.

POD activity was performed using the procedure described by Gajewska et al. [33] following the increase in absorbance at 470 nm (2 min) using 30 mM guaiacol and 4 mM H_2O_2 as substrates. One unit of POD was defined as the consumption of 1 μmol of guaiacol min^{-1} mL^{-1} of enzyme extract, at room temperature using a molar absorptivity (ε) for tetraguaiacol of 26.6 $mM^{-1}cm^{-1}$. Results were expressed as Ug^{-1} of FW.

2.3. Olive oil extraction

Olive oils were extracted in a laboratory Abencor equipment, under previously optimized conditions [34]. The olives were crushed with a hammer mill equipped with a 4 mm sieve at 3000 rpm. Malaxation of the pastes was performed at 28-30 ºC for 30 min and centrifugation at 3500 rpm (3 min). After centrifugation, the olive oil was separated by settling in a graduated cylinder; water traces were removed with anhydrous sodium sulfate, filtered through a cellulose filter and stored in amber glass bottles at 4 ºC until analysis [34].

2.4. Olive oil analysis

Samples of olive oils were filtered and total phenols were determined within one day after extraction. Total phenols were extracted by solid phase extraction (SPE) columns filled with 1g of octadecyl (C_{18}) material from J.T. Baker, US and determined using Folin-Ciocalteau reactive with quantification by VIS spectroscopy at 765 nm [35]. The results were expressed in gallic acid equivalents (mg GAE kg^{-1}). Acidity, peroxide value (PV) and UV specific absorbances (K_{232} and K_{270}) were carried out following the analytical methods described in EEC/2568/91 EU Regulation [36]. Fatty acid composition was evaluated as fatty acid methyl esters by GC-FID, in a Hewlett Packard 6890, SP

column 2380^{TM} Supelco (60 m x 0.25 mm x 0.20 μm). Phenolic compounds were extracted from olive oil by liquid-liquid extraction using the procedure proposed by Pirisi et al. [37]. The phenolic extracts were analyzed by HPLC in an Agilent 1100 HPLC system, consisting of a degasser, a quaternary pump, an autosampler and a diode array detector (DAD). The stationary phase was a Purospher C18 analytical column (150 mm x 3.9 mm x 4 μm). The mobile phase was (A) 0.2 % H_3PO_4 (v/v) (B) methanol (C) acetonitrile and fed at a constant flow rate of 1 mL min^{-1}. The gradient program was the one proposed by the International Olive Oil Council (COI/T20/DOC.29) [38]. Quantification of phenolic compounds was carried out using the area values measurements at 280 nm for gallic acid, hydroxytyrosol, tyrosol, vanillic acid, caffeic acid, vanillin, ferulic acid, *o*-coumaric acid, at 320 nm for *p*-coumaric acid and at 360 nm for luteolin and apigenin. The quantification of individual phenols was only carried out for those compounds we had the standards. Quantitative assays were achieved using external calibration curves for all phenol standards (Sigma-Aldrich). Chlorophyll pigments were evaluated by VIS spectroscopy based on the IUPAC method proposed by Pokorny et al. [39]. Tocopherols were determined by HPLC in an Agilent 1100 Series chromatograph, using fluorescence detection with excitation set at 290 nm and emission set at 330 nm. A solution of oil in hexane was analyzed on a Lichrosorb Si 60 column (5μm) at room temperature, which was eluted with *n*-hexane and 2-propanol (99.5:0.5, v/v), with a flow rate of 1.2 mL min^{-1}. Organoleptic assessment, according to Regulation Nº 1343/2013 [40], as well as specific absorbances at 225 nm, according to Gutiérrez -Rosales et al. [41] were performed for the olive oils obtained in the highest ripening stage of each cultivar (RI 4).

2.5. Statistical analysis

Statistical analysis was performed using the software StatisticaTM", version 6, from Statsoft, Tulsa, OK, USA. Data was analyzed by univariate procedures (ANOVA) in order to identify the differences for each FA between the olive oils from both cultivars, as well as to evaluate the presence of significant differences for each variable among groups of cultivars and ripening stages (Duncan test). Multivariate data were processed by principal component analysis (PCA), cluster analysis (CA) and discriminant analysis (DA). Both

PCA and CA are unsupervised techniques used to characterize samples in a hyperspace and eventually to identify groups of similar samples. By PCA, the original hyperspace defined by m axes, corresponding each axis to an independent variable, will be replaced by a new system where the new axes are the principal components. Since principal components (PC) show decreasing variance, it is possible to represent the original samples on a smaller space defined by the first principal components without considerable loss of information [42]. The identification of possible groups of similar samples, as well as the identification of the most important original variables on the description of each set of samples is sometimes possible by PCA. After, the hierarchic cluster analysis was used to investigate the existence of the groups of samples suggested by PCA. The average Euclidean distance was used to measure the distance between samples and the single linkage method was used for aggregation [43].

PCA and CA were performed on (i) a matrix containing the EVOO samples defined by 13 fatty acids and (ii) a matrix containing the EVOO samples described by the enzymatic activities in fruit mesocarp and in seeds, ripening index of olives, tocopherols, total phenol content, hydroxytyrosol, tyrosol, vanillic acid, vanillin, *p*-coumaric acid, luteolin, apigenin, tocopherols (α, β and γ) and chlorophyll pigments of the extracted olive oils.

Discriminant analysis (DA) is a supervised pattern recognition technique because the groups of samples are *a priori* defined. DA was used on a 20 x 15 matrix, containing all the 20 samples characterized by their oxidoreductases activity (PPO in pulp and POD in seed), total phenols, hydroxytyrosol, tyrosol, vanillic acid, vanillin, *p*-coumaric acid, luteolin, apigenin, tocopherols (α, β and γ) and chlorophyll pigments in the olive oils. DA was performed to determine which of these compounds discriminate among the four groups of olive oils obtained from olives with similar ripening stage (RS1, RS2, RS3 and RS4). The discrimination model was built by forward stepwise analysis using the following options: tolerance of 0.010; F to enter equal to 1.00 and F to remove equal to 0.00. At each step, the contribution of each variable to discriminate among groups was evaluated.

3. Results and discussion

3.1. Olives characterization

During the harvest period, moisture content of the fruits of 'Cobrançosa' and 'Galega Vulgar' ranged between 53 and 60 % (Table 1). At each harvest moment, 'Cobrançosa' olives had always a lower ripening index than 'Galega Vulgar' fruits (Table 1). The mean values for oil content (DW) for both 'Cobrançosa' and 'Galega Vulgar' were 32.4 and 33.9 %, respectively.

Table 1. Ripening index, moisture, fat content (DW) of 'Galega Vulgar' and 'Cobrançosa' olive samples

	'Cobrançosa'		´Galega Vulgar'	
	Mean	Std.Dev.	Mean	Std.Dev
Ripening Index	2.3	0.95	3.2	0.84
Moisture (%)	57.3	1.88	55.9	2.79
Fat content (d.w.) (%)	32.4	3.96	33.9	4.94

3.2. Fatty acids composition and quality criteria for olive oils

The average fatty acid (FA) composition observed for olive oils from each cultivar is presented in Table 2.

Table 2. Fatty acid (%, w/w) composition (mean and standard deviation) for the 'Cobrançosa' and 'Galega Vulgar' olive oils.

Fatty Acids	'Cobrançosa'		´Galega Vulgar'	
	Mean	Std.Dev.	Mean	Std.Dev
C14:0	0.007	0.0005	0.010	0.0009
C16:0	13.12	0.644	15.19	0.641
C16:1	0.97	0.056	2.18	0.089
C17:0	0.14	0.005	0.12	0.012
C17:1	0.30	0.006	0.32	0.024
C18:0	3.41	0.304	1.70	0.064
C18:1	73.56	0.450	74.87	0.998
C18:2	6.83	0.527	4.13	0.361
C18:3	0.82	0.055	0.66	0.062
C20:0	0.43	0.023	0.31	0.019
C20:1	0.20	0.008	0.25	0.0169
C22:0	0.11	0.005	0.09	0.012
C24:0	0.06	0.009	0.05	0.010
MUFA	75.1	0.46	77.7	0.94
PUFA	7.6	0.50	4.8	0.43
SFA	17.3	0.435	17.5	0.70

For all olive oil samples, the fatty acids values are in accordance with EU Regulation Nº 1343/2013 [40]. A PCA was performed on a matrix containing 25 olive oil samples (13 Galega Vulgar, 12 Cobrançosa) characterized by 13 fatty acids. For each sample, at least the average value of 2 samples was considered. The PCA of this matrix of 25x13 shows that the initial hyperspace of 13 dimensions (one for each FA) can be reduced to a plane defined by the first two PC. This plane accounts for more than 90% of the variance of the original data. The projections of the loadings of the FA and of olive oil samples onto this plane are shown in Figure 1.

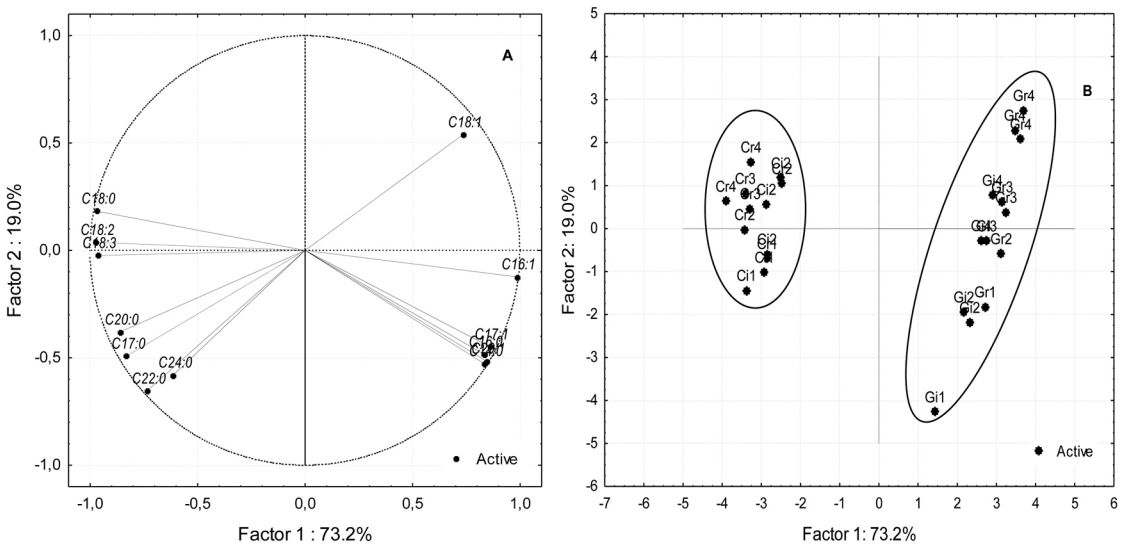

Figure 1. PCA analysis- projection of the loadings of fatty acid composition (A) and of the samples of the olive oils (B) from 'Galega Vulgar' (G) and 'Cobrançosa' (C) cultivars grown in orchard r or i, on the plane defined by the first two PC (factors 1 and 2).

The PCA of these results suggests that VOO samples can be grouped by cultivar. 'Cobrançosa' olive oils can be distinguished from 'Galega Vulgar' oils by the higher contents of PUFA (linoleic (C18:2) and linolenic (C18:3) fatty acids) and some SFA (margaric (C17:0), stearic (C18:0), araquidic (C20:0), behenic (C22:0) and lignoceric (C24:0) fatty acids). 'Galega Vulgar' olive oils are characterized by higher contents of oleic (18:1), palmitoleic (16:1), palmitic (C16:0), miristic (C14:0), margaroleic (C17:1) and eicosenoic (C20:1) fatty acids (Table 2). The separation of these olive oils by cultivar, as a function of their fatty acid composition, was confirmed by cluster analysis, where these groups are formed by cultivar, at a distance linkage of 1 (Figure 2). In fact, ANOVA showed

significant differences between cultivars for all fatty acids and groups, except for the sum of saturated fatty acids.

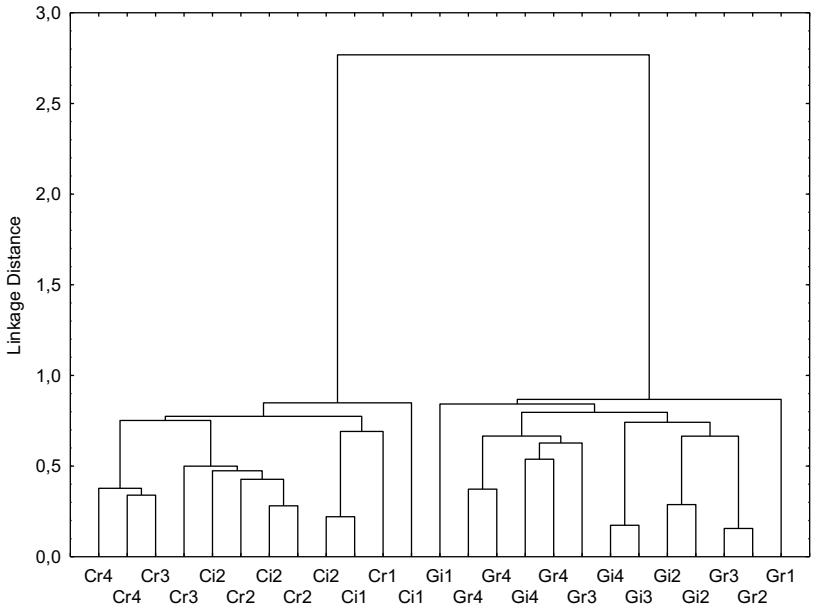

Figure 2. Cluster analysis of the samples of olive oils from‘Cobrançosa’ and ‘Galega Vulgar’ cultivars, characterized by their fatty acid composition, harvested in two olive groves (r and i)

In what concerns ‘Cobrançosa’ olive oils, the fatty acid composition has several consequences in the quality criteria of the oils. The higher content of PUFA (more susceptibility to oxidation) conducts to higher values for the oxidation indexes (PV and UV absorbances) (Table 3).

Table 3. Peroxide value, acidity and UV specific absorbances (K_{270} and K_{232}) of ‘Cobrançosa’ and ‘Galega Vulgar ‘olive oils.

Quality criteria	‘Cobrançosa’		‘Galega Vulgar’	
	Mean	Std.Dev	Mean	Std.Dev
Peroxide Value (meq O_2/kg)	10.2	3.67	7.4	3.36
Acidity (% oleic acid)	0.24	0.030	0.17	0.038
K_{270}	0.179	0.0203	0.116	0.0174
K_{232}	1.460	0.1207	1.339	0.0926

The fatty acid composition will also influence the type of volatile compounds that will be formed in the LOX pathway [44-46]. In fact, C6 aldehydes and alcohols and the corresponding esters, which are the most important compounds in the VOO aroma, are synthesized from polyunsaturated fatty acids containing a (Z,Z)-

1,4-pentadiene structure such as linoleic and linolenic acids [47]. Moreover, the degree of unsaturation of the lipid matrix can also influence the sensory perception, because pungency and bitterness are more pronounced in monounsaturated matrices than in polyunsaturated ones [48]. All quality criteria evaluated for olive oils are in conformity with EVOO category (Table 3).

3.3. PPO and POD activities in fruits and bioactive compounds in olive oils

Table 4 shows the average values for phenols, tocopherols, and chlorophyll pigments contents in olive oils and oxidoreductase activity (in mesocarp and seed) of olive fruits of 'Galega Vulgar' and 'Cobrançosa', grouped according to ripening stages.

PPO mesocarp activity was higher in 'Cobrançosa' olives than in 'Galega Vulgar' fruits. POD activity was detected predominantly in the seed, while no PPO activity was detected in the seed, as already described for other cultivars [11, 15].

POD activity measured in the seeds of 'Galega' and 'Cobrançosa' were significantly higher for the RS4 ripening stage. PPO activity measured in the fruit mesocarp for each cultivar was not statistically different between ripening stages, showing that relatively constant values were achieved during the sampling period. This was also reported for Arbequina and Picual olive fruits after 28-30 weeks after flowering (WAF) [15] but not in other reported results [49] where PPO activity values, found in Arbequina fruit, decreased along ripening (between 24 and 28 weeks WAF).

However, it does not explain the total phenol content, since the content of the main hydrophilic phenols found in VOO is closely related to the activity of enzymes responsible for the hydrolysis of the main phenolic glycosides initially present in the olive tissue. Several experiments concluded that, in addition to PPO and POD, olive β-glucosidase is a key enzyme to determine the phenolic profile of VOO [49, 50]. So, a complex enzymatic system is involved in shaping the phenol profile of olive oils. The presence of hydrophilic phenols in VOO is related not only with the initial phenolic content of each cultivar but also with the

Table 4. Average values of phenols, tocopherols, chlorophyll pigments in olive oils and oxidoreductases activity (in mesocarp and seed) of olive fruits of 'Galega Vulgar' and 'Cobrançosa', grouped according to ripening stages (RS1, RS2, RS3 and RS4). In each row superscript indexes indicate differences based on Duncan test. Cobrançosa seed samples at RS3 were irreversibly lost.

Ripening stage (harvest month)	'Galega Vulgar'				'Cobrançosa'			
	RS1 (Oct)	RS2 (Oct)	RS3 (Oct-Nov)	RS4 (Nov)	RS1 (Oct)	RS2 (Oct-Nov)	RS3 (Nov)	RS4 (Nov)
Chlorophyll Pigments (mg kg^{-1})	76.6 ^{a}	42.4 ^{b}	21.04 ^{cd}	5.3 ^{d}	76.87 ^{a}	63.4 ^{a}	37.91 ^{bc}	12.0 ^{d}
Total Phenols (mg GAE kg^{-1})	810.3 ^{abc}	663.6 ^{bc}	657.2 ^{bc}	577.40 ^{c}	960.57 ^{a}	893.7 ^{ab}	937.2 ^{a}	723.0 ^{abc}
Hydroxytyrosol (mg kg^{-1})	1.86^{d}	2.52^{d}	2.04^{d}	2.67^{d}	11.73^{a}	8.41^{bc}	9.16^{b}	7.35^{c}
Tyrosol (mg kg^{-1})	2.42^{c}	2.75^{bc}	2.42^{c}	4.17^{ab}	5.41^{a}	4.81^{a}	4.70^{a}	4.89^{a}
Vanillic acid (mg kg^{-1})	0.61^{a}	0.37^{bc}	0.40^{bc}	0.54^{abc}	0.31^{c}	0.61^{ab}	0.33^{c}	0.42^{bc}
Vanillin (mg kg^{-1})	0.64^{ab}	0.70^{ab}	0.78^{b}	0.64^{ab}	0.35^{cd}	0.12^{d}	0.51^{bc}	1.20^{a}
p-coumaric acid (mg kg^{-1})	0.30^{cde}	0.24^{de}	0.22^{e}	0.22^{d}	0.40^{ab}	0.44^{a}	0.32^{bc}	0.38^{abc}
Luteolin (mg kg^{-1})	0.20^{c}	0.34^{c}	0.40^{c}	0.47^{c}	1.98^{b}	1.66^{b}	1.81^{b}	3.3^{a}
Apigenin (mg kg^{-1})	0.12^{d}	0.12^{d}	0.11^{d}	0.21^{ab}	0.21^{abc}	0.19^{bc}	0.15^{cd}	0.25^{a}
α-tocopherol (mg kg^{-1})	224.46^{ab}	205.72 ^{ab}	219.40 ^{ab}	151.97 ^{b}	243.38 ^{a}	195.49 ^{ab}	168.59 ^{ab}	210.25 ^{ab}
β-tocopherol (mg kg^{-1})	4.74 ^{a}	3.29 ^{cd}	3.35 ^{cd}	2.94 ^{d}	4.64 ^{a}	4.02 ^{b}	3.46 ^{c}	3.29 ^{cd}
γ-tocopherol (mg kg^{-1})	8.26 ^{cd}	9.82 ^{b}	11.54 ^{a}	11.41 ^{a}	7.77 ^{cd}	7.36 ^{d}	8.20 ^{cd}	9.32 ^{bc}
PPO mes activity (Ug^{-1}FW)	72.1 ^{c}	129.2 ^{bc}	140.0 ^{ab}	108.2 ^{bc}	154.2 ^{ab}	166.1 ^{ab}	159.9 ^{ab}	175.7 ^{a}
POD seed (Ug^{-1}FW)	7.03 ^{c}	9.61 ^{bc}	11.9 ^{abc}	13.6 ^{ab}	6.96 ^{c}	6.7 ^{c}	-	17.6 ^{a}
Ripening Index	1.4^{d}	2.5^{c}	3.4 ^{b}	4.0 ^{a}	1.1^{d}	2.2^{c}	3.2^{b}	3.7^{ab}

activity of hydrolytic and oxidative enzymes acting during milling and malaxation processes [50].

As previously referred, the olives used in this study were picked in early ripening stages (RI < 4.5) which can also be shown by the high mean values of chlorophyll pigments present in the olive oils from both cultivars (Table 4). However, a significant decrease in chlorophyll pigments with RS for both cultivars was observed, although it was more pronounced at the RS4 stage for 'Galega Vulgar' olive oil (chlorophyll pigments <10 mg kg^{-1}). This is very important from the consumer point of view, since the presence of high contents of chlorophyll pigments in olive oils produced from early harvest olives can be perceived as very negative by the consumer. Current consumers of EVOO are not used to olive oils with such high intensity of green color [51].

Total phenol contents, as well as tocopherols, during the harvest season were very high denoting the high quality of olives from which the olive oils were extracted. The results for tocopherols are in the range of those reported by other studies (84-463 mg kg^{-1}, for α -tocopherol) [8, 52]. α-tocopherol is the most important lipophilic phenol in both oils, contributing to more than 90 % of total tocopherol. γ-tocopherol content is higher in 'Galega Vulgar' olive oils than in 'Cobrançosa' olive oils which can contribute for the high oxidative stability of 'Galega Vulgar' olive oils [16]. This is particularly important when the phenol content is low, as the antioxidant activity increases from α to γ-tocopherol at room temperature [53].

Higher decrease in phenol content for "Galega Vulgar" olive oils in the last ripening stages was observed. When "Galega Vulgar" and "Cobrançosa" monovarietal olive oils from fruits in the last ripening stage (RS4) were sensory evaluated, they presented bitter taste intensities with median of 3 and 7, respectively. These values are in accordance with the specific absorbance at 225 nm (K_{225}) of 0.197 found for 'Galega Vulgar' oils and of 0.323 for 'Cobrançosa' oils.

From the profile of phenols assessed by HPLC, 'Cobrançosa' and 'Galega Vulgar' oils showed similar chromatograms where it was possible to identify and quantify seven compounds: hydroxytyrosol (HYT), tyrosol (TYR), vanillic acid, vanillin, *p*-

coumaric acid, luteolin and apigenin. The phenolic acids (gallic, ferulic, caffeic or *o*-coumaric) were not detected in both oils. As previously reported, no caffeic acid was found in 'Hojiblanca' and 'Picudo' olive oils, while ferulic and *o*-coumaric acids were present in 'Picual' and Hojiblanca' olive oils obtained from olives in different ripening stages [54, 55]. The presence of phenolic acids in olive oils would represent a good feature since these compounds have a potential protective effect against diseases related with oxidative damage, in addition to the presence of positive sensory evaluation [4, 55]. In our samples, vanillic and *p*-coumaric acids were present in both oils in low quantities, as well as vanillin. Higher contents of vanillin were observed in 'Cobrançosa' oils obtained from olives at later ripening stage RS4. This was also observed in Picudo olive oils [54].

By comparison of our results with those reported in the literature, it was possible to identify the dialdehydic form of elenolic acid linked to hydroxytyrosol (3,4-DHPEA-EDA), as the most important phenolic compound in both oils. This compound represents 20-35 % of total phenols peak areas for 'Cobrançosa' oils and 23 – 43 % for 'Galega Vulgar' oils (data not shown). In fact, Servilli et al. [21] states that 3,4-DHPEA-EDA and 3,4-DHPEA-EA are the main phenolic compounds in VOO characterized by high phenolic concentration. The quantification of those compounds was not possible because of lack of standards.

Hydroxytyrosol and tyrosol, as well as the flavonoid luteolin were present in higher contents in 'Cobrançosa' EVOO than in "Galega Vulgar" EVOO. HYT and TYR form esters with elenolic acid derivatives to give glycosylated secoiridoids, such as oleuropein and ligstroside, namely 3,4-DHPEA-EDA and 3,4-DHPEA-EA. These compounds have antimicrobial, anti inflammatory and hypoglycemic effects and are considered to be responsible for the antioxidant properties of EVOO [3, 4, 5, 7, 21]. So, although higher contents were found for HYT and TYR in 'Cobrançosa' EVOO, only the evaluation of HYT and its derivatives (oleuropein complex and tyrosol) can be used for labelling purposes for health and nutrition claims [3].

From the point of view of flavonoids, luteolin is a good marker for 'Cobrançosa' oils, as in all ripening stages the contents in these oils are significantly higher ($p < 0.05$) than in 'Galega Vulgar' oils. The contents of luteolin are especially high in

Turkish oils (29-76 mg kg^{-1}) [25]. Our results for 'Cobrançosa' oils are close to the ones of Spanish oils of Picudo cultivar (3-5 mg kg^{-1}) [50].

To search for eventual relationships among the enzymatic activities, phenol composition and ripening index for the various samples of 'Cobrançosa' and 'Galega Vulgar' oils, a PCA was performed. 15 variables were considered: the activities of PPO in fruit mesocarp (PPOp), POD in seed (PODs), ripening index of olives (RI), α-tocopherol (AT), β-tocopherol (BT), γ-tocopherol (GT), total phenols (TP), hydroxytyrosol (HYT), tyrosol (TYR), vanillic acid (ACV), vanillin (VAN), *p*-coumaric acid (PCM), luteolin (LUT), apigenin (APG) and chlorophyll pigments (CP) of the extracted olive oils.

Again, the original data-set can be represented on a plane defined by the first and second principal components explaining 60.8 % of the variance of the original data (Figure 3).

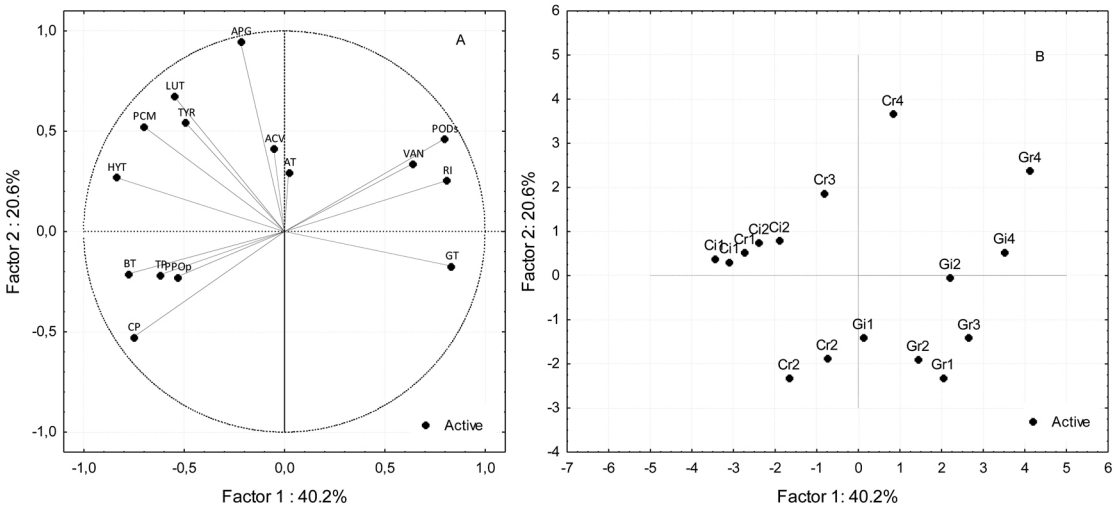

Figure 3. PCA analysis- projection of the loadings of olives (RI, PPO in pulp and POD in seed) and of olive oils (tocopherols, phenols and chlorophyll pigments) (A) and of the samples of the olive oils from Galega (G) and Cobrançosa (C) cultivars grown in orchard r and i, at different ripening stages (1 to 4), on the plane defined by the first two PC (factors 1 and 2). RI-ripening index; PPO-polyphenol oxidase activity in pulp; POD-peroxidase activity in seed; (AT) α-tocopherol; (BT) β-tocopherol; (GT) γ-tocopherol; (TP) total phenols; (HYT) hydroxytyrosol; (TYR) tyrosol; (ACV) vanillic acid; (VAN) vanillin; (PCM) *p*-coumaric acid; (LUT) luteolin; (APG) apigenin and (CP) chlorophyll pigments.

The factor 1 is mainly defined by the ripening index (RI) (loading of 0.8), γ–tocopherol (loading of 0.8), PODs (loading of 0.8) and VAN (loading of 0.6) which are correlated with the positive side of the axis, and by total phenols (loading of -0.6), CP (loading of -0.8) and β-tocopherol (loading of -0.8), HYT (loading of -

0.8), PCM (loading of - 0.7), correlated with the negative side of factor 1. Thus, as RI increases, the POD activity in seeds increases, as well as vanillin and gamma-tocopherol content in olive oil. On the contrary, early ripening stages correspond to high levels of green pigments, BT, HYT, PCM and LUT. The second PC (factor 2) is highly and positively correlated with apigenin content (loading of 0.9). Therefore, it can be identified as the apigenin axis. The PCA projection of olive oil samples obtained from both cultivars suggested the presence of groups that could be related not only with the cultivars, but also with the ripening stage of the samples. 'Galega Vulgar' olive oil samples are projected on the first and fourth quadrants (positive side of the first axis) while 'Cobrançosa' olive oils are located on the second and third quadrants, corresponding to olive oils extracted from olives with lower ripening indexes. The 'Cobrançosa' olive oils present higher contents of HYT, BT, PCM, LUT, TYR and CP than 'Galega Vulgar' olive oils. In addition, both 'Cobrançosa' and 'Galega Vulgar' olive oil samples are spread along the first axis according to increasing ripening index.

In order to investigate the existence of groups in the original hyperspace, a CA was performed on the same data set used for PCA. However, the presence of groups of olive oils extracted from olives of different cultivars at different ripening stages was not easily identified (Figure not shown).

Therefore, to evaluate the presence of groups of olive oils from fruits at different ripening stages, four groups (RS1, RS2, RS3 and RS4) according to the ripening index of the olives were defined. In each group, samples were joined by ripening index and not by cultivar. A DA was performed on the original data, considering these four groups *a priori* defined, in order to assess the existence of clusters and to identify the variables that could discriminate amongst these groups. Concerning the ripening stage, the variables POD, total phenols, tocopherols (β and γ), chlorophyll pigments, tyrosol and vanillin showed to have discriminant power among samples. Table 5 presents the coefficients of the linear classification functions, derived by a stepwise discriminant analysis, describing each group of samples defined by a specific ripening stage, independently from the cultivar. These polynomial functions can be used to determine to which group each sample most likely belongs. However, due to the dependence of olive oil composition on edapho-climatic conditions and cultivars, these classification

functions found by DA cannot be easily generalized. Figure 4 shows the score plots of EVOO samples on the planes defined by the canonical roots 1 and 2, after performing DA. The clustering of the samples into the groups previously defined is well illustrated.

Table 5. Coefficients of the linear classification functions, derived by stepwise discriminant analysis, describing each ripening stage (RS1, RS2, RS3 and RS4).

Variables	RS1	RS2	RS3	RS4
Chlorophyll Pigments (mg kg^{-1})	-2,81	-2,61	-3,14	-3,55
Tyrosol (mg kg^{-1})	60,19	56,32	63,34	79,72
Peroxidase activity (seed)($Ug^{-1}FW$)	17,7	16,58	18,36	23,4
Vanillin (mg kg^{-1})	215,28	198,26	223,67	272,87
β-tocopherol (mg kg^{-1})	348,73	314,53	333,13	355,35
γ-tocopherol (mg kg^{-1})	48,59	43,94	46,69	49,57
Total phenols (mg GAE kg^{-1})	0,37	0,34	0,38	0,43
Constant	-1282,7	-1058,2	-1219,51	-1548,22

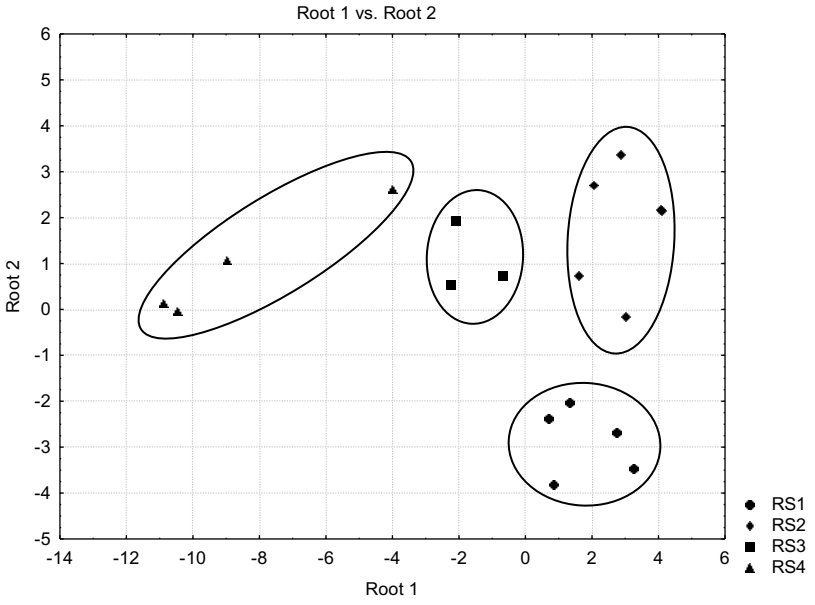

Figure 4. EVOO samples plotted on the planes defined by the canonical roots 1 and 2, after performing DA.

These results show that by choosing the adequate ripening stage of the fruits, it is possible to obtain olive oils from different cultivars with similar polyphenol and tocopherol contents, and therefore similar functional properties.

4. Conclusions

This study shows that most of the characteristics of EVOO are determined by the chemical composition and biochemical status of the olive fruit. Concerning the fatty acid composition profile, the olive oil samples of 'Galega Vulgar' and 'Cobrançosa' cultivars were very well separated in two groups by cultivar. The higher contents of polyunsaturated fatty acids of 'Cobrançosa' EVOO correspond to a more susceptible oil to oxidation (higher indexes of oxidation) than 'Galega Vulgar' olive oil.

Enzymatic activity of PPO and POD may contribute to phenol degradation in fruits but, in our study, they were not reflected in the total phenol contents of 'Galega Vulgar' and 'Cobrançosa' olive oils. The POD activity was mainly detected in seeds of both cultivars and PPO activity was only detected in the fruit mesocarp. In the present study, for ripening stages of 4.0 in fruits, 'Galega Vulgar' olive oils showed lower contents of total phenols and chlorophyll pigments than 'Cobrançosa' oils. Therefore, the decision about the harvesting date will allow the production of virgin olive oils with different sensory properties and nutritional value. The ripening stage, other than the cultivar, showed to be a key factor on the amount of bioactive compounds (mainly phenolic compounds) of olive oil from 'Galega Vulgar' or 'Cobrançosa' cultivars.

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References

1. Beauchamp GK, Keast RSJ, Morel D, Lin J, Pika J, Han Q, Lee C-H, Smith AB, Breslin PAS (2005) Phytochemistry: Ibuprofen-like activity in extra-virgin olive oil. Nature 437:45-46

2. Andrewes P, Busch JLHC, Joode T, Groenewegen A, Alexandre H (2003) Sensory Properties of Virgin Olive Oil Polyphenols: Identification of Deacetoxy-ligstroside Aglycon as a Key Contributor to Pungency. Journal of Agricultural and Food Chemistry 51:1415-1420

3. Martín-Peláez S, Covas MI, Fitó M, Kušar A, Pravst I (2013) Health effects of olive oil polyphenols: Recent advances and possibilities for the use of health claims. Molecular Nutrition & Food Research 57:760-771

4. Bendini A, Cerretini L, Carrasco-Pancorbo A, Caravaca AMG, Segura-Carretero A, Fernández-Gutiérrez A, Lercker G (2007) Phenolic Molecules in Virgin Olive Oils: a Survey of Their Sensory Properties, Health Effects, Antioxidant Activity and Analytical Methods. An Overview of the Last Decade. Molecules 12:1679-1719

5. Servili M, Montedoro G (2002) Contribution of phenolic compounds to virgin olive oil quality. European Journal of Lipid Science and Technology 104:602-613

6. Carrasco-Pancorbo A, Gómez-Caravaca AM, Cerretani L, Bendini A, Segura-Carretero A, Fernández-Gutiérrez A (2006) A simple and rapid electrophoretic method to characterize simple phenols, lignans, complex phenols, phenolic acids, and flavonoids in extra-virgin olive oil. Journal of Separation Science 29:2221-2233

7. Kanakis P, Termentzi A, Michel T, Gikas E, Halabalaki M, Skaltsounis A-L (2013) From Olive Drupes to Olive Oil. An HPLC-Orbitrap-based Qualitative and Quantitative Exploration of Olive Key Metabolites. Planta Med 79:1576-1587

8. Beltrán G, Jiménez A, Rio C, Sánchez S, Martínez L, Uceda M, Aguilera MP (2010) Variability of vitamin E in virgin olive oil by agronomical and genetic factors. Journal of Food Composition and Analysis 23:633-639

9. Servili M, Baldioli M, Minati E, Montedoro GF (1996) Antioxidant activity of new phenolic compounds extracted from virgin olive oil and their interaction with α-tocopherol and β-carotene. Rivista Italiana delle Sostanze Grasse 73:55-59

10. Deiana M, Rosa A, Cao CF, Pirisi FM, Bandino G, Dessì MA (2002) Novel Approach to Study Oxidative Stability of Extra Virgin Olive Oils: Importance of α-Tocopherol Concentration. Journal of Agricultural and Food Chemistry 50:4342-4346

11. Servili M, Baldioli M, Begliomini AL, Selvaggini R, Montedoro GF (2000) The phenolic and volatile compounds of virgin olive oil:relationships with the endogenous oxidorreductases during the mechanical oil extraction process. in Lanzotti V, Taglialatela-Scafati O (eds) Flavour and Fragrance Chemistry. Kluwer Academic Publishers, Dordrecht, pp. 163-173.

12. Servili M, Taticchi A, Esposto S, Urbani S, Selvaggini R, Montedoro G (2007) Effect of Olive Stoning on the Volatile and Phenolic Composition of Virgin Olive Oil. Journal of Agricultural and Food Chemistry 55:7028-7035

13. Yoruk R, Marshal R (2003) Physicochemical properties and function of plant polyphenol oxidase: a review. J. Food Biochem 27:361-422

14. Tomás-Barberan FA, Espin JC (2001) Phenolic compounds and related enzymes as determinants of quality of fruits and vegetables. J. Sci. Food Agric. 81:853-876

15. García-Rodríguez R, Romero-Segura C, Sanz C, Sánchez-Ortiz A, Pérez AG (2011) Role of polyphenol oxidase and peroxidase in shaping the phenolic profile of virgin olive oil. Food Research International 44:629-635

16. Peres F, Luz JP, Fragoso P, Gouveia C, Vitorino C, Amaro C, Henriques L, Coutinho J, Pintado CM, Peres C, Ferreira A (2010) Integrated Production and Quality of Galega Olive Oil. IOBC Bulletin:145-149

17. García-Rodríguez R, Romero-Segura C, Sanz C, Pérez AG (2015) Modulating oxidoreductase activity modifies the phenolic content of virgin olive oil. Food Chemistry 171:364-369

18. Taticchi A, Esposto S, Veneziani G, Urbani S, Selvaggini R, Servili M (2013) The influence of the malaxation temperature on the activity of polyphenoloxidase and

peroxidase and on the phenolic composition of virgin olive oil. Food Chemistry 136:975-983

19. Clodoveo ML, Hbaieb RH, Kotti F, Mugnozza GS, Gargouri M (2014) Mechanical Strategies to Increase Nutritional and Sensory Quality of Virgin Olive Oil by Modulating the Endogenous Enzyme Activities. Comprehensive Reviews in Food Science and Food Safety 13:135-154

20. Servili M, Taticchi A, Esposto S, Sordini B, Urbani S (2012). Technological Aspects of Olive Oil Production, Olive Germplasm - The Olive Cultivation, Table Olive and Olive Oil Industry in Italy, I. Muzzalupo (Ed.), ISBN: 978-953-51-0883-2, InTech, DOI: 10.5772/52141. Available from: http://www.intechopen.com/books/olive-germplasm-the-olive-cultivation-table-olive-and-olive-oil-industry-in-italy/technological-aspects-of-olive-oil-production

21. Servili M, Selvaggini R, Esposto S, Taticchi A, Montedoro GF, Morozzib G (2004) Health and sensory properties of virgin olive oil hydrophilic phenols: agronomic and technological aspects of production that affect their occurrence in the oil. Journal of Chromatography A 1054:113-127

22. Gómez-Rico A, Salvador MD, Moriana A, Pérez D, Olmedilla N, Ribas F, Fregapane G (2007) Influence of different irrigation strategies in a traditional Cornicabra cv. olive orchard on virgin olive oil composition and quality. Food Chemistry 100:568-578

23. Casas JS, De Miguel Gordillo C, Osorio Bueno E, Marín Expósito J, Mendoza MF, Hierro TA, González LG, Martínez Cano M (2009) Characteristics of Virgin Olive Oils from the Olive Zone of Extremadura (Spain), and an Approximation to their Varietal Origin. Journal of the American Oil Chemists' Society 86:933-940

24. El Riachy M, Priego-Capote F, Rallo L, Luque-de Castro MD, León L (2013) Phenolic composition of virgin olive oils in cultivars for narrow hedgerow olive orchards. European Journal of Lipid Science and Technology 115:800-810

25. Yorulmaz A, Erinc H, Tekin A (2013) Changes in Olive and Olive Oil Characteristics During Maturation, Journal of the American Oil Chemists' Society 90:647-658

26. Ortega-García F, Peragón J (2009) Phenylalanine Ammonia-Lyase, Polyphenol Oxidase, and Phenol Concentration in Fruits of Olea europaea L. cv. Picual, Verdial, Arbequina, and Frantoio during Ripening. Journal of Agricultural and Food Chemistry 57:10331-10340

27. Ortega-Garcia F, Blanco S, Peinado MA, Peragon J (2008) Polyphenol oxidase and its relationship with oleuropein concentration in fruits and leaves of olive (Olea europaea) cv. 'Picual' trees during fruit ripening. Tree Physiol 28:45-54

28. Moyano MJ, Heredia FJ, Meléndez-Martínez AJ (2010) The Color of Olive Oils: The Pigments and Their Likely Health Benefits and Visual and Instrumental Methods of Analysis. Comprehensive Reviews in Food Science and Food Safety 9:278-291

29. Hermoso M, Uceda M, Frias L, Beltran G (1997) Maduración. In: Barranco D, Fernandez-Escobar D, Rallo L (eds) El Cultivo del Olivo. Ediciones Mundi-Prensa, Madrid, pp. 137- 153

30. Saraiva JA, Nunes CS, Coimbra MA (2007) Purification and characterization of olive (Olea europaea L.) peroxidase – Evidence for the occurrence of a pectin binding peroxidase. Food Chemistry 101:1571-1579

31. Lester GE, Hodges DM, Meyer RD, Munro KD (2004) Pre-extraction Preparation (Fresh, Frozen, Freeze-Dried, or Acetone Powdered) and Long-Term Storage of Fruit and Vegetable Tissues: Effects on Antioxidant Enzyme Activity. Journal of Agricultural and Food Chemistry 52:2167-2173

32. Oktay M, Kufrevioglu I, Kocaçaliskan I, Sakiroglu H (1995) Polyphenoloxidase from Amasya Apple. Journal of Food Science 60:494-496

33. Gajewska E, Skłodowska M, Słaba M, Mazur J (2006) Effect of nickel on antioxidative enzyme activities, proline and chlorophyll contents in wheat shoots. Biologia Plantarum 50:653-659

34. Peres F, Martins LL, Ferreira-Dias S (2014) Laboratory-scale optimization of olive oil extraction: Simultaneous addition of enzymes and microtalc improves the yield. European Journal of Lipid Science and Technology 116:1054-1062

35. Favati F, Caporale G, Bertuccioli M (1994) Rapid determination of phenol content in virgin olive oil. Grasas y Aceites 45:68-70

36. Commission Regulation (EEC) No 2568/91 on the characteristics of olive oil and olive-residue oil and on the relevant methods of analysis.

37. Pirisi FM, Cabras P, Cao CF, Migliorini M, Muggelli M (2000) Phenolic Compounds in Virgin Olive Oil. 2. Reappraisal of the Extraction, HPLC Separation, and Quantification Procedures. Journal of Agricultural and Food Chemistry 48:1191-1196

38. COI (2009) Determination of Biophenols in olive oils by HPLC. COI/T20/DOC.29

39. Pokorny J, Kalinová L, Dysseler P (1995) Determination of Chlorophyll pigments in Crude Vegetable Oils. Pure Appl. Chem. 67:1781-1787

40. Commission Implementing Regulation Nº 1343/2013 amending Regulation (EEC) No 2568/91 on the characteristics of olive oil and olive-residue oil and on the relevant methods of analysis

41. Gutiérrez -Rosales F, Perdiguero S, Gutiérrez R, Olias JM (1992) Evaluation of the bitter taste in virgin olive oil. Journal of the American Oil Chemists' Society 69: 1558-9331

42. Hoffman DL, Young FW (1983) Quantitative analysis of qualitative data: Applications in food research. In: Martens H, Russwurm H (eds) Food Research and Data Analysis. Elsevier, pp. 69-93.

43. Miller JC, Miller JN (1993) Statistics for Analytical Chemistry. Ellis Horwood and Prentice Hall, pp.169-201

44. Angerosa F, Servili M, Selvaggini R, Taticchi A, Esposto S, Montedoro G (2004) Volatile compounds in virgin olive oil: occurrence and their relationship with the quality. Journal of Chromatography A 1054:17-31

45. Peres F, Jeleń HH, Majcher MM, Arraias M, Martins LL, Ferreira-Dias S (2013) Characterization of aroma compounds in Portuguese extra virgin olive oils from Galega Vulgar and Cobrançosa cultivars using GC–O and GCxGC–ToFMS. Food Research International 54:1979-1986

46. Kalua CM, Allen MS, Bedgood JDR, Bishop AG, Prenzler PD, Robards K (2007) Olive oil volatile compounds, flavour development and quality: A critical review. Food Chemistry 100:273-286

47. Olias JM, Perez AG, Rios JJ, Sanz LC (1993) Aroma of virgin olive oil: Biogenesis of the "green" odor notes. Journal of Agricultural and Food Chemistry 41: 2368 – 2373

48. García-Mesa J, Pereira-Caro G, Fernández-Hernández A, Civantos CGO, Mateos R (2008) Influence of lipid matrix in the bitterness perception of virgin olive oil. Food Quality and Preference 19:421-430

49. Hbaieb RH, Kotti F, García-Rodríguez R, Gargouri M, Sanz C, Pérez AG (2015) Monitoring endogenous enzymes during olive fruit ripening and storage: Correlation with virgin olive oil phenolic profiles. Food Chemistry 174:240-247

50. Romero-Segura C, García-Rodríguez R, Sánchez-Ortiz A, Sanz C, Pérez AG (2012) The role of olive β-glucosidase in shaping the phenolic profile of virgin olive oil. Food Research International 45:191-196

51. Tuorila H, Recchia A (2014) Sensory Perception and Other Factors Affecting Consumer Choice of Olive Oil.In: Monteleone, E Langstaff, S. Olive Oil Sensory Science. John Wiley & Sons, Ltd, pp. 55-80.

52. Cunha SC, Amaral JS, Fernandes JO, Oliveira MBP (2006) Quantification of Tocopherols and Tocotrienols in Portuguese Olive Oils Using HPLC with Three Different Detection Systems. J. Agric. Food Chem. 54:3351-3356

53. Belitz H, Grosh DW, Shieberle P (2009) Food Chemistry. Springer-Verlag Berlin Heidelberg, Leipzig, 4ª ed. pp.158-244

54. Jiménez, B, Sánchez-Ortiz, A, Lorenzo, M L, & Rivas, A (2013) Influence of fruit ripening on agronomic parameters, quality indices, sensory attributes and phenolic compounds of Picudo olive oils. Food Research International, 54: 1860-1867

55. Rivas, A, Sanchez-Ortiz, A, Jimenez, B, García-Moyano, J, & Lorenzo, ML (2013). Phenolic acid content and sensory properties of two Spanish monovarietal virgin olive oils. European Journal of Lipid Science and Technology, 155:621-63

Chapter 6

Phenolic compounds of ‘Galega Vulgar’ and ‘Cobrançosa’ olive oils along early ripening stages

Submitted

Abstract

The main antioxidants in virgin olive oil (VOO) are phenolic compounds. In this study it was evaluated the lipophilic and hydrophilic phenol composition of VOO produced from the two most important Portuguese cultivars (‘Galega Vulgar’ and ‘Cobrançosa’) obtained from olives harvested at different ripening stages and under two irrigation schemes (rain fed and irrigated). Polyphenol oxidase (PPO) and peroxidase (POD) activities in fruits mesocarp and in seeds were also determined. The degradation level of virgin olive oils was evaluated by measuring the acidity, peroxide value, and spectroscopic indices K_{232} and K_{270}. Phenolic alcohols (hydroxytyrosol and tyrosol) phenolic acids and derivatives and flavonoids (luteolin and apigenin) as well as tocopherols were quantified. Fatty acid characterization was also performed. Lipophilic (>300 mg/kg) and hydrophilic phenols (>600 mg/kg) were present in high contents in both VOO, for early ripening stages. Total phenols showed a decrease between ripening index 2.5 and 3.5. Higher PPO mesocarp activity as well as POD seed activity in the last ripening stages of ´Galega Vulgar’ olives corresponds to lower phenol contents. The dialdehydic form of elenolic acid linked to hydroxytyrosol (3,4-DHPEA-EDA), also known as oleacein, was the major phenolic compound identified in both oils. The concentration of free hydroxytyrosol and tyrosol in both VOO is very low while their esterified derivatives, like 3,4-DHPEA-EDA and *p*-HPEA- EDA are much more abundant.

Keywords: oleacein, oxidative stability, oxidoreductases, tocopherols

1. Introduction

The benefits of consuming olive oil were traditionally attributed to its high content in oleic acid (Gurr, 2000). However, it is now well known that these benefits may also be ascribed to the phenol compounds of extra virgin olive oil (EVOO) due to their anti-oxidant, anti-inflammatory and anti-microbial activities. For some activities of EVOO phenolic compounds, the scientific evidence is already strong enough to enable the legal use of health claims on labelling (Martín-Peláez et al., 2013).

Lipophilic and hydrophilic phenols are the most important antioxidants in EVOO. Lipophilic phenols in EVOO are tocopherols, which are heteroacids of high molecular weight. Among them, α-tocopherol is the most abundant (90%) but β- and γ-tocopherols are also present (Beltrán et al., 2010). Claims have been made for the preventive activity of tocopherols against reactive oxygen species (ROS) in biological systems, namely their positive effect on cell aging, some cancer types, immune system maintenance and cardiovascular diseases (Bramley et al., 2000). Moreover, apart from their action as lipid radical scavengers, they also inhibit the photooxidation by reacting with singlet oxygen (Velasco and Dobarganes, 2002). Variability in tocopherol contents by crop year is explained by the rainfall levels, showing that oils from drier crop years have higher tocopherol content (Beltrán et al., 2010). However, the content of tocopherols in virgin olive oils is relatively low when compared with several seed oils. In fact, hydrophilic phenols are the compounds that most differentiate EVOO from other vegetable oils. The most important phenolic compounds that have been identified in olive oil are phenolic alcohols (hydroxytyrosol and tyrosol), secoiridoid derivatives, such as the dialdehydic form of elenolic acid linked to hydroxytyrosol (3,4-DHPEA-EDA) (oleacein), the dialdehydic form of elenolic acid linked to tyrosol (p-HPEA-EDA) (oleocanthal), the aldehydic form of elenolic acid linked to tyrosol (p-HPEA-EA), 4-(acetoxyethyl)-1,2-dihydroxybenzene (3,4-DHPEA-AC), oleuropein aglycone (3,4-DHPEA-EA) and its methylated form (methyl 3,4-DHPEA-EA), phenolic acids and derivatives (such as vanillic acid and vanillin, respectively), lignans (pinoresinol and acetoxypinoresinol) and flavonoids such as luteolin and apigenin (Ryan et al., 1999; Servili et al., 1999; Servili and

Montedoro, 2002; Bendini et al., 2007; Servili et al., 2009; Kanakis et al., 2013; Pizarro et al., 2013).

Olive oil phenol composition is quite different from that of the olive drupe and of the olive paste (Kanakis et al., 2013). In contrast to olive fruits, olive oil contains neither anthocyanins nor flavonols. During the extraction process the glycosidic oleuropein, demethyloleuropein and ligstroside are hydrolyzed by endogenous β-glycosidases to form aldehydic aglycones. The aglycones become soluble in the oil phase, whereas the glycosides remain in the water phase (Servili and Montedoro, 2002). The main source of lignans was demonstrated to be the stone and not the pulp (Oliveras López et al., 2008).

EVOO phenolic compounds play also an important role in organoleptic properties namely in attributes related to bitterness and pungency (Andrewes et al., 2003; Beauchamp et al., 2005; Peyrot des Gachons et al., 2011). The phenolic compounds 3,4-DHPEA-EA, *p*-HPEA-EA, 3,4-DHPEA-EDA, *p*-HPEA-EDA, elenolic acid (EA), and elenolic acid methyl ester (EAME) showed high correlations with bitterness and pungency (Dierkes et al., 2012). Moreover, oleocanthal causes a pungency perceived as an unusual irritation in the pharynx, consequence of both the specificity of this molecule for a single sensory receptor and the anatomical restriction of this sensory receptor to the pharynx (Peyrot des Gachons et al., 2011).

Olive endogenous enzymes such as oxidoreductases, polyphenol oxidase (PPO) and peroxidase (POD), which oxidase phenolic compounds may be a biochemical factor affecting the phenol content of VOO (García-Rodríguez et al., 2011; Hbaieb et al., 2015).

The ripening stage of olives has a high impact on the oil's yield, quality, stability and sensory characteristics. Irrigation also plays an important role in the productivity of olives and consequently in fruit ripening, and therefore in phenol and volatile composition (Gómez-Rico et al., 2009). Moreover, when early frosts occur, oils extracted from frosted fruits develop sensory defects (Guillaume et al., 2010). So, in the last years a lot of attention has been drawn to the main changes on the characteristics of olives and olive oils along fruit ripening, in order to decide the best harvest time (Rotondi et al., 2004; Kalua et al., 2005; Gómez-Rico et al., 2008; Jiménez et al., 2013; Dag et al., 2014) .

Early ripening has been a recommendation in the center of Portugal (Beira Baixa) for organic olive growing. The predominance of 'Galega Vulgar' cv., which is highly susceptible to pests and diseases, is the main reason for this procedure (Peres et al., 2009; Peres et al., 2010). However early ripening corresponds to lower yields, so it is crucial to determine how early the harvest can be, in order to have good quality, high nutritional value and sensory scores and a reasonable yield.

The aim of the present study was to investigate the effect of early harvest corresponding to olive ripening index lower than 4.5, on phenol compounds levels in virgin olive oil from 'Galega Vulgar' and 'Cobrançosa' fruits, two of the most important Portuguese cultivars for olive oil extraction.

2. Materials and methods

2.1. Olives Characterization

Portuguese olive fruits (*Olea europaea* L.) of 'Cobrançosa' and 'Galega Vulgar' cultivars used in this study were produced according to the Integrated Production rules, in Beira Baixa Region, in two types of farming: rainfed orchard (RF) (39º 49' N, 7º 27'W) and an irrigated orchard (IR) (39º 50´N, 7º 42'W). 'Galega Vulgar' orchards corresponds to 100-123 trees/ha while 'Cobrançosa' orchards corresponds to 200-300 trees/ha. For the irrigated orchards the irrigation drip system is performed as a function of soil humidity and climacteric conditions. Olive fruits were picked from the beginning of October till the 2nd fortnight of November. The annual accumulated precipitation of the year under study (2011) was 737.5 mm, very similar to the period 1981-2010 of the region (783.2 mm). Their ripening indices (RI) were determined following the guidelines of Estación de Olivicultura y Elaiotecnia, Jaén, Spain (Hermoso et al., 1997); moisture and fat content (by Soxtec) of the fruits were also evaluated. Only healthy fruits were selected for fruit characterization and for olive oil extraction.

2.2. Enzymatic activity assays

Extracts were prepared by homogenizing olive pulp and seeds with cold acetone (-20 °C) in an ultraturrax homogeneizer (2 min), followed by filtration in fiber glass filters, washing the pellet with cold acetone (-20 °C) until total removal of

pigments, and by drying samples at room temperature with N_2 (Roche et al., 2000; Saraiva et al., 2007). For enzymatic assay 0.4 g of acetone powder were suspended in 5 mL of extraction buffer (0.05 M potassium phosphate, pH 6.2 containing 1M KCl) (Servili et al., 2007) and 2% (w/w) of PVP and stirred for 30 min, 4ºC, 400 rpm; the suspension was centrifuged at 12000 rpm for 30 min and filtered (0.45 μm). PPO activity was evaluated using catechol (30 mM) as substrate, following the increase in absorbance at 420 nm, during 1 min (Oktay et al., 1995). One unit of PPO was defined as the quantity of enzyme that causes the absorbance variation of 0.001 min^{-1} mL^{-1} of enzyme extract, at room temperature. Results were expressed as Ug^{-1} FW (fresh weight).

POD activity was performed following the increase in absorbance at 470 nm (2 min) using 30 mM guaiacol and 4 mM H_2O_2 as substrates (Gajewska et al., 2006). One unit of POD was defined as the consumption of 1 μmol of guaiacol min^{-1} mL^{-1} of enzyme extract, at room temperature using a molar absorptivity (ε) for tetraguaiacol of 26.6 mM^{-1} cm^{-1}. Results were expressed as Ug^{-1} FW.

2.3. Olive oil extraction

Olive oils were extracted in a laboratory oil extraction system (Abencor analyser; MC2 Ingenieria y Sistemas S.L., Seville, Spain) under optimized conditions (Peres et al., 2014). The olives were crushed with a hammer mill equipped with a 4 mm sieve at 3000 rpm. Malaxation of the pastes was performed at 27-30 ºC, during 30 min, and centrifugation at 3500 rpm (3 min). After centrifugation olive oil was separated by settling in a graduated cylinder. Water traces in the oil were removed with anhydrous sodium sulfate, filtered through a cellulose filter and stored in amber glass bottles at 4 ºC. From each batch three independent extractions were performed.

2.4. Olive oil characterization

European Union chemical quality criteria (acidity value, peroxide value (PV) and UV specific absorbances (K_{232} and K_{270}) was carried out following the analytical methods described in EEC/2568/91 EU Regulation. Fatty acid methyl esters were evaluated by gas chromatography with flame ionization detector (GC-FID), in a Hewlett Packard 6890, SP column 2380™ Supelco (60m x 0.25mm x 0.20μm).

Samples of olive oils were also sensory evaluated by a panel test with more than 10 years of experience in olive oil tasting according to the methodology of Regulation Nº 1343/2013, using a profile sheet with an unstructured scale adapted from Cerretani et al. (2008). Chlorophyll pigments were evaluated by VIS spectroscopy (Pokorny et al., 1995). Oxidative stability was measured using a Metrohm Rancimat model 670 (temperature of 120 ºC; air flow of 20 Lh^{-1}).

2.5. Phenol composition evaluation

For tocopherols determination, a solution of oil in hexane (8 % (w/v)), filtered with Pall Gelman Acrodisc® syringe filters (0.45 µm, 25 mm, GHP membrane) was analyzed by high-performance liquid chromatography (HPLC) in an Agilent 1100 Series chromatograph. Fluorescence detection with excitation set at 290 nm and emission set at 330 nm and a Lichrosorb Si 60 column (250mm x 4.6 mm x 5µm) at room temperature were used. Total phenol compounds were extracted by solid phase extraction (SPE) columns filled with 1g of octadecyl (C_{18}) material from J.T. Baker, and evaluated by VIS spectroscopy (Peres et al., 2014).

The profile of phenolic compounds was evaluated by HPLC according to International Olive Council method with some modifications (IOOC, 2009). The phenolic compounds were recovered from olive oil by liquid-liquid extraction using the procedure proposed by (Pirisi et al., 2000). An Agilent 1100 HPLC system, consisting of a degasser, a quaternary pump, an autosampler and a diode array detector (DAD) was used. The stationary phase was a Purospher C18 analytical column (150 mm x 3.9 mm x 4 µm). The mobile phase was (A) 0.2 % H_3PO_4 (v/v) (B) methanol (C) acetonitrile at a constant flow rate of 1 mL min^{-1}. The gradient program was the one of IOC document (IOC, 2009). Quantification of phenolic compounds was carried out using the area values measurements at 280 nm for gallic acid, hydroxytyrosol, tyrosol, vanillic acid, caffeic acid, vanillin, *o*-coumaric acid, at 320 nm for *p*-coumaric acid and at 360 nm for luteolin and apigenin. Quantitative assays were achieved using external calibration curves for all standard phenols. Confirmation of phenolic compounds was achieved with an LC-ESI-MS Agilent 1200 series equipped with a triple quadrupole mass spectrometer Agilent 6400. A Zorbax SB-C18 (50mm x 4.6 mm i.d. x 1.8 µm particle diameter – Agilent tecnologies) column

was used for the separation at a flow rate of 0.7 mL min^{-1}, at 30 °C. Elution was performed by means of a gradient of 0.1% formic acid (eluent A) and acetonitrile (eluent B). The gradient was as follows: started at 10% B, 20% B at 10 min, 40% B at 40min, 60% B at 60 min, 90% B at 80 min, at 81 min return to initial conditions and stabilized for 9 min. ESI operated with a nitrogen flow of 10 L min^{-1} at 300 °C. MS detector operated in MS2-Scan scan type in the range 80–1000Da, and negative mode was selected. The capillary voltage set to 4.0 kV, the quadrupole temperatures were 100 °C, fragmentor energy was 145, and cell accelerator voltage was 7. Data were acquired and analysed using Masshunter Workstation Software (version B.04.00) from Agilent technologies. Characterization of the phenolic compounds with LC-ESI-MS was reached after results from several samples were compared. Positive characterization was achieved when a phenolic compound showed the same fragmentation pattern and a similar pattern with data from literature. Standards of α-tocopherol, β-tocopherol, γ-tocopherol, δ-tocopherol, hydroxytyrosol, tyrosol, vanillic acid, vanillin, cafeic acid, ferulic acid, *o*-coumaric, *p*-coumaric, apigenin, verbascoside, were purchased from Sigma-Aldrich and oleuropein and luteolin from Extrasyntese.

2.5. Statistical analysis

Statistical analysis was performed using the software Statistica™, version 6, from Statsoft, Tulsa, OK, USA. Data was analyzed by univariate procedures (ANOVA, Tukey test, $p<0.05$) in order to identify the differences between the olive oils from both cultivars and the two orchards.

3. Results and discussion

3.1. Olives characterization

The characterization of olives in different ripening dates is presented in Table 1. For each sampling date the olives from ‘Cobrançosa’ cultivar had always a lower ripening index than ‘Galega Vulgar’ fruits (Table1).

Table 1. Ripening index, moisture content, fat content, oxidoreductases activity (PPO and POD) of olive fruits 'Galega Vulgar' and 'Cobrançosa', in two olive groves (RF - rain fed; IR - irrigated). In each row superscript indexes indicate differences based on Tukey test.

	'Galega Vulgar'				'Cobrançosa'			
Olive grove	RF		IR		RF		IR	
Harvest month	Oct	Nov	Oct	Nov	Oct	Nov	Oct	Nov
Ripening Index	2.8 ^{bc}	4.2 ^{a}	2.1 ^{c}	3.5 ^{ab}	2.1 ^{c}	3.6 ^{ab}	1.1 ^{d}	2.7 ^{c}
Moisture (%)	50.8 ^{d}	61.6 ^{a}	51.8 ^{cd}	62.3 ^{a}	53.8 ^{c}	58.1 ^{b}	53.1 ^{cd}	59.3 ^{b}
Fat content (% DW)	36.0 ^{c}	46.2 ^{a}	29.1 ^{d}	38.9 ^{bc}	37.9 ^{bc}	41.5 ^{b}	28.9 ^{d}	40.9 ^{b}
PPO mesocarp activity ($Ug^{-1}FW$)	84.8 ^{bc}	220.2 ^{a}	59.8 ^{c}	124.3 ^{bc}	111.2 ^{bc}	147.9 ^{ab}	44.6 ^{c}	124.8 ^{bc}
POD mesocarp activity ($Ug^{-1}FW$)	<1.0	2.0	<1.0	1.1	<1.0	1.9	<1.0	2.4
POD seed activity ($Ug^{-1}FW$)	8.4 ^{ab}	10.1 ^{ab}	9.8 ^{ab}	12.7 ^{a}	7.9 ^{ab}	7.8 ^{ab}	6.5 ^{b}	3.8 ^{b}

Also, the olives from the rain fed orchard presented always higher RI than those from irrigated one, mainly related with the lower tree load of the trees due to water shortage (Inglese et al., 1996). Moisture content of the fruits of both cultivars were quite similar, in each harvest date, reflecting the changes related with climateric modifications, ie., more rain in November. Significantly higher fat contents were achieved in November for all the cultivars and orchards. The olives from the rain fed orchard showed higher fat content, especially for 'Galega Vulgar' in November. A lower tree load (more accumulation of fat and higher ripening index) may explain this difference. Only the RF orchard for 'Cobrançosa' represent a reasonable yield lost when comparing October or November harvest.

3.2. PPO and POD activities of 'Galega Vulgar' and 'Cobrançosa' olives

Phenolic compounds are enzymatically oxidized by PPO, which results in color changes of olive pastes as soon as the rupture of olive fruit tissues begins by crushing. PPO activity was found in the fruit mesocarp (Table 1) but no PPO activity was detected in the seed, which is in agreement with other authors (Servili et al., 2000; García-Rodríguez et al., 2011) and our own previous results (Peres et al., 2011). PPO mesocarp activity increases with RI and is lower in IR orchards probably because olives from RF orchard presented always a higher ripening index. No significant differences were found for PPO activity in 'Cobrançosa' olives in November for both olive orchards, which can show that for this cultivar a stabilization of PPO activity occurs at lower ripening indexes.

The results for POD showed that its activity is detected predominantly in the seed and 'Galega Vulgar' fruits showed higher values. POD mesocarp activity was also detected at values higher than 1 $Ug^{-1}FW$ at higher ripening stages, corresponding to November harvests. The impact of olive crushing in the VOO phenolic compounds can be related to the different distribution of the endogenous oxidoreductases and phenolic compounds in the pulp and seed of the olive fruit. POD occurs in high amounts in the olive seed. On the contrary, the hydrophilic phenols are largely concentrated in the pulp, whereas the seed contain only small quantities of these substances; tocopherols are in both parts of the fruit, although higher contents are found in the seed (Lavelli and Bondesan, 2005; Servili et al., 2007).

3.3. Olive oil characterization

According to quality criteria defined by the European Union (Regulation (EU) Nº 1343/2013) for acidity, peroxide value and UV absorbances, all the samples are classified as "Extra Virgin Olive Oil" (Table 2). The VOO from the two cultivars were characterized by high levels of saturated fatty acids (palmitic acid higher than 14%) as well as oleic acid (higher than 70%). 'Cobrançosa' olive oils can be distinguished from 'Galega Vulgar' oils by the higher contents of PUFA and in stearic acid (C18:0). In turn, 'Galega Vulgar' olive oils are characterized by higher contents of oleic (18:1), palmitoleic (16:1), palmitic (C16:0), and gadoleic (C20:1) fatty acids. Margaric, margaroleic and behenic acids were significantly ($p<0.05$) lower in rain fed orchards. The levels of linoleic and stearic acids for Cobrançosa olive oils also differed between orchards.

Table 2. Fatty acid composition (%) and quality criteria (acidity, peroxide value and UV absorbances) of 'Galega Vulgar' and 'Cobrançosa' virgin olive oils (mean ± standard deviation), two olive orchards (RF and IR). In each row superscript indexes indicate differences based on Tukey test.

	'Galega Vulgar'		'Cobrançosa'	
Olive orchard	RF	IR	RF	IR
Miristic acid (C14:0)	0.01±0.000 [a]	0.01±0.001 [a]	0.008±0.000 [a]	0.009±0.001 [b]
Palmitic acid (C16:0)	15.93 ± 0.19 [a]	16.35 ± 0.28 [a]	14.55 ± 0.91 [b]	14.54 ± 0.30 [b]
Palmitoleic acid (C16:1)	2.31 ± 0.11 [a]	2.15 ± 0.15 [a]	1.21 ± 0.17 [b]	1.03 ± 0.04 [b]
Margaric acid (C17:0)	0.10 ± 0.00 [b]	0.13 ± 0.02 [a]	0.11 ± 0.01 [b]	0.16 ± 0.01 [a]
Margaroleic acid (C17:1)	0.28 ± 0.01 [b]	0.35 ± 0.04 [a]	0.22 ± 0.01 [b]	0.25 ± 0.00 [a]
Stearic acid (C18:0)	1.69 ± 0.04 [c]	1.84± 0.06 [c]	3.04 ± 0.31 [b]	3.33 ± 0.14 [a]
Oleic acid (C18:1)	74.01 ± 0.30 [a]	73.37 ± 0.21 [a]	70.25 ± 1.61 [b]	70.78 ± 0.14 [b]
Linoleic acid (C18:2)	4.16 ± 0.26 [c]	4.10 ± 0.15 [c]	9.03 ± 1.18 [a]	8.04 ± 0.39 [b]
Linolenic acid (C18:3)	0.66 ± 0.05 [c]	0.69 ± 0.03 [c]	0.75 ± 0.04 [b]	0.89 ± 0.02 [a]
Arachidic acid (C20:0)	0.32 ± 0.06 [a]	0.41 ± 0.09 [a]	0.44 ± 0.08 [a]	0.44 ± 0.03 [a]
Gadoleic acid (C20:1)	0.25 ± 0.01 [b]	0.27 ± 0.02 [a]	0.20 ± 0.00 [c]	0.21 ± 0.01 [c]
Behenic acid (C22:0)	0.10 ± 0.01 [c]	0.11 ± 0.01 [b]	0.11 ± 0.01 [b]	0.13 ± 0.00 [a]
MUFA	76.87±0.24 [a]	76.17±0.30 [a]	71.90±1.54 [b]	72.31±0.14 [b]
PUFA	4.82±0.22 [c]	4.79±0.13 [c]	9.78±1.17 [a]	8.91±0.42 [b]
SFA	18.19±0.17 [c]	18.92±0.42 [a]	18.33±0.59 [bc]	18.70±0.33 [ab]
Acidity (% oleic acid)	0.24 ± 0.04 [b]	0.22 ± 0.04 [b]	0.31 ± 0.02 [a]	0.33 ± 0.05 [a]
Peroxide value (meq O_2 kg^{-1})	5.80 ± 1.57 [bc]	5.16 ± 0.92 [c]	7.93 ± 1.73 [a]	7.14 ± 1.81 [ab]
K_{270}	0.130 ± 0.030 [b]	0.136 ± 0.021 [b]	0.198 ± 0.024 [a]	0.206 ± 0.009 [a]
K_{232}	1.28 ± 0.08 [b]	1.28 ± 0.06 [b]	1.46 ± 0.08 [a]	1.41 ± 0.06 [a]

3.4. Phenol contents of 'Galega Vulgar' and 'Cobrançosa' olive oils

The evolution of total phenols in 'Galega Vulgar' and 'Cobrançosa' VOO along fruit ripening is represented in Figure 1. Total phenols were very high, in all ripening stages, denoting the high quality of the fruits and the fact that no damaged fruits were processed. 'Cobrançosa' oils showed higher total phenol content than 'Galega Vulgar' for all ripening indexes. Within the same cultivar, olive oils obtained from RF olives have also higher total phenol content. It seems that the decrease in total phenols begins between 2.5 and 3 of ripening index. Such a high content of total phenols resulted in high bitter intensity detected by sensory evaluation. The only exception was the VOO obtained from 'Galega Vulgar' with the highest ripening index (RI > 4.0) that showed almost disappearance of the bitter taste (Figure 2). For 'Cobrançosa' oils, high scores of green flavours as well as quite astringent and pungent notes, were sensory evaluated in all samples, even for ripening index of 4.0. Furthermore, for each cultivar and for each ripening stage the higher content of phenols has as consequence a high oxidative stability (OS) (Table 3).

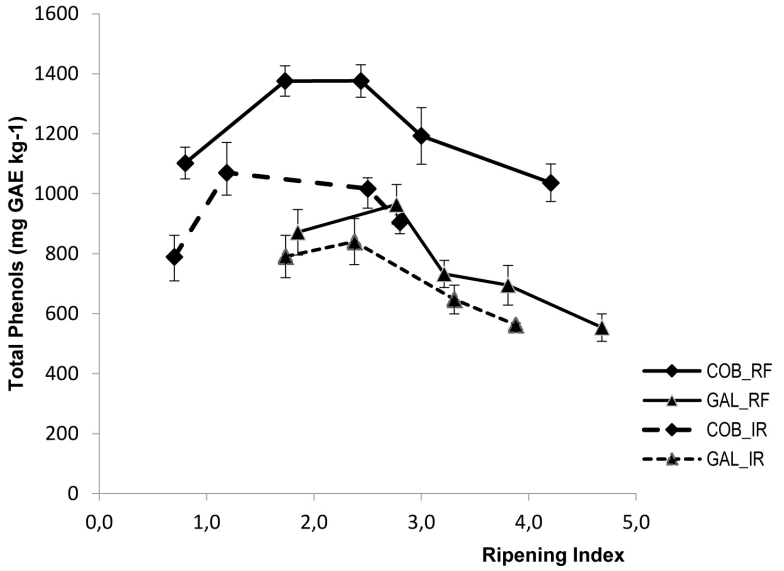

Figure 1. Evolution of total phenols in early ripening stages for 'Galega Vulgar' and 'Cobrançosa' virgin olive oils in two orchards (rain fed-RF and irrigated-IR).

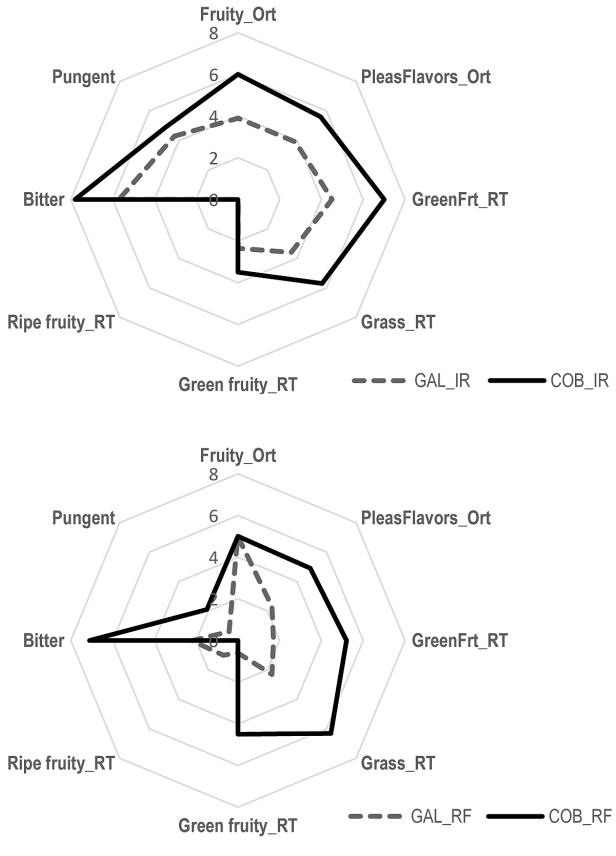

Figure 2. Sensory profiles of 'Galega Vulgar' and 'Cobrançosa' VOO from olives produced in the orchard IR (Gal_IR and COB_IR, with ripening index of 3.0) and in orchard RF (Gal_RF and COB_RF, with ripening index 4.0). Fruity_ORT- orthonasal olive fruity; PleasFlavors_ORT- other pleasant flavours; GreenFrt_RT – retronasal green fruity; Grass_RT- retronasal grass; Ripe fruity_RT- retronasal ripe flavours.

Table 3. Tocopherols, phenols, chlorophyll pigments and oxidative stability of 'Galega Vulgar' and 'Cobrançosa' olive oils, in two olive orchards (RF and IR). In each row superscript indexes indicate differences based on Tukey test (four independent samples per group of olive oils with similar ripening index).

	'Galega Vulgar'				'Cobrançosa'			
Olive orchard	RF		IR		RF		IR	
Harvest month	Oct	Nov	Oct	Nov	Oct	Nov	Oct	Nov
Chlorophyll pigments (mg kg^{-1})	55.54^{ab}	3.01^{c}	76.3^{ab}	9.12^{c}	53.9^{b}	10.6^{c}	79.3^{a}	51.2^{b}
α-Tocopherol (mg kg^{-1})	342.52^{c}	285.07^{d}	393.89^{b}	293.25^{d}	370.69^{bc}	290.25^{d}	448.67^{a}	293.34^{d}
β-Tocopherol (mg kg^{-1})	3.46^{c}	4.27^{bc}	4.90^{ab}	5.16^{a}	3.64^{cd}	3.89^{cd}	4.95^{ab}	3.97^{cd}
γ-Tocopherol (mg kg^{-1})	13.85^{b}	16.00^{b}	9.49^{c}	20.15^{a}	5.99^{de}	8.68^{c}	5.77^{e}	8.18^{cd}
Hydroxytyrosol (mg kg^{-1})	1.20^{b}	1.20^{b}	1.60^{b}	1.48^{b}	1.41^{b}	1.35^{b}	2.43^{a}	3.17^{a}
Tyrosol (mg kg^{-1})	4.28^{a}	3.27^{ab}	1.69^{c}	1.92^{bc}	1.20^{c}	1.33^{c}	2.19^{bc}	2.13^{bc}
Vanillic acid (mg kg^{-1})	0.38^{a}	0.30^{a}	0.33^{a}	0.32^{a}	0.19^{a}	0.25^{a}	0.39^{a}	0.37^{a}
Vanillin (mg kg^{-1})	0.69^{bc}	0.44^{c}	0.62^{bc}	0.56^{c}	0.92^{b}	1.54^{a}	0.75^{bc}	0.73^{bc}
p-Coumaric acid (mg kg^{-1})	0.22^{b}	0.19^{b}	0.26^{ab}	0.22^{b}	0.18^{b}	0.17^{b}	0.20^{b}	0.38^{a}
Luteolin (mg kg^{-1})	0.32^{d}	0.73^{cd}	0.29^{d}	0.43^{d}	1.18^{bc}	2.25^{a}	1.56^{ab}	1.42^{abc}
Apigenin (mg kg^{-1})	0.08^{c}	0.07^{c}	0.07^{c}	0.11^{c}	0.41^{b}	0.65^{a}	0.75^{a}	0.89^{a}
Oxidative stability (h)	42^{a}	33^{b}	38^{a}	33^{b}	32^{b}	23^{c}	25^{c}	27^{c}

The phenol compounds quantified of 'Galega Vulgar' and 'Cobrançosa' VOO are presented in Table 3. For both cultivars, VOO α-tocopherol content decreased during the ripening process and although γ-tocopherol showed an increase for the last harvesting dates, this trend is explained by Beltrán et al. (2010) to be related to the chlorophyll losses in the oil. The presence of high contents of α-tocopherol in early ripening stages (October harvest) represents a good antioxidant protection during storage for both VOO, without contributing, as hydrophilic phenols do, for bitter taste (Peri, 2014). In the present harvest, γ-tocopherol contents of 'Galega Vulgar' VOO were significantly higher than those of Cobrançosa VOO. This is considered to be a good characteristic for 'Galega Vulgar' VOO, as this compound provides different antioxidant activities in food and in-vitro studies and showed higher activity in trapping lipophilic electrophiles and reactive nitrogen and oxygen species (Wagner et al., 2004).

Chromatographic determination of the phenolic compounds in VOO by HPLC showed a similar profile for both monovarietal oils which included seven compounds that were possible to identify and quantify: hydroxytyrosol, tyrosol, vanillic acid, vanillin, *p*-coumaric, luteolin and apigenin (Table 3 and Figure 3). Oleuropein was not quantified because in 'Cobrançosa' chromatograms had a bad resolution (<1.0). Verbascoside (retention time of 27 min), as was expected,

was not present in VOO, but was present in olive extracts of ‘Cobrançosa’ olives (Sousa et al., 2014).

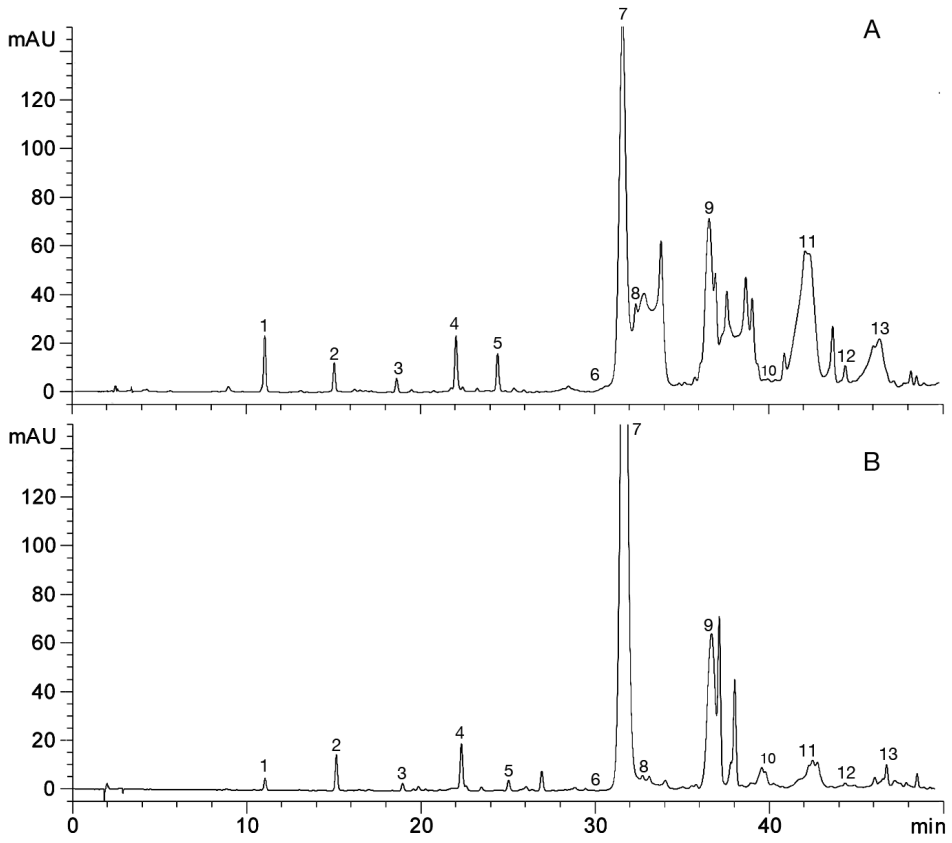

Figure 3. HPLC chromatograms (at 280 nm) of phenolic extracts of ‘Cobrançosa’ (A) and ‘Galega Vulgar’ (B) virgin olive oils, at the same ripening index (RI=3) in the same grove (IR). 1- hydroxytyrosol; 2- tyrosol; 3-vanillic acid; 4- vanillin; 5- *p*-coumaric acid; 6- *o*-coumaric acid; 7- 3,4-DHPEA-EDA; 8-oleuropein; 9- *p*-HPEA-EDA; 10- luteolin; 11- 3,4-DHPEA-EA; 12- apigenin; 13- *p*-HPEA-EA

Olive oils showed low amounts of phenolic acids and phenolic alcohols, and the prevalent phenolic compound was the dialdehydic form of elenolic acid linked to hydroxytyrosol (3,4-DHPEA-EDA), confirmed by LC-MS, which in some samples of ‘Galega Vulgar’ oils corresponds to more than 50% of total area. It was also confirmed by LC/MS, the presence of the secoiridoids *p*-HPEA-EDA, 3,4-DHPEA-EA and 3,4-*p*-HPEA-EA. The phenolic alcohols detected were hydroxytyrosol and tyrosol (Table 3), the first one was in higher content in ‘Cobrançosa’ VOO from IR orchard and the second one in ‘Galega Vulgar’ VOO from RF orchard, but no significant differences between values in early ripening stages were found. Such low contents in hydroxytyrosol and tyrosol are expected in fresh oils, because during the storage of the olive oils an increase in these

phenolic alcohols occurs, explained by the breakdown of the secoiridoids 3,4-DHPEA-EDA, *p*-HPEA-EDA, 3,4-DHPEA-EA, and 3,4-DHPEA-AC (Brenes et al., 2001; Morelló et al., 2004). The results obtained for these phenolic alcohols are quite similar of the ones obtained for other Portuguese cultivars (Negrinha do Freixo and Carrasquinha) in early ripening stages (Garcia et al., 2012). In what concerns phenolic acids no caffeic, ferulic or gallic acid were detected by HPLC-DAD or LC-MS. However, caffeic acid was identified in 'Picual' as well as in Moraiolo, Frantoio and Leccino VOO; ferulic and gallic acids were detected in 'Picual' and 'Hojiblanca' olive oils (Servili and Montedoro, 2002; Rivas et al., 2013). No significant differences ($p>0.05$) between ripening stages were achieved for vanillic acid for all cultivars, orchards and ripening stages. For the phenolic acid derivative, vanillin, higher contents in 'Cobrançosa' olive oils were found, with significantly higher values ($p<0.05$) in the olive oils produced from olives from the RF orchard, showing for these oils a significant increase along fruit ripening, like in cultivar 'Picudo' (Jiménez et al., 2013); this was also reported in previous year with the same cultivars (*submitted*). The content of *p*-coumaric acid was higher in 'Cobraçosa' olive oils from IR orchard for higher ripening index, and *o*-coumaric acid was not detected. The flavonoids, luteolin and apigenin, were in significantly ($p<0.05$) higher contents in 'Cobrançosa' VOO. For all the phenol compounds quantified in this work, the observed contents were in the range of values referred by "Phenol Explorer", although different cultivar and different modes of olive oil extraction were used (Neveu et al., 2010).

García-Rodríguez et al. (2011) showed that verbascoside, compared to oleuropein and demethyloleuropein, was the preferred substrate for olive POD, which seems to confirm that the best substrates for these enzymes are those having the highest number of hydroxyl groups in the benzoic ring. The same study showed that purified PPO, although active towards both substrates, has slightly higher oxidation rates for verbascoside than for oleuropein and that the highest oxidation rate of VOO secoiridoids by PPO was observed for 3,4-DHPEA-EDA and 3,4- DHPEA-EA (ortho diphenolic secoiridoids) while almost no activity was observed towards monophenolic secoiridoids. In the present study, 'Galega Vulgar' PPO was probably more active towards verbascoside and oleuropein than 'Cobrançosa' PPO, because no verbascoside and low contents of

oleuropein were present in ‘Galega Vulgar’ oils (Figure 3). Higher PPO activity for higher ripening indexes as well as seed POD activity in ‘Galega Vulgar’ olives may explain the lower phenol content of ‘Galega Vulgar’ oils vs. ‘Cobrançosa’ oils. However, beta-glycosidase activity can also mask the phenolic glycosides oxidative degradation (Romero-Segura et al., 2012).

Phenols are not the only virgin olive oil compounds with impact on taste and aroma. Bitterness enhanced by the presence of cut grass odorant is an example of taste and smell interactions (Caporale et al., 2004). The odorants of ‘Galega Vulgar’ and ‘Cobrançosa’ VOO evaluated in the present study have already been studied (Peres et al., 2013): in early ripening the volatile compounds for the cut grass sensations were present, which in conjunction with the high phenol content, explain the very high bitter taste scores given by the panelists (Figure 2).

4. Conclusion

Phenol composition of EVOO can give important information on their quality because it has an important impact on organoleptic evaluation and on the nutritional value of the product. ‘Galega Vulgar’ and ‘Cobrançosa’ olive oils obtained from fruits in early ripening stages showed very high contents in phenolic compounds. This study shows that harvesting in different ripening stages, in each orchard, for each cultivar will produce several types of olive oils from green and pungent oil with high levels of phenol compounds to golden, mild and fruity oil. Therefore, the decision of the harvesting date will allow the production of virgin olive oils with different taste notes and functional value. Moreover, for the production of olive oil with high shelf life, harvesting in early ripening stages can be a good decision. This is the case for ‘Galega Vulgar’ oils that can have improved their nutritional and sensory characteristics as well as improved the shelf life (higher oxidative stability). Especially the activity of PPO can also dictate the profile of phenol compounds in the final olive oil due to different substrate specificity.

However, very early ripening stages can also result in oils with a strong green colour that some consumers are not used to, especially for ‘Cobrançosa’, quite astringent and pungent olive oils, that are not balanced at all. From the point of

view of the olive grower, a very low yield obtained from very green olives is always another reason for delaying the harvest.

The productivity of the olive orchard, resulting from different agronomical practices can influence the ripening progress and consequently the biosynthesis of the different phenol compounds.

Further studies on these two important cultivars for Portugal, will be needed to evaluate the influence of environment and ripening in the phenol content and the influence of other enzymes on the composition of the final oil, in order to have a better knowledge for the Protected Designations of Origin (PDO) where they are most closely linked.

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References

Andrewes, P., J. L. H. C. Busch, T. Joode, A. Groenewegen and H. Alexandre (2003). "Sensory Properties of Virgin Olive Oil Polyphenols: Identification of Deacetoxy-ligstroside Aglycon as a Key Contributor to Pungency." Journal of Agricultural and Food Chemistry **51**(5): 1415-1420.

Beauchamp, G. K., R. S. J. Keast, D. Morel, J. Lin, J. Pika, Q. Han, C.-H. Lee, A. B. Smith and P. A. S. Breslin (2005). "Phytochemistry: Ibuprofen-like activity in extra-virgin olive oil." Nature **437**(7055): 45-46.

Beltrán, G., A. Jiménez, C. Rio, S. Sánchez, L. Martínez, M. Uceda and M. P. Aguilera (2010). "Variability of vitamin E in virgin olive oil by agronomical and genetic factors." Journal of Food Composition and Analysis **23**(6): 633-639.

Bendini, A., L. Cerretini, A. Carrasco-Pancorbo, A. M. G. Caravaca, A. Segura-Carretero, A. Fernández-Gutiérrez and G. Lercker (2007). "Phenolic Molecules in Virgin Olive Oils: a Survey of Their Sensory Properties, Health Effects, Antioxidant Activity and Analytical Methods. An Overview of the Last Decade." Molecules **12**(8): 1679-1719.

Bramley, P. M., I. Elmadfa, A. Kafatos, K. F. J., Y. Manios, H. E. Roxborough, W. Schuch, P. J. A. Sheehy and K. H. Wagner (2000). "Vitamin E." Journal of the Science of Food and Agriculture **80**(7): 913-938.

Brenes, M., A. García, P. García and A. Garrido (2001). "Acid Hydrolysis of Secoiridoid Aglycons during Storage of Virgin Olive Oil." Journal of Agricultural and Food Chemistry **49**(11): 5609-5614.

Caporale, G., S. Policastro and E. Monteleone (2004). "Bitterness enhancement induced by cut grass odorant (cis-3-hexen-l-ol) in a model olive oil." Food Quality and Preference **15**(3): 219-227.

Cerretani, L., M. D. Salvador, A. Bendini and G. Fregapane (2008). "Relationship Between Sensory Evaluation Performed by Italian and Spanish Official Panels and Volatile and Phenolic Profiles of Virgin Olive Oils." Chemosensory Perception **1**(4): 258-267.

Dag, A., G. Harlev, S. Lavee, I. Zipori and Z. Kerem (2014). "Optimizing olive harvest time under hot climatic conditions of Jordan Valley, Israel." European Journal of Lipid Science and Technology **116**(2): 169-176.

Dierkes, G., S. Krieger, R. Duck, A. Bongartz, O. J. Schmitz and H. Hayen (2012). "High-Performance Liquid Chromatography-Mass Spectrometry Profiling of Phenolic Compounds for Evaluation of Olive Oil Bitterness and Pungency." Journal of Agricultural and Food Chemistry **60**(31): 7597-7606.

Gajewska, E., M. Skłodowska, M. Słaba and J. Mazur (2006). "Effect of nickel on antioxidative enzyme activities, proline and chlorophyll contents in wheat shoots." Biologia Plantarum **50**(4): 653-659.

Garcia, B., J. Magalhães, G. Fregapane, M. D. Salvador and F. Paiva-Martins (2012). "Potential of selected Portuguese cultivars for the production of high quality monovarietal virgin olive oil." European Journal of Lipid Science and Technology **114**(9): 1070-1082.

García-Rodríguez, R., C. Romero-Segura, C. Sanz, A. Sánchez-Ortiz and A. G. Pérez (2011). "Role of polyphenol oxidase and peroxidase in shaping the phenolic profile of virgin olive oil." Food Research International **44**(2): 629-635.

Gómez-Rico, A., G. Fregapane and M. D. Salvador (2008). "Effect of cultivar and ripening on minor components in Spanish olive fruits and their corresponding virgin olive oils." Food Research International **41**(4): 433-440.

Gómez-Rico, A., M. D. Salvador and G. Fregapane (2009). "Virgin olive oil and olive fruit minor constituents as affected by irrigation management based on SWP and TDF as compared to ETc in medium-density young olive orchards (Olea europaea L. cv. Cornicabra and Morisca)." Food Research International **42**(8): 1067-1076.

Guillaume, C., L. Ravetti and S. Gwyn (2010). "Characterisation of Phenolic Compounds in Oils Produced from Frosted Olives." Journal of the American Oil Chemists' Society **87**(3): 247-254.

Gurr, M. I. (2000). The Role of Lipids in Human Nutrition. Handbook of Olive Oil. J. H. R. Aparicio. Maryland, Aspen Publishers, Inc.**:** 521-563.

Hbaieb, R. H., F. Kotti, R. García-Rodríguez, M. Gargouri, C. Sanz and A. G. Pérez (2015). "Monitoring endogenous enzymes during olive fruit ripening and storage: Correlation with virgin olive oil phenolic profiles." Food Chemistry **174**(0): 240-247.

Hermoso, M., M. Uceda, L. Frias and G. Beltran (1997). Maduración. El Cultivo del Olivo. D. Barranco, D. Fernandez-Escobar and L. Rallo. Madrid, Ediciones Mundi-Prensa**:** 137-153.

Inglese, P., E. Barone and G. Gullo (1996). "The effect of complementary irrigation on fruit growth, ripening pattern and oil characteristics of olive (Olea europaea L.) cv. Carolea." Journal of Horticultural Science **71**(2): 257-264.

IOC (2009). Determination of Biophenols in olive oils by HPLC. COI/T20/DOC.29

Jiménez, B., A. Sánchez-Ortiz, M. L. Lorenzo and A. Rivas (2013). "Influence of fruit ripening on agronomic parameters, quality indices, sensory attributes and phenolic compounds of Picudo olive oils." Food Research International **54**(2): 1860-1867.

Kalua, C. M., M. S. Allen, D. R. Bedgood, A. G. Bishop and P. D. Prenzler (2005). "Discrimination of Olive Oils and Fruits into Cultivars and Maturity Stages Based on Phenolic and Volatile Compounds." Journal of Agricultural and Food Chemistry **53**(20): 8054-8062.

Kanakis, P., A. Termentzi, T. Michel, E. Gikas, M. Halabalaki and A.-L. Skaltsounis (2013). "From Olive Drupes to Olive Oil. An HPLC-Orbitrap-based Qualitative and Quantitative Exploration of Olive Key Metabolites." Planta Med **79**(16): 1576-1587.

Lavelli, V. and L. Bondesan (2005). "Secoiridoids, Tocopherols, and Antioxidant Activity of Monovarietal Extra Virgin Olive Oils Extracted from Destoned Fruits." Journal of Agricultural and Food Chemistry **53**(4): 1102-1107.

Martín-Peláez, S., M. I. Covas, M. Fitó, A. Kušar and I. Pravst (2013). "Health effects of olive oil polyphenols: Recent advances and possibilities for the use of health claims." Molecular Nutrition & Food Research **57**(5): 760-771.

Morelló, J.-R., M.-J. Motilva, M.-J. Tovar and M.-P. Romero (2004). "Changes in commercial virgin olive oil (cv Arbequina) during storage, with special emphasis on the phenolic fraction." Food Chemistry **85**(3): 357-364.

Neveu, V., J. Perez-Jiménez, F. Vos, V. Crespy, L. du Chaffaut, L. Mennen, C. Knox, R. Eisner, J. Cruz, D. Wishart and A. Scalbert (2010). "Phenol-Explorer: an online comprehensive database on polyphenol contents in foods." Database **2010**.

Oktay, M., I. Kufrevioglu, I. Kocaçaliskan and H. Sakiroglu (1995). "Polyphenoloxidase from Amasya Apple." Journal of Food Science **60**(3): 494-496.

Oliveras López, M. J., M. Innocenti, F. Ieri, C. Giaccherini, A. n. Romani and N. Mulinacci (2008). "HPLC/DAD/ESI/MS detection of lignans from Spanish and Italian Olea europaea L. fruits." Journal of Food Composition and Analysis **21**(1): 62-70.

Peres, F., L. Henriques, C. Gouveia, C. Vitorino and J. Coutinho (2009). Effect of mass trapping of olive fruit fly (Bactrocera oleae) on the quality of organic virgin olive oil. 3rd Scientific Conference of Institute of Tropics and Subtropics, Sustainable use of Natural Resources in tropics and subtropics, Czech University of Life Sciences, Institute of Tropics and Subtropics (Ed.), Czech Republic.

Peres, F., H. H. Jeleń, M. M. Majcher, M. Arraias, L. L. Martins and S. Ferreira-Dias (2013). "Characterization of aroma compounds in Portuguese extra virgin olive oils from Galega Vulgar and Cobrançosa cultivars using GC–O and GCxGC–ToFMS." Food Research International **54**(2): 1979-1986.

Peres, F., J. P. Luz, P. Fragoso, C. Gouveia, C. Vitorino, C. Amaro, L. Henriques, J. Coutinho, C. M. Pintado, C. Peres and A. Ferreira (2010). "Integrated Production and Quality of Galega Olive Oil." IOBC Bulletin(53): 145-149.

Peres, F., L. L. Martins and S. Ferreira-Dias (2014). "Laboratory-scale optimization of olive oil extraction: Simultaneous addition of enzymes and microtalc improves the yield." European Journal of Lipid Science and Technology **116**(8): 1054-1062.

Peres, F., L. L. Martins, M. Mourato and S. Ferreira-Dias (2011). Changes in virgin olive oil antioxidants, polyphenol oxidase and peroxidase activities during fruit ripening. 4th International Conference Olivebioteq 2011, Chania.

Peri, C. (2014). Quality Excellence in Extra Virgin Olive Oils. Olive Oil Sensory Science. E. S. L. Monteleone, John Wiley & Sons, Ltd**:** 1-32.

Peyrot des Gachons, C., K. Uchida, B. Bryant, A. Shima, J. B. Sperry, L. Dankulich-Nagrudny, M. Tominaga, A. B. Smith, G. K. Beauchamp and P. A. S. Breslin (2011). "Unusual Pungency from Extra-Virgin Olive Oil Is Attributable to Restricted Spatial Expression of the Receptor of Oleocanthal." The Journal of Neuroscience **31**(3): 999-1009.

Pirisi, F. M., P. Cabras, C. F. Cao, M. Migliorini and M. Muggelli (2000). "Phenolic Compounds in Virgin Olive Oil. 2. Reappraisal of the Extraction, HPLC Separation, and Quantification Procedures." Journal of Agricultural and Food Chemistry **48**(4): 1191-1196.

Pizarro, M. L., M. Becerra, A. Sayago, M. Beltrán and R. Beltrán (2013). "Comparison of Different Extraction Methods to Determine Phenolic Compounds in Virgin Olive Oil." Food Analytical Methods **6**(1): 123-132.

Pokorny, J., L. Kalinová and P. Dysseler (1995). "Determination of Chlorophyll pigments in Crude Vegetable Oils " Pure Appl. Chem. **67**(10): 1781-1787.

Rivas, A., A. Sanchez-Ortiz, B. Jimenez, J. García-Moyano and M. L. Lorenzo (2013). "Phenolic acid content and sensory properties of two Spanish monovarietal virgin olive oils." European Journal of Lipid Science and Technology **115**(6): 621-630.

Roche, H. M., M. J. Gibney, A. Kafatos, A. Zampelas and C. M. Williams (2000). "Beneficial properties of olive oil." Food Research International **33**(3-4): 227-231.

Romero-Segura, C., R. García-Rodríguez, A. Sánchez-Ortiz, C. Sanz and A. G. Pérez (2012). "The role of olive β-glucosidase in shaping the phenolic profile of virgin olive oil." Food Research International **45**(1): 191-196.

Rotondi, A., A. Bendini, L. Cerretani, M. Mari, G. Lercker and T. G. Toschi (2004). "Effect of Olive Ripening Degree on the Oxidative Stability and Organoleptic Properties of Cv. Nostrana di Brisighella Extra Virgin Olive Oil." Journal of Agricultural and Food Chemistry **52**(11): 3649-3654.

Ryan, D., K. Robards and S. Lavee (1999). "Changes in phenolic content of olive during maturation." International Journal of Food Science & Technology **34**(3): 265-274.

Saraiva, J. A., C. S. Nunes and M. A. Coimbra (2007). "Purification and characterization of olive (Olea europaea L.) peroxidase – Evidence for the occurrence of a pectin binding peroxidase " Food Chemistry **101**(4): 1571-1579.

Servili, M., M. Baldioli, A. L. Begliomini, R. Selvaggini and G. F. Montedoro (2000). The phenolic and volatile compounds of virgin olive oil:relationships with the endogenous oxidorreductases during the mechanical oil extraction process. Flavour and Fragrance Chemistry. V. Lanzotti and O. Taglialatela-Scafati. Dordrecht, Kluwer Academic Publishers**:** 163-173.

Servili, M., M. Baldioli, R. Selvaggini, E. Miniati, A. Macchioni and G. F. Montedoro (1999). "High-performance liquid chromatography evaluation of phenols in olive fruit, virgin olive oil, vegetation waters, and pomace and 1D- and 2D-nuclear magnetic resonance characterization " Journal of the American Oil Chemists' Society **76**(7): 1558-9331

Servili, M., S. Esposto, R. Fabiani, S. Urbani, A. Taticchi, F. Mariucci, R. Selvaggini and G. Montedoro (2009). "Phenolic compounds in olive oil: antioxidant, health and organoleptic activities according to their chemical structure." Inflammopharmacology **17**(2): 76-84.

Servili, M. and G. F. Montedoro (2002). "Contribution of phenolic compounds to virgin olive oil quality." European Journal of Lipid Science and Technology **104**(9-10): 602-613.

Servili, M., A. Taticchi, S. Esposto, S. Urbani, R. Selvaggini and G. Montedoro (2007). "Effect of Olive Stoning on the Volatile and Phenolic Composition of Virgin Olive Oil." Journal of Agricultural and Food Chemistry **55**(17): 7028-7035.

Sousa, A., R. Malheiro, S. Casal, A. Bento and J. A. Pereira (2014). "Antioxidant activity and phenolic composition of Cv. Cobrançosa olives affected through the maturation process." Journal of Functional Foods **11**(0): 20-29.

Velasco, J. and C. Dobarganes (2002). "Oxidative stability of virgin olive oil." European Journal of Lipid Science and Technology **104**(9-10): 661-676.

Wagner, K.-H., A. Kamal-Eldin and I. Elmadfa (2004). "Gamma-Tocopherol - An Underestimated Vitamin? ." Ann Nutr Metab **48**(169-188).

Chapter 7

General conclusions and perspectives of future research

1. General Conclusions

A better knowledge of the cultivars for olive oil production, namely their ripening profile, the adaptation to mechanical harvest, the susceptibility to pests and diseases and to frost damage, as well as the optimization of yield and olive oil quality (higher oxidative stability, nutritional value and sensory characteristics) are the challenges to promote progress and innovation in olive oil technology. From the olive grower point of view, it will be the productivity and extraction yield the decisive aspects for the choice of the cultivar and cultural practices that enhance these factors, like irrigation and fertilization. Concerning productivity, fruit susceptibility to pests and diseases/frost damage and adaptability to mechanical harvesting are important factors to consider. For industry, yield is the first concern but the olive oil shelf life and its sensory characteristics are equally important. From the consumer point of view, price and sensory characteristics are the choice drivers for extra virgin olive oil, but environment and health aspects can also be decisive (eg. organic agriculture). The recent authorization of the European Food Safety Authority (EFSA) for health claims on olive oil labeling can be a future important issue both from the consumer and industrial points of view.

To study yield improvement and olive oil characteristics, Abencor extraction system was used in the present work. An optimization of the extraction process was established, using a Placket-Burman design to select significant technological variables followed by a CCRD to optimize the process as a function of these selected factors. Based on the results of Placket-Burman and CCRD, the optimized Abencor operation conditions were: no water addition in 30 min malaxation and 14 % (w/w) water addition at 50 ºC in centrifugation. To study the effect of adjuvants in yield and in olive oil quality obtained from ‘Cobrançosa’ and ‘Galega Vulgar’ fruits, a CCRD was also followed for the optimization of extraction with simultaneous addition of enzymes (E) (Endozym Olea) and natural microtalc (MT) (FC8-Aw). The results of the effect of E and/or MT addition showed to depend on the cultivar. However, an improvement in extractability was always found for both cultivars. This study shows that for ‘Cobrançosa’ olives, considering both olive oil yield and economical aspects, the chosen conditions are 0.05-0.15 % of microtalc in the absence of added enzymes. For ‘Galega Vulgar’ olives, the highest extractability is observed under the presence of

amounts of enzyme preparations higher than 0.10 % and microtalc amount of 0.4-0.5 %. However, these results changed with the ripening index of the olives. No influence was found on the concentration of total phenols, chlorophyll pigments and K_{225}, as well as on chemical quality criteria parameters of the extracted olive oil after the combined effects of MT and of E.

Nowadays, for olive oil technology improving sensory characteristics is one of the main goals. To study the sensory characteristics it is important to know the compounds that have impact on flavour. Our aim in the present study was to select the main compounds responsible for the major odour notes in Galega Vulgar and Cobrançosa olive oils using gas chromatography – olfactometry (GC-O), and then to quantify the detected odourants, as well as other compounds known from the literature to have influence on olive oil flavour. Galega and Cobrançosa olive oils obtained from olives in early ripening stages showed a very similar aroma profile by GC-O. The majority of the volatile compounds identified by GC × GC-ToF-MS system could not be sensory detected, probably due to their low concentrations and/or high odor threshold. So, relatively few odourants can explain the aromatic profiles of each monovarietal olive oil. Under optimized conditions, 22 volatile compounds were quantified in all samples. *Trans*-2-hexenal was the most abundant of the quantified compounds with an odour threshold of 420 μg kg^{-1}. Both trans-2-hexenal and hexanal, with an odour threshold of 80 μg kg^{-1}, are the main compounds responsible for the green attributes of 'Galega Vulgar' and 'Cobrançosa' VOO. For 10 compounds, no quantitative differences were detected between the two monovarietal olive oils, while the contents of 12 compounds differed among oils. Concerning the harvesting time and cultivar, nine volatiles showed to have discriminant power among samples, namely heptanal, *trans*-2-hexenal, 1-octen-3-ol, nonanal, 2,3-butanedione, ethyl-2-methylbutyrate, hexanal, *cis*-3-hexenylacetate and 3-methylbuthylacetate.

Volatile and phenolic compounds are the main compounds that affect the aroma and taste of olive oil. Apart sensorial characteristics, the presence of different bioactive compounds in virgin olive oil influences its nutritional and oxidative stability. Phenolic compounds are olive endogenous bioactive compounds highly susceptible to degradation. Olive endogenous oxidoreductases, mainly

polyphenol oxidases (PPO) and peroxidases (POD), may play an important role on the profile of bioactive compounds in olive oil by promoting oxidation of phenolic compounds. The aim of this study was to evaluate if changes on PPO and POD activities in olive fruits from *Olea europaea*, 'Cobrançosa' and 'Galega Vulgar' cvs. are related with the composition of their olive oils, especially phenolic compounds. Pattern recognition techniques (Principal Component Analysis, PCA, Cluster Analysis, CA, and Discriminant Analysis, DA) were used for multivariate data analysis. Olive oils characterized by their fatty acid composition were grouped by cultivar. When olive oils were characterized by their phenolic composition, green pigments, and enzymatic activities in fruits, they could be discriminated by olive ripening stage. Along ripening, PPO activity was only detected in the fruit mesocarp of both cultivars and POD activity was mainly detected in the seeds. Higher contents of alpha tocopherol were observed in the beginning of the harvest period and Galega oils have higher contents of gamma-tocopherol. Secoridoid derivatives that have significant biological activities, like oleacein (3,4-DHPEA-EDA) represent the highest percentage of phenol compounds in both olive oils. Cobrançosa had higher contents of flavonoids. The ripening stage of fruits showed to be a key factor on the amount and profile of bioactive compounds of olive oil.

2. Perspectives of future research

The thesis provided background information from which several future works can be suggested:

− The application of adjuvants for other cultivars/ripening index based on previous optimization of malaxation conditions.

All the trials performed showed that adjuvants have a positive effect on yield. Therefore, research must go on testing different adjuvants not only in 'Cobrançosa' and 'Galega vulgar' but also in other cultivars, based in specific ripening indices and in previous crushing and malaxation optimization. RSM proved to be a good methodology to establish the best extraction conditions.

A survey of the scientific literature showed that volatile compounds are usually not studied in adjuvants addition trials. Thus, the effect of adjuvants on volatile compounds release in VOO should be evaluated.

− Optimization conditions in confined malaxer as well as inside the crusher (control of O_2 and CO_2 concentration).

The atmosphere control in the malaxation step has been studied for several researchers. However, the control of atmosphere composition inside the mill could as well have a special relevance for enzymatic control.

− Effect of fruit stoning in Galega Vulgar and Cobrançosa olive oils quality and composition.

The qualitative and quantitative composition of the volatile and phenolic compounds of high quality virgin olive oils depends closely on the enzymes activity released by crushing and accelerated in malaxation. Genetic characteristics fix the contents of different enzymes, which are therefore responsible for the qualitative composition of volatile and phenolic compounds. However, the accumulation of the different volatile and phenolic compounds in fruits and VOO is connected with enzymes activities, which in VOO of good quality, are related to the ripening degree of the fruits.

In the present study the presence of POD was detected in the seed of both cultivars and consequently it should be very important to evaluate the behaviour of olive oils obtained by stoned mills. This could be of special importance to lower the level of bitter/pungent/cut grass sensations namely for Cobrançosa oils extracted from fruits in very early ripening stages.

− Identify limiting factors for the biosynthesis of VOO aroma compounds

For volatile compounds, these findings must be investigated for olive oils, from Portuguese cultivars because in the present study LOX activity was evaluated in early ripening stages but we could not find any relationship with the presence of volatile compounds. Other enzyme activities from the LOX pathway should be studied, in order to explain the presence or absence of some compounds. Moreover, the study of each of the LOX pathway enzymes is very important in order to promote/inhibit their activities during olive oil extraction.

− To evaluate other biochemical factors directly affecting the phenolic profile of virgin olive oil (VOO) such as the β-glucosidase activity present in the olive fruit.

In the present study, ‘Galega Vulgar’ PPO was probably more active towards verbascoside and oleuropein than ‘Cobrançosa’ PPO, because no verbascoside and low contents of oleuropein were present in ‘Galega Vulgar’ oils. Higher PPO activity for higher ripening indexes as well as seed POD activity in ‘Galega Vulgar’ olives may explain the lower phenol content of ‘Galega Vulgar’ oils vs. ‘Cobrançosa’ oils. However, beta-glucosidase activity can also mask the phenolic glucosides oxidative degradation. So, this must be object of research.

− Development of new types of EVOO.

VOO is unique amongst all edible oils because it is an ingredient which has the potential to complement the aroma and taste of the food. Thus, the development of different types of VOO that matches several food pairings needs more research from the technological point of view. Cultivar characteristics, ripening profile, olive orchard and technology can altogether influence the enzymatic behaviour of olives, pastes and olive oil, contributing to different compounds that will give several sensory notes.

− The application of new emerging technologies, such as pulsed electric field, ultrasound and microwave in olive oil extraction.

Such green technologies may be tested in order to increase extraction yield and improve nutritional value of VOO.

Finally, further studies on these two important cultivars for Portugal, will be needed to evaluate the influence of environment vs. ripening in the phenol and volatile compounds as well as the influence of other enzymes on the composition of the final oil. This knowledge is extremely important for the Protected Designations of Origin (PDO).

Thus, knowledge about enzyme behaviour in fruits of Portuguese cultivars during ripening, post-harvest and during the extraction process, with the aim of improving not only the yield but also nutritional and sensory characteristics of virgin olive oil, is a crucial research issue for ensuring relevant results in terms of technology transfer in the field of olive oil technology.