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OLIVE RIPENING AND OIL FLAVOUR IN VENETO: MOLECULAR AND TECHNOLOGICAL ASPECTS

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Declaration

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgement has been made in the text.

January 31st, 2012 Alice Vezzaro

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Table of contents

Summary

Extra-virgin olive oil is a very appreciated vegetable fat used mainly for food purposes due to its pleasant flavour and taste and to the demonstrated and well-known health promoting effects, which include anti-inflammatory and antioxidant functions and protection against cardiovascular diseases.

Olive oil quality depends on many factors, among which environmental (climate and soil), genetic (variety), agronomic and technological factors play important roles. Among the agronomic factors, the maintenance and use of healthy fruits, and, most of all, the choice of the best ripening stage of olive fruits represent the factors having a prominent role in determining olive oil quality.

The ideal cultivation area for olive tree is the Mediterranean region, which usually has mild winters and long dry summers. Despite these climatic limitations, olive trees grown in marginal areas such as Northern Italy are known to yield extra-virgin olive oils with a pleasant flavour and an overall high quality profile. However, the often unfavourable climatic conditions in this area make necessary optimising the choice of the best harvesting period and setting up technological factors in order to improve oil quality.

Many different approaches have been suggested so far for the definition of a simple and reliable way to assess the right harvesting time and to maximise oil quality. More in detail, physiological and biochemical changes that occur throughout olive ripening have been taken into account, such as loss of firmness, skin colour changes, pigment content or ratio, sugar content, oil accumulation, decrease in phenolics and some others. Results were however often conflicting due to the great variability among cultivars, growing areas, and technological aspects. It is therefore necessary to study all these factors in every specific growing area.

In this dissertation different approaches have been chosen to characterise a cultivar grown locally in Veneto, i.e. cv. Grignano, yielding a unique oil with lemon peel aromatic notes and to evaluate factors involved in the definition of oil quality also in comparison with Frantoio, a common Italian variety.

First of all, a study on anoxic oil extraction has been performed in order to understand the influence of the absence of oxygen, which can oxidise olive oil components but is also a required reagent for the biosynthesis of flavour compounds, on the global volatile profile of

oils extracted from olives harvested at four consecutive ripening stages across *véraison*. The analysis of the volatile components of oils has been performed by means of PTR-MS (Proton Transfer Reaction-Mass Spectrometry), a spectrometric technique that allows an evaluation of the global volatile fingerprint of samples without any preparatory step. This "real-time" evaluation of the spectral signature of extra-virgin olive oils from cv. Grignano coupled with unsupervised multivariate analyses, i.e. principal component analysis and hierarchical clustering, allowed to explore the complex spectra by means of the construction of a heat map. Results showed that the ripening stage has a predominant effect on the definition of the global volatile profile compared to anoxic extraction, and that the progression of ripening is characterised by the biosynthesis of specific groups of volatiles that seem to be "expressed" only transiently. Moreover, the progression of fruit ripening causes a saturation effect in the volatile profile.

Consequently, given that ripening is predominant over technological factors, an in depth study regarding olive ripening in Veneto region has been carried out focusing on fruit firmness, oil accumulation, chlorophyll *a* content, colorimetric analyses (calculating colour indices), and gene expression profiles. Results demonstrated that in Grignano and Frantoio cvs. ripening-related events were different, since Grignano showed an earlier and faster ripening course, both in loss of firmness and in oil accumulation. These findings suggest that monitoring cv. Grignano ripening stages is more difficult, since changes in ripening-related physiological parameters occur very quickly. Moreover, differences among two consecutive harvest seasons were observed resulting in a complex reading of the overall ripening profile. Statistical analyses have been performed, and principal component analysis established that cultivar Frantoio shows a greater variability in oil accumulation data, confirming that cv. Grignano ripening course is somehow more compact. One of the colour indices calculated in this study, namely CI, was suitable to distinguish drupe samples picked at different ripening stages.

Then, to investigate on the peculiar flavour features of Grignano oil, a study on the terpenic metabolism in olive has been carried out isolating and characterising for the first time three enzymes responsible for the biosynthesis of terpenes (terpene synthases) in this species. To this end, a thorough search for putative terpene synthases in a published olive EST (expressed sequence tag) database was performed. Fragments have been then amplified and

the full length genes have been used to deduce the amino acidic sequence to be heterologously expressed in *E. coli*. Expressed proteins have been checked for enzymatic activity and one of them turned out to be an active geraniol synthase, whilst the other two terpene synthases seemed inactive, a phenomenon not unusual in terpene metabolism. qPCR demonstrated that the three isolated terpene synthase genes have a ripening-dependent trend in transcript accumulation, suggesting a role in flavour development.

Finally, gene expression analyses performed in the previous works emphasised the absence of validated reference genes suitable for gene expression studies in olive. Therefore, an extensive search for candidate reference genes to be used for olive gene expression studies has been performed using the above-mentioned EST database and retrieving studies regarding validated reference genes in other plant species. 13 candidate genes were identified for *Olea europaea*, which were tested both in olive drupes and leaves for their transcriptional stability. Stability analyses performed with genorm^{PLUS} and NormFinder software packages showed that 12 over the 13 genes in this work represented actually good reference genes and that, among these genes, the optimum number that must be used for good data normalisation was two. The best two reference genes identified in the study, specifically suitable for normalisation of gene expression data in fruit samples at different developmental stages, were GAPDH2 and PP2A1.

Riassunto

L'olio extravergine di oliva è un olio vegetale molto apprezzato utilizzato principalmente per scopi alimentari a causa del profilo aromatico piacevole e a effetti salutistici noti e dimostrati, che includono proprietà antinfiammatorie e antiossidanti e una protezione nei confronti di alcune malattie cardiovascolari.

La qualità dell'olio di oliva dipende da molti fattori, e tra questi fattori ambientali (clima e suolo), genetici (cultivar), agronomici e tecnologici hanno un ruolo importante. Tra i fattori agronomici, la gestione e l'uso di frutti sani e, soprattutto, la scelta dello stadio di maturazione adatto dei frutti rappresentano le variabili che hanno un ruolo fondamentale nella determinazione della qualità globale dell'olio di oliva.

Le zone adatte per la coltivazione dell'olivo sono le regioni mediterranee, che sono caratterizzate solitamente da inverni miti ed estati lunghe e secche. Nonostante queste limitazioni climatiche, olivi cresciuti in zone marginali come l'Italia settentrionale conferiscono oli extravergini con profili aromatici gradevoli e di alta qualità. Le condizioni climatiche spesso avverse in queste aree rendono però necessarie un'ottimizzazione della scelta del miglior periodo di raccolta e l'implementazione di processi tecnologici allo scopo di migliorare la qualità dell'olio.

Molti approcci diversi sono stati proposti finora per la definizione di un modo semplice e affidabile per valutare il periodo di raccolta corretto e massimizzare la qualità dell'olio estratto. Più in dettaglio, i cambiamenti fisiologici e biochimici che si verificano durante la maturazione dell'oliva sono stati presi in considerazione, come ad esempio la perdita di consistenza, cambiamenti del colore superficiale delle drupe, contenuto di pigmenti o loro rapporti, contenuto in zuccheri, inolizione, diminuzione di componenti fenoliche e altri. I risultati si sono dimostrati però molto spesso discordanti, a causa della grande variabilità presente tra diverse cultivar, aree di coltivazione e aspetti tecnologici. Si rende perciò necessario uno studio di questi fattori in ogni singola area di coltivazione.

In questa tesi diversi approcci sono stati adottati per la caratterizzazione di una varietà coltivata localmente in Veneto, la cultivar Grignano, da cui si estrae un olio dal profilo sensoriale unico con note di buccia di limone, e per la valutazione di fattori coinvolti nella determinazione della qualità dell'olio anche attraverso un confronto con la cultivar Frantoio, una varietà diffusa comunemente in tutta Italia.

Inizialmente è stato condotto uno studio che prevedeva l'estrazione di olio in atmosfera priva di ossigeno, che può essere responsabile dell'ossidazione di componenti dell'olio ma è anche un reagente richiesto nella biosintesi di composti aromatici, per valutare l'effetto dell'estrazione in atmosfera controllata sul profilo volatile globale di oli ottenuti da olive raccolte in quattro stadi consecutivi di maturazione a cavallo dell'invaiatura. L'analisi delle componenti volatili è stata condotta mediante PTR-MS (spettrometria di massa con reazione di trasferimento di protoni), una tecnica di spettrometria che permette una valutazione del *fingerprint* globale dei volatili senza fasi preliminari o trattamento dei campioni. Questa valutazione "*real-time*" dei profili spettrali di oli extravergini da cultivar Grignano unita a analisi multivariata dei dati, mediante analisi delle componenti principali e *clustering* gerarchico, ha permesso di proiettare i complessi spettri ottenuti tramite l'analisi spettrometrica in una *heat map*. I risultati hanno dimostrato che lo stadio di maturazione ha un effetto predominante sulla definizione del profilo volatile rispetto all'estrazione in atmosfera controllata, e che il progredire della maturazione è caratterizzato dalla biosintesi di specifici gruppi di molecole volatili che sembrano quindi "espresse" in modo transiente. Inoltre, è stato osservato un effetto saturazione nel profilo volatile con il progredire della maturazione.

Conseguentemente, dato che lo stadio di maturazione ha un effetto prevalente rispetto ai fattori tecnologici studiati, uno studio approfondito della fisiologia della maturazione in Veneto è stato condotto ponendo particolare attenzione a perdita di consistenza, inolizione, contenuto in clorofilla *a*, dati colorimetrici (calcolo di indici di colore) e dati di espressione genica. I risultati hanno dimostrato che gli eventi collegati alla maturazione sono differenti nelle cultivar Grignano e Frantoio, dato che Grignano manifesta una dinamica di maturazione più precoce e rapida, sia considerando la perdita di consistenza che l'accumulo di olio nella drupa. Questi risultati suggeriscono come monitorare la maturazione in Grignano sia più difficile, dato che i cambiamenti nei parametri di maturazione si verificano molto velocemente. Inoltre, sono state osservate differenze importanti nei parametri elencati in due stagioni successive, da cui consegue una notevole difficoltà nella comprensione del profilo globale di maturazione. Sono state condotte analisi statistiche sui campioni descritti e l'analisi delle componenti principali ha stabilito che la cultivar Frantoio mostra maggiore variabilità nei dati di inolizione, confermando che il decorso di

maturazione della cultivar Grignano è in qualche modo più compatto. Tra gli indici di colore calcolati nello studio, uno, cioè CI, sembra appropriato per distinguere campioni raccolti in diversi stadi di maturazione.

Successivamente, per approfondire le caratteristiche aromatiche tipiche della cultivar Grignano, è stato condotto uno studio riguardante il metabolismo terpenico in olivo, con l'isolamento e la caratterizzazione di tre enzimi responsabili della biosintesi di terpeni (terpene sintasi) per la prima volta in questa specie. A questo scopo è stata condotta una ricerca approfondita di putative terpene sintasi in un database di EST (*expressed sequence tag*) di olivo recentemente pubblicato. I frammenti selezionati sono stati amplificati permettendo di ottenere i geni *full length*, le cui sequenze sono state utilizzate per dedurre le sequenze aminoacidiche che sono state espresse in *E. coli*. Le proteine espresse sono state sottoposte a saggi enzimatici per determinarne l'attività e una di queste si è dimostrata una geraniolo sintasi attiva, mentre le altre due proteine non hanno dato prodotti anche se la presenza di terpene sintasi inattive non è così infrequente. Analisi qPCR hanno dimostrato come le tre terpene sintasi isolate abbiano un andamento legato alla maturazione nell'espressione genica, ed è possibile perciò un loro ruolo nella determinazione del profilo aromatico.

Infine, l'utilizzo della qPCR come analisi di espressione genica nei lavori precedenti ha evidenziato come geni *reference* validati e adatti per studi di espressione genica in olivo non siano disponibili in letteratura. Una ricerca di geni *reference* candidati per questo scopo è stata quindi condotta utilizzando il database di EST citato sopra e cercando in letteratura studi riguardanti la validazione di geni *reference* in altre specie vegetali. Sono stati quindi identificati 13 geni candidati in *Olea europaea*, la cui stabilità dei trascritti è stata testata sia in drupe che in foglie di olivo. Analisi di stabilità condotte mediante gli strumenti genorm^{PLUS} e NormFinder hanno dimostrato che 12 tra questi geni sono effettivamente geni *reference* funzionali e utilizzabili per studi di espressione genica in olivo e che, tra questi, il numero di geni ottimale da utilizzare per una buona normalizzazione dei dati corrisponde a due. I migliori geni *reference* identificati in questo studio, adatti per analisi di espressione genica in frutti a diversi stadi di sviluppo, sono stati GAPDH2 e PP2A1.

Chapter I

General introduction

Introduction

Olive (*Olea europaea* L.) is a perennial evergreen subtropical species of utmost importance in the Mediterranean basin. *O. europaea* is a member of the Oleaceae family, containing approximately 600 species spread worldwide. The olive tree was domesticated in the east Mediterranean area approximately 1,500-3,000 BC (Riley, 2002) and its cultivation was spread to all the Mediterranean area by the Greeks and Romans. The European colonisation brought this species in the Americas starting from the sixteenth century, whilst introduction of olive in South Africa, Australia and other countries with Mediterranean-like climate is far more recent. Olive in fact can grow in limited areas due to its climate needs: long and dry summers, without extremely high temperature or abundant rainfall, but also a definite amount of cold for vernalisation. Therefore, besides Mediterranean countries, olive growing is at present confined in California, some areas of Peru, Chile, Argentina, South Africa, Australia, New Zealand.

Since olive plants suffer from low temperatures, the northern limit for olive growing consists of Veneto region in Italy and the lower valley of Rhone river, in France (Figure 1).

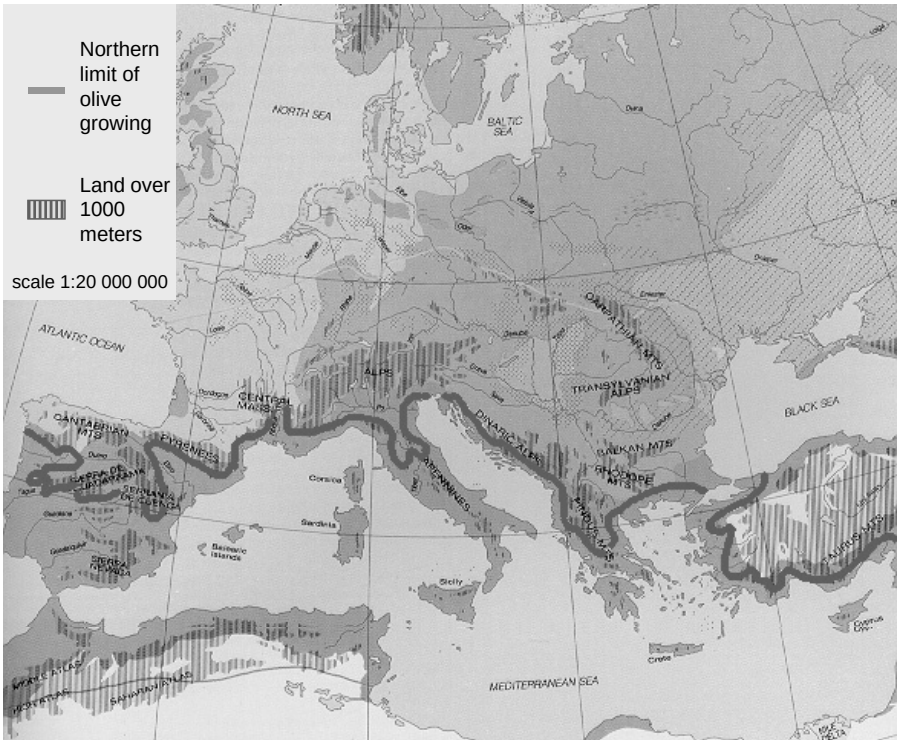

Figure 1. Europe map with the northern limit for olive growing marked by a dark grey line (modified from http://faculty.maxwell.syr.edu/gaddis/hst310/Aug30/Default.htm)

Olive oil importance

Olive is one of the main fruit crops in the world, with an estimated production of more than 19 million tonnes of olives (FAOSTAT, 2009). Olive cultivation has always been considered a source of nutritional fat and income, but has also had and still has a strong social, cultural, and landscape importance.

Notwithstanding olive fruits can be harvested for the production of table olives, in the Mediterranean area olives are mainly appreciated as a source of oil. Olive oil has been used historically for different purposes: as a fuel, for oil lamps, as a lubricant, in soap making and in cosmetics. Olive oil is sacred in Judaism, Christianity and Islam, and bore many symbolic meanings in ancient civilisations. Nowadays the most common use of olive oil (about 90% of oil production, Grigg, 2001) is the culinary one, both as an ingredient, a dressing, a frying fat, a preserving medium, and a lubricant.

World data on production of olive oil can be easily accessed in FAO (Food and Agriculture Organisation) databases. As shown in Table 1, the main olive oil producing countries are Spain, Italy and Greece, which alone account for 72% of world olive oil production (FAOSTAT, 2009).

Country	Production (tonnes)
Spain	1199200
Italy	587700
Greece	332600
Syrian Arab Republic	168163
Tunisia	150000
Turkey	143600
Morocco	95300
Algeria	56000
Portugal	53300
Argentina	22700
Lebanon	19700
Jordan	16760
Libya	15000
Egypt	7300
France	6300
Israel	6000
Croatia	5800
Australia	5639
Occupied Palestinian Territory	5000
Chile	4050

United States of America	2700
Cyprus	2014
The former Yugoslav Republic of Macedonia	1700
Albania	1200
El Salvador	1179
Iran (Islamic Republic of)	850
Peru	406
Slovenia	300
Mexico	238
Montenegro	187
Afghanistan	122
Azerbaijan	105
Malta	2

Table 1. Production (in tonnes) of olive oil in the major producing countries in the world (2009 data).

According to Grigg (2001), production and consumption of olive oil are concentrated mainly in Mediterranean countries, and olive oil is not an important part of the world's diet in terms of quantity since it accounts only for 3.7% of the annual consumption of vegetable oils in the world (1995-1999 period; Grigg, 2001).

Olive growing and importance in Italy and Veneto, Northern Italy

As shown in Table 1, Italy ranked second among world's olive oil producing countries in 2009. Olive growing and olive oil production in Italy are however very diverse, since many different growing and production realities are found throughout the territory. Table 2 shows the production areas, the production of olive oil in tonnes and the ranking according to the latter parameter in Italian regions in which olive growing and oil extraction are spread. Data are up-to-date as of 2010.

Regions	Production area (ha)	Olive oil production (t)	Ranking
Apulia	374450	161078,3	1°
Calabria	192376	157918,4	2°
Sicily	159442	48467,3	3°
Campania	71581	39605,8	4°
Lazio	85611	28555,3	5°
Abruzzo	43496	18748	6°
Tuscany	92756	18448,9	7°
Umbria	27808	10340	8°
Molise	19949	6248,8	9°
Basilicata	29004	6219,8	10°

Sardinia	35418	6066,3	11°
Marche	9352	4408,5	12°
Liguria	15680	3557,3	13°
Veneto	4580	1308,6	14°
Lombardy	2333	999,1	15°
Emilia-Romagna	2600	785,4	16°
Trentino-Alto Adige (only Trento)	380	263,8	17°
Friuli-Venezia Giulia	95	34,3	18°
Piedmont	31	13,3	19°
Italy	**1166942**	**513067,2**	

Table 2. Production areas in hectares and olive oil production in tonnes of Italian regions. Areas that don't produce oil are not listed here (from ISTAT, 2010)

Olive growing and oil extraction in Italy are characterised by a high fragmentation of production due mainly to the orographical structure of the Italian territory. According to D'Auria (2001), 67% of the surface used for olive cultivation is situated in hill areas, and 11% is situated in mountain areas. In some regions, such as Apulia, olive growing is mostly spread in flat areas. Olive growing and oil production are more common in Central and Southern Italy (Table 2), which have the suitable climatic characteristics for olive tree cultivation. These areas show mild winters and long and dry summers. According to Cimato *et al.* (2001), olive trees need specific temperature patterns for optimal growth, which are known empirically by the growers. Temperature needs can be roughly defined as about +2-4°C/+14-18°C for fruit set, 25-28°C for optimal photosynthesis and growth during summer and autumn and winter temperatures not lower than -5°C for prolonged periods. The absence of early and late frosts in necessary. Besides Central and Southern Italy, other cultivation areas can be therefore suitable in Italy, in northern regions where hill areas or the presence of lakes can mitigate the climatic conditions. In fact, olive growing in Northern Italy has a millennia-old olive growing tradition, and first reports date back to approximately the 1st century AD. In the following centuries, Veneto region next to Garda Lake (Verona province) became one of the most important olive oil production areas during the early Middle Ages (Fabbri, 2006). This region, together with some minor oil producing areas that can be found in Vicenza, Padova, and Treviso provinces, is still an oil producing area, as described in Table 3, even if in a small amount compared to oil production in Central and Southern Italy.

Province	Production area (ha)	Olive oil production (t)
Verona	3510	1077,2
Vicenza	420	102,4
Padua	285	83,3
Treviso	365	45,7
Belluno	0	0
Venice	0	0
Rovigo	0	0
Veneto	**4580**	**1308,6**

Table 3. Detailed production areas in hectares and olive oil production in tonnes of Veneto provinces (from ISTAT, 2010).

Olive growing and oil production in Veneto region have had changing fortunes through the centuries, due for instance to the new rail infrastructures built in 19th century that increased Tuscan and Apulian oil diffusion and the appearance of the olive fruit fly around 1840. Recently, a new development of olive growing in Veneto can be observed, with growing practices that resemble the ones followed in Tuscany hill areas (Fabbri, 2006).

Even if their contribution to the total Italian olive oil production is quite low, as shown in Table 2, extra-virgin olive oils produced in Veneto have typical features and aromatic notes that are considered very palatable and valuable by consumers (Peretti, 2007). In Veneto region a Protected Designation of Origin (PDO) has been established in 2001 for some quality extra-virgin olive oils, comprising the three sub-designations Veneto Valpolicella, Veneto Euganei e Berici, and Veneto del Grappa.

Notwithstanding Venetian olive oils appeal is rising, in this region olive growing is still marginal and not competitive compared to other producing areas. For this reason, olive growers need to focus on quality to obtain high-grade oils. This can be possible only with a thorough understanding of factors involved in oil quality determination and the clever use of native varieties. Among these varieties for instance Grignano, typical of Garda zone, is well known for its cold resistance, early production and resistance to some pests such as *Spilocaea oleaginea*, responsible for olive leaf spot, and *Pseudomonas savastanoi*, which causes olive knot (Bargioni, 2007).

Olive oil, not only a fruit juice

Olive oil, and especially extra-virgin olive oil, is often preferred by consumers for its features that include a peculiar chemical composition, very pleasant flavour and smell and demonstrated health effects.

Chemical composition of olive oil

Olive oil is chemically composed by a saponifiable fraction, which accounts for usually more than 98% of all components, and an unsaponifiable fraction, which is usually less than 2% (Morales and León-Camacho, 2000). The first fraction is made up mainly of triglycerides, which include C_{14} to C_{24} fatty acids and whose content for the "extra-virgin" category is regulated by Regulation EC No. 1989/2003 as shown in Table 4:

Fatty acid	EEC Regulation limit (%)
C14:0 (Myristic acid)	≤ 0.05
C16:0 (Palmitic acid)	7.5 - 20.0
C16:1 (Palmitoleic acid)	0.3 - 3.5
C17:0 (Heptadecanoic acid)	≤ 0.3
C17:1 (Heptadecenoic acid)	≤ 0.3
C18:0 (Stearic acid)	0.5 - 5.0
C18:1 (Oleic acid)	55.0 - 83.0
C18:2 (Linoleic acid)	3.5 - 21.0
C18:3 (Linolenic acid)	≤ 1.0
C20:0 (Arachidic acid)	≤ 0.6
C20:1 (Eicosenoic acid)	≤ 0.4
C22:0 (Behenic acid)	≤ 0.2
C24:0 (Lignoceric acid)	≤ 0.2

Table 4. Qualitative and quantitative fatty acid profile allowed in extra-virgin olive oils (Regulation EC No. 1989/2003)

As seen in Table 4, olive oil is mainly made up of esters of oleic acid, a monounsaturated omega-9 fatty acid, which is responsible for some anti-inflammatory and immune functions of olive oil. Moreover, MUFA (monounsaturated fatty acids) may be responsible for lowering total cholesterol and LDL (low-density lipoprotein) levels (Harwood and Yaqoob,

2002). Other components of this fraction are partial glycerides, esters of fatty acids like waxes and sterol esters, terpenic alcohols and nonesterified fatty acids.
The unsaponifiable fraction includes interesting compounds that can be used to characterise oils and can be listed as follows:

- hydrocarbons, paraffins, terpenic hydrocarbons, steroid hydrocarbons;
- tocopherols, among which α-tocopherol, a form of vitamin E, is the most common;
- triterpenic alcohols like cycloarthenol;
- 4,4-methyl-sterols;
- sterols;
- terpenic dialcohols;
- phenolics and flavonoids;
- pigments;
- volatile compounds (VOCs).

Among these components, phenolic compounds and volatile compounds are responsible for the appreciated sensory notes of olive oil, which can be defined as follows (IOOC, 1987): fruity, which can be grassy/leafy or aromatic depending on the ripening stage of olive fruits; bitter, similar to aqueous solutions of quinine, caffeine and other alkaloids; and pungent, a tactile sensation of “biting”.

Sensory attributes of virgin olive oils

García-González *et al.* (2010) stated that the pleasant attributes of virgin olive oil are given by phenols (for what concerns taste) and volatile compounds (for the definition of aroma), which are found in the oil as a result of the mechanic extraction from olive drupes.

Phenolic compounds

Phenols are secondary metabolites synthesised through the shikimic acid pathway in olive fruits. In olive oil new phenolic compounds can also be present as a consequence of the release of cellular components during olive crushing and malaxation in oil extraction and of subsequent chemical reactions. Notwithstanding the high content of phenolics in olive

drupes, many of these compounds are water-soluble and are not found in olive oils (Cimato *et al.*, 2001), therefore not contributing to virgin olive oil quality.

A recent review has given a thorough overview of phenolic compounds in virgin olive oils, describing this group of molecules qualitatively and summarising their health effects, sensory attributes, and analytical approaches for their characterisation (Bendini *et al.*, 2007). Phenolic compounds in virgin olive oil can be divided in the following classes: phenolic acids (gentisic acid, vanillic acid, gallic acid, 3-hydroxybenzoic acid), cinnamic acids (such as *p*-coumaric acid), phenyl ethyl alcohols (hydroxytyrosol and tyrosol), hydroxy-isochromans (such as 1-phenyl-6,7-dihydroxyisochroman), flavonoids (taxifolin, apigenin, luteolin, and the pigment components anthocyanidins), lignans (such as pinoresinol) and secoiridoids, the latter being found only among *Oleaceae* and representing the main fraction of phenols in virgin olive oils. Secoiridoids are responsible for the typical bitter and pungent sensory notes highly appreciated in virgin olive oil, and are synthesised from phenyl ethyl alcohols and elenolic acid and may have a glucosidic residue (yielding compounds such as oleuropein). Aglycons may be also found in olive oil as a consequence of the action of endogenous β-glucosidases during olive oil extraction (Bendini *et al.*, 2007).

The common basic structure and some common phenolics found in olive drupes and olive oils are shown in Figure 2.

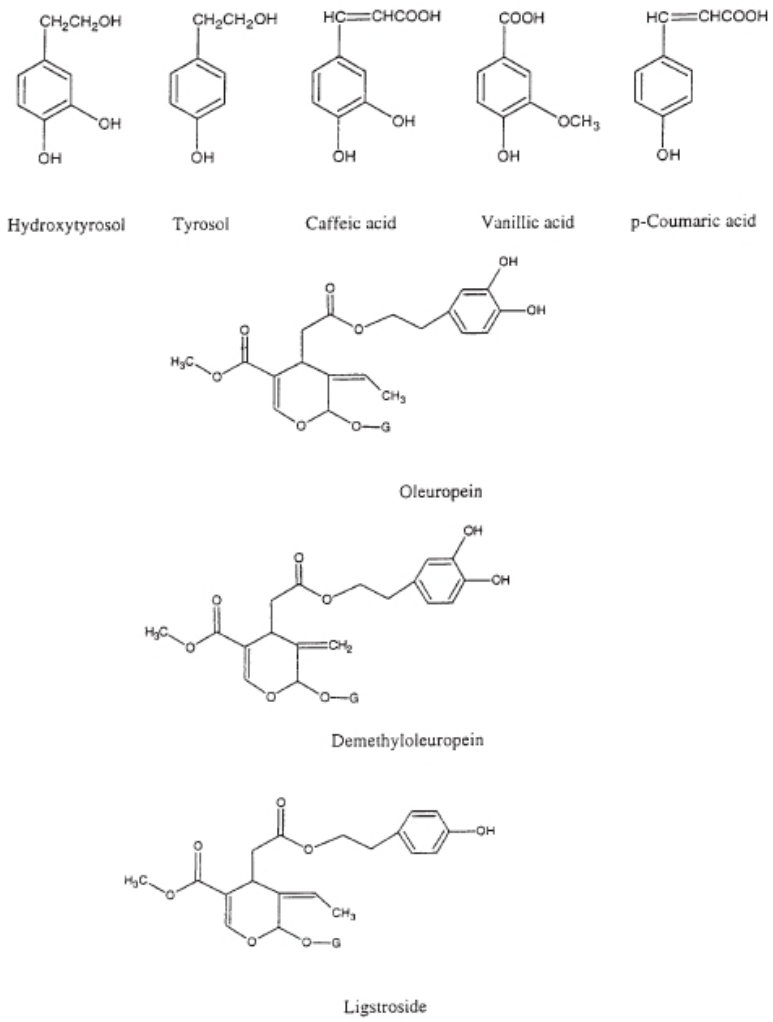

Figure 2. Chemical structure of representative phenolics found in olive fruit and olive oil (from Ryan *et al.*, 1999).

According to Bendini *et al.* (2007), the most abundant secoiridoids in virgin olive oil are *3,4*-DHPEA-EA (oleuropein aglycon), *3,4*-DHPEA-EDA, and *p*-HPEA-EDA (respectively the dialdehydic forms of elenolic acid linked to hydroxytyrosol and tyrosol; the latter is also known as oleocanthal). Bitterness and pungency, which are considered pleasant attributes in olive oils, when not excessive, are strictly linked to the phenolic profile of oils. More in detail a relationship between pungency and the concentration of oleocanthal has been found, whilst bitterness can be caused also by *3,4*-DHPEA-EA and *3,4*-DHPEA-EDA.

The phenolic content in virgin olive oils can be greatly influenced by genetic factors, pedoclimatic and agronomic conditions and especially fruit ripening, since it has been

observed that total phenols reach a maximum level during the “purple” stage (Rotondi *et al.*, 2004).

Volatile compounds

Volatile compounds are characterised by low molecular weight, less than 300 Da, and high vapour pressure at room temperature that allows an easy vaporisation in the headspace of the sample. These compounds are found in virgin olive oils as a result of different metabolic pathways and may have therefore different chemical nature. Volatile components of olive oil flavour have been described thoroughly in recent works and reviews (Kiritsakis, 1998; Angerosa *et al.*, 2004; Kalua *et al.*, 2007). These components include aldehydes, alcohols, esters, ketones, carboxylic acids, terpenoids and other minor compounds, and probably others that still remain unidentified. According to Sánchez and Harwood (2002), the most abundant volatile components (up to 80% of the total VOCs) in olive oil are C6 compounds, which are synthesised through the so-called LOX (lipoxygenase) pathway, as shown in Figure 3, and more in detail hexanal, 3-*Z*-hexenal, 2-*Z*-hexenal, hexanol, 3-*Z*-hexenol, 2-*Z*-hexenol, hexyl acetate, and 3-*Z*-hexenyl acetate. The LOX pathway reactions take place when olive fruits are crushed during oil extraction. The first step is the release of free fatty acids by means of acyl hydrolase, yielding α-linolenic and linoleic acid. Lipoxygenase is a dioxigenase that subsequently oxidises both fatty acids giving mainly 13-hydroperoxides, with a strong preference for α-linolenic acid (Salas *et al.*, 1999) that results in a higher biosynthesis of C6 compounds. The following step is the cleavage of 13-hydroperoxides by means of HPL (hydroperoxide lyase), yielding aldehydes and oxoacids. Aldehydes can be reduced to the respective alcohols by ADH (alcohol dehydrogenase), and further chemical reactions may take place since alcohols can be esterified in presence of acetyl-CoA by AAT (alcohol acetyl transferase). The less studied step of the LOX pathway in olive is the enal isomerisation, a reaction catalysed by enal isomerase that yields 2-*Z*-hexenal from 3-*Z*-hexenal, since the latter is very unstable (Sánchez and Harwood, 2002).

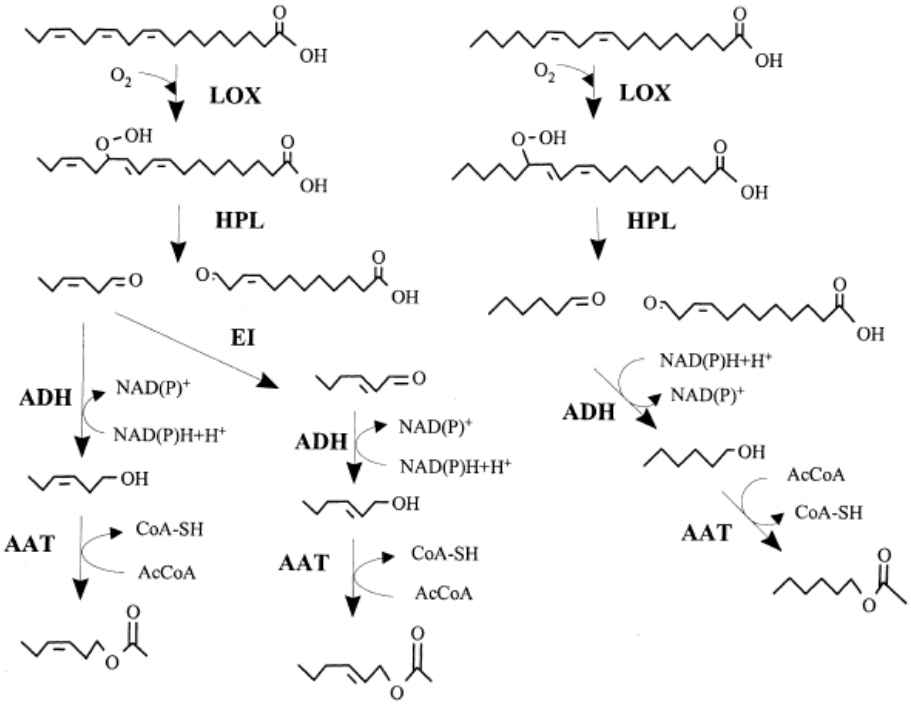

Figure 3. LOX pathway, responsible for the biosynthesis of the major volatile compounds in virgin olive oil (from Sánchez and Harwood, 2002). LOX: lipoxygenase; HPL: hydroperoxide lyase; ADH: alcohol dehydrogenase; AAT: alcohol acetyl transferase; EI: enal isomerase.

Other important components of VOCs found in olive oil are terpenes. The heterogeneous class of terpenes, a term widely used to cover not only terpenes with basic structure but also their modified derivatives terpenoids, is a large group of compounds that include an estimated number of about 30,000 components. Terpenes have many different physiological and ecological functions, as many of them are volatile and may therefore be emitted to attract insects, discourage herbivores, and contribute to aroma and flavour of edible portions of some plants. Terpenes are synthesised starting from isopentenyl diphosphate (IPP), a C5 compound, and dimethylallyl disphosphate (DMAPP). An overview of plant terpene biosynthesis is given in Figure 4. Terpenes are classified according to the number of isoprene units found in their structure; the most common terpenes in plants are monoterpenes, which have 10 carbon atoms and 2 isoprene units, sesquiterpenes, with 15 carbon atoms and 3 isoprene units, and diterpenes, which have 20 carbon atoms and 4 isoprene units. Terpenes are synthesised by specific enzymes called terpene synthases (TPSs), which are included in a large superfamily comprising hundreds of *TPS* genes. Some TPS can yield one product, but many of them are capable to synthesise

more than one compound resulting in the huge complexity of terpenic compounds class (Bohlmann *et al.*, 1997; Tholl, 2006).

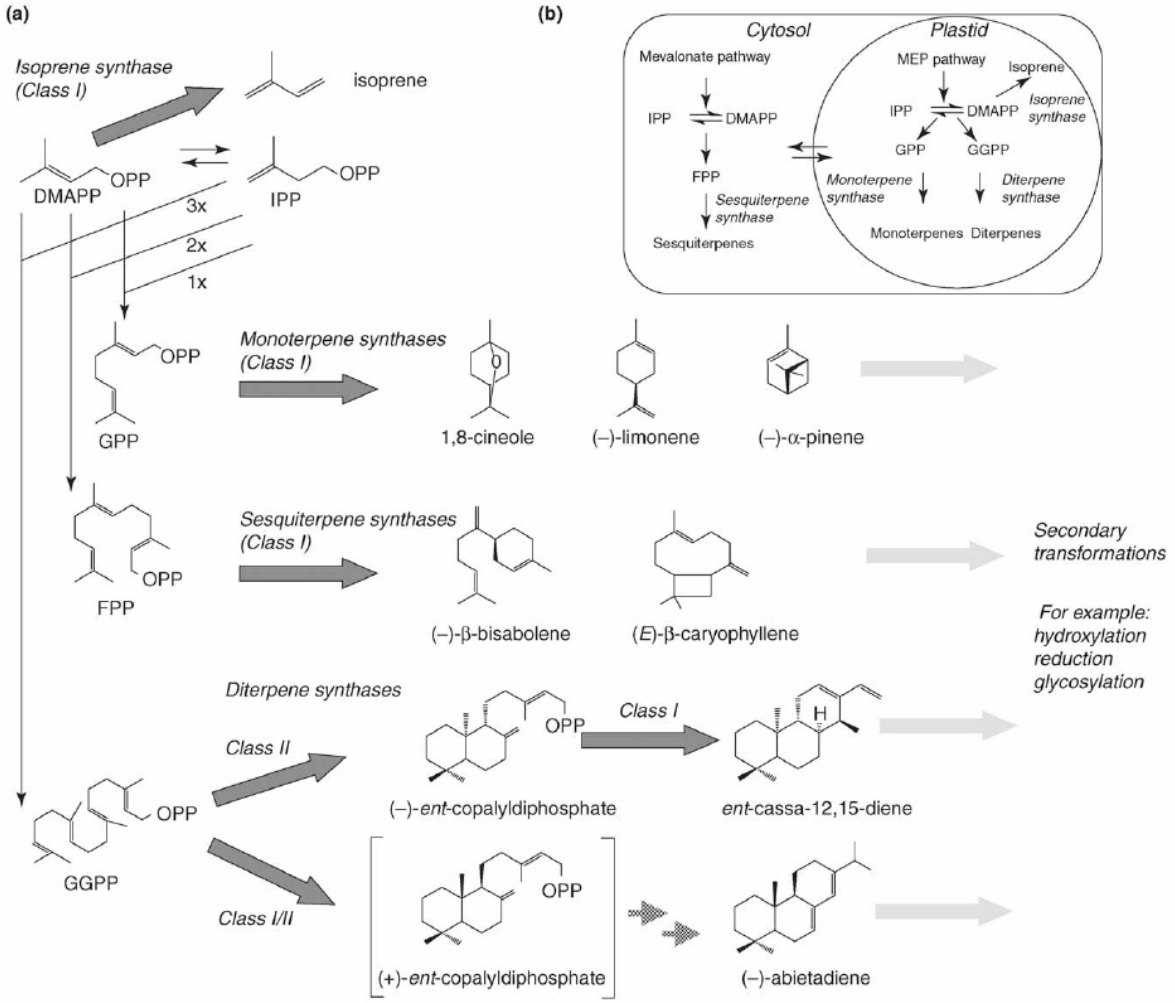

Figure 4. Biosynthesis of plant terpenes (a). The substrates for the synthesis of mono-, sesqui-, and diterpenes are respectively geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP). Many products are subjected to secondary transformation, resulting in a very high number of different structures. (b) Cellular compartmentation of terpene biosynthesis. IPP is formed through the mevalonate pathway, whereas DMAPP is synthesised through the MEP (methylerythritol phosphate) pathway. Sesquiterpene metabolism occurs in the cytosol, whilst mono- and diterpenes are synthesised in plastids (from Tholl, 2006).

Terpene synthases have been widely studied in plant systems such as conifers, where for instance monoterpenes are solvents for resin, *Lamiaceae* family, which have terpene-rich essential oils, in model plants such as *Arabidopsis thaliana* and also in edible fruit-bearing plants as *Vitis vinifera* (grape) and *Citrus* since terpenes are major components of flavour and aroma (Bohlmann *et al.*, 1997; Tholl, 2006).

Notwithstanding olive oil aroma is well studied and very appreciated, no studies regarding olive TPSs are at present available. The presence of this class of compounds in olive oil has been anyway frequently reported in literature, even if in low concentration, and some of them are listed as flavour components such as the monoterpenes 1,8 cineole (eucalyptol), linalool and α-terpineol (Flath *et al.*, 1973). The major terpenes found in the volatile fraction of virgin olive oils are the sesquiterpenes *E,E*-α-farnesene and α-copaene and the monoterpene *E*-β-ocimene (Flamini *et al.*, 2003; Cavalli *et al.*, 2003). Minor terpenes found in oils extracted from Italian, French, Spanish, and Tunisian olives include the sesquiterpenes α-muurolene, γ-cadinene, curcumene, β-caryophyllene, valencene, and α-bergamotene, and the monoterpenes limonene, α/γ-terpinene, δ-3-carene, and α/β-pinene (Berlioz *et al.*, 2006; Cavalli *et al.*, 2003; Cavalli *et al.*, 2004; Vichi *et al.*, 2010). The presence of terpenes in oils is strongly dependent on cultivar, geographical area and growing conditions, but not on technological factors (Vichi *et al.*, 2006) and many terpenes are therefore found in different amounts or not found at all with regards to the studied variety. This strong dependence on variety, together with a dependence on geographical area, makes terpenes good candidates suitable for the discrimination of oils with different geographical origin. Zunin *et al.* (2005) reported for instance that the amounts of α-copaene, α-muurolene, and α-farnesene can be used to build a decisional tree that successfully classifies West-Liguria extra-virgin olive oils from other Mediterranean oils. Vichi *et al.* (2010) stated for the first time that the accumulation of sesquiterpene hydrocarbons is ripening-dependent in olive, and therefore that ripening must be taken carefully into account when studying terpenes for characterisation or authentication of samples.

Olive oil VOCs are usually identified by means of mass spectrometry techniques, among which the most frequently used is GC-MS (gas chromatography-mass spectrometry), sometimes coupled with SPME (solid phase microextraction). GC-MS analysis with SPME extraction is capable to detect more than 130 different compounds (Angerosa *et al.*, 2004), and is used as a routine analysis due to its simplicity, repeatability, and low cost (Cavalli *et al.*, 2003). For this reasons this technique is nowadays well established and acknowledged for giving good results in olive oil volatiles analyses, detecting the major volatiles

responsible for flavours and off-flavours in olive oils, even if some troubles can occur in sample concentration, absorption and desorption, also considering different temperatures, sample size and artefacts. Some relatively new techniques have been therefore applied to the analysis of volatile compounds, among which PTR-MS (proton transfer reaction-mass spectrometry; Lindinger *et al.*, 1998), has been used for different purposes in previous works dealing with olive oil. This spectrometry technique has been successfully used for geographical classification of olive oil, allowing to distinguish samples according to their country of origin (Araghipour *et al.*, 2008), to evaluate olive oil oxidation, and for separating defective samples from good ones (Aprea *et al.*, 2006). This technique, although not allowing an unambiguous identification of all the spectral data and a subsequent assignment to specific compounds, provides a very sensitive online global fingerprint of samples' headspace and does not need any prior steps nor volatile concentration processes.

Olive oil health effects

In recent years olive oil has attracted interest from the scientific community since it is a major component of the so-called Mediterranean diet. This kind of diet, even if quite various among Mediterranean countries, has some cornerstones: the use of olive oil, a moderate intake of wine, a high intake of fruit and vegetables, a low intake of red meat and animal derived fats.

Since some positive health effects have been demonstrated as a result of the use of olive oil as a cooking fat or a dressing, much scientific literature has been devoted to understand the physiological mechanisms responsible for the well-known low incidence of some chronic diseases in the Mediterranean region (e.g. Pérez-Jiménez *et al.*, 2007).

According to these studies, a regular intake of virgin olive oil increases HDL (high-density lipoprotein) cholesterol levels and lowers LDL cholesterol levels, improves glucose metabolism in diabetes, has antioxidant and anti-inflammatory effects, and may improve vasodilatation, reduce blood pressure and thrombosis-provoking factors. All these effects considered together point out that virgin olive oil can lower cardiovascular diseases risk and prevent some kinds of cancer.

Olive oil quality

Olive oil quality is a major issue, especially considering that the best flavour and major health effects are found in extra-virgin olive oil and that, according to Luchetti (1993), only 6% of the total amount of olive oils produced in the world meets the quality requirements for the "extra-virgin" definition. Minimum requirements needed to fulfill olive oil standards are set in the Regulation EC No. 1989/2003 and are shown in Table 5. This regulation sets thresholds for the chemical composition of olive oils, in terms of free acidity (expressed as oleic acid), peroxide value (an indicator of the quantity of hydroperoxides obtained by oil oxidation), absorbances at 232 and 270 nm (detection of hydroperoxides, dienes and trienes as markers of preservation and refining processes of oils), and sensorial attributes (absence of sensory defects and presence of fruity notes for the "extra-virgin" definition) (Table 5).

Category	Free acidity %	Peroxide value	Absorbance		Sensorial - negative*	Sensorial - fruity*
			K_{232}	K_{270}		
Extra-virgin	<0.8	≤20	≤2.50	≤0.22	0	>0
Virgin	≤2.0	≤20	≤2.60	≤0.25	≤2.5	>0
Lampante	>2.0	-	-	-	>2.5	-

* expressed as medians

Table 5. (modified from Escuderos *et al.*, 2007)

The overall composition of extra-virgin olive oil depends on the characteristics of the olive fruits and on the technological factors involved in oil production starting from harvest to all the phases of oil mechanical extraction.

Briefly, factors that have a strong impact on olive oil quality can be summarised as:

- fruit health
- geographical and climatic aspects
- cultivar (genotype)
- technological aspects (harvesting, storage, extraction)
- fruit ripening.

Fruit health

A basic point of olive oil quality is the use of healthy fruits for oil extraction (Angerosa *et al.*, 2004). Among olive pests, the most common and dangerous for olive oil quality is a fruit fly, *Bactrocera oleae*, which can attack fruits until harvest time. This fly causes damage in the fruit by piercing the exocarp. Attacked fruits can yield poor quality oils, affecting the volatile profile and the phenolic content. Moreover, serious infestations cause an increase in carbonyl compounds and alcohols (Angerosa, 2002). In a study performed on the Portuguese olive cultivars Cobrançosa, Madural and Verdeal Transmontana (Pereira *et al.*, 2004) it has been shown that *Bactrocera oleae* infestation can increase acidity values in oils, decrease total tocopherol content, and affect globally olive oil quality. Tamendjari *et al.* (2009) demonstrated that cv. Chemlal olives attacked by *B. oleae* yield oils with poor quality, which show a loss of phenolic compounds and an organoleptic decay that are more evident in advanced stages of ripening.

Geographical and climatic aspects

Geographical origin of olives can result in different volatile profiles of oils (Angerosa *et al.*, 2004), and it is common knowledge that environmental factors (edaphic/climatic) can influence the overall quality and chemical composition of olive oil. Cimato *et al.* (2001) have reviewed environmental factors influencing olive oil quality, and these factors are temperature, wind, light, and water availability. Angerosa *et al.* (1999), studying VOCs occurrence in olive oils obtained by different cultivars, pointed out that climatic factors have an indirect influence on oil quality since they act primarily on ripening physiology. These factors can have an impact on the acidic composition of oil, therefore causing potential differences in organoleptic profiles, on oil accumulation, and on other components. For instance, 2-*Z*-hexenal is abundant in European olive oils but not in oils from other areas; Italian oils are richer in C6 aldehydes, whilst Moroccan ones are richer in esters (Kalua *et al.*, 2007).

Criado *et al.* (2004) studied differences in pigments, phenols and minor components in olive oils from cv. Arbequina trees cultivated in three regions of Spain with different climatic and edaphic conditions (Lleida, Jaén and Tarragona). Results showed that there are

significant differences in pigments content and ratios and in colorimetric values according to the CIELAB chromatic space, in phenolic compounds and stability.

Considering these differences, in previous years there has been an increasing interest in the possibility of discriminating olive oils according to their geographical origin, with the purpose of ensuring authenticity and quality. One example is the already cited work by Araghipour *et al.* (2008); Zunin *et al.* (2005) identified three terpenoid hydrocarbons, α-copaene, α-muurolene, α-farnesene, which can be used to discriminate West Liguria olive oils from a pool of Mediterranean oils. Mannina *et al.* (2001) successfully discriminated olive oils from different Italian regions by means of analysis of variance, clustering analyses and linear discriminant analysis of ^{1}H high-field nuclear magnetic resonance spectroscopy spectra. Benincasa *et al.* (2007) stated that trace elements such as iron, magnesium, strontium, calcium and arsenic can discriminate samples coming from five different Italian growing regions.

Cultivar

Genotypic aspects (cultivar), together with fruit ripening, are the most important factors determining olive oil overall attributes and, as a consequence, quality. It is interesting to notice that, as suggested by Angerosa *et al.* (1999), levels and activities of enzymes involved in the LOX pathway are genetically determined, even if the amount of products can be affected by ripening stage and storage.

Tura *et al.* (2008), in a study performed for 4 years on 18 local olive cultivars from western Garda Lake, demonstrated that the volatile profiles and the sensory attributes of oils are greatly influenced by cultivar, and this is true mainly for ethanol, 2-methyl-propan-2-ol, pentan-1-ol, 2-*Z*-penten-1-ol, 3-*Z*-hexen-1-ol and octan-1-ol for what concerns VOCs and "floral", "fruity", "apple" and others for sensory notes.

Tura *et al.* (2007) also claimed that olive cultivar has a strong influence on oil antioxidant pool, since significant changes with regards to variety can be noticed for tocopherols but also partly for fatty acid composition. Significant differences in C6 alcohols were found among six Spanish cultivars (Gómez-Rico *et al.*, 2008), and also the ratio between phenols content in olive and in extracted oils had significant variations according to cultivar.

Technological aspects

Technological operations that olive fruits undergo from the orchard to the extraction plant can strongly influence oil quality, as well as oil storage. All these crucial phases will be discussed separately below: olive harvesting, olive storage, oil extraction procedures, oil storage.

Olive harvesting

Olives are usually picked by means of mechanical devices, such as pickers or shakers that remove the olives from branches and allow them to fall in nets spread out on the ground or in harvesting umbrellas. Many olive growers still prefer handpicking, sometimes with the aid of proper combs. Whilst hand harvesting is more expensive, mechanical harvesting can cause damage on fruit. Olive fruit bruising may cause an increase in free fatty acids content in olive oil, which is associated with a qualitative decay (Rana and Ahmed, 1981). After harvesting, olives are dirty with soil, dust, extraneous components and are usually picked together with leaves that fall during harvest operations. Olives need therefore to be washed before oil extraction, and leaves need to be removed since their crushing together with fruits increase the “green” and “leaf” sensory attributes in extracted oils, which may not be accepted by consumers if too pronounced (Di Giovacchino *et al.*, 2002b). Moreover, in areas where the habit of keeping olives on the ground for long periods is frequent, sensorial defects such as “mouldy” and “earthy” can be found in oils (Angerosa *et al.*, 2004).

Olive storage prior to oil extraction

The storage of olives prior to oil extraction operations is strongly not recommended. Olives stored in bags and subsequently piled can reach high levels of temperature and humidity, promoting therefore bacterial and mould fermentation processes. Negative effects caused by olive improper storage have been reviewed by Angerosa *et al.* (2004). *Clostridium* and *Pseudomonas* genera can synthesise branched aldehydes, alcohols and acids that are responsible for the “fusty” oil defect. Yeasts and *Acetobacter* may produce ethanol and acetic acid respectively, which can give the oil the negative attributes of “winey” and “vinegary”. Also moulds (*Penicillium*, *Aspergillus*) can develop on stored olives, reducing

the synthesis of C6 compounds and producing C8 compounds and of course causing drupes rotting.

Low temperature storage has been performed on Koroneiki olives for 30 and 60 days (Kiritsakis *et al.*, 1998), and oil was obtained subsequently in an attempt to find the best condition to preserve olives from the negative effects of storage listed above. Results showed that olives stored at 7.5°C are attacked by moulds and have high values of oil acidity, whilst olives stored at 0°C have acceptable quality parameters but show discoloration and loss of phenols due to chilling injury. The latter phenomenon has also been observed by Yousfi *et al.* (2008) during cold storage of Picual, Manzanilla and Verdial olives, accompanied by a decrease in oil bitterness.

Oil extraction process

Olive oil extraction consists of physical operations that include crushing the drupes to disrupt tissues and release oil, kneading the obtained paste to aggregate oil drops and then separating the solid waste, the vegetation water and the oil ready to be stored or consumed.

The first phase of oil extraction can be performed in a traditional way, by means of stone mills, or using metallic crushers such as blades, discs, hammers and others. As reported by Di Giovacchino *et al.* (2002b), the use of metallic crushers causes a violent disruption of drupes, resulting in the release of more phenolic compounds that will be found in the oil, whilst for cultivars that are known to give very bitter and pungent oils the use of stone mills is advisable since milder oils will be obtained. On the contrary, crushing by means of metallic crushers seems to reduce the amount of volatile compounds in extracted oils, especially 2-*E*-hexenal that is responsible for positive “green, grass” notes, because a strong disruption of drupes causes an increase in paste temperature and a subsequent reduction of HPL activity, as pointed out by Angerosa *et al.* (2004).

Malaxation, or kneading of olive paste in proper chambers, is a crucial step in olive oil extraction, since in this phase variations in temperature and time can lead to important changes in oil quality. It is generally acknowledged that increasing the temperature inside malaxing chambers can yield more oil, due to a lower viscosity (Kalua *et al.*, 2007), but also that this condition may lead to poor quality oils: an inactivation of HPL can be observed, lipolysis and lipid oxidation can accelerate, fermentation processes are more

likely to occur and polyphenol oxidase (PPO) and peroxidase (POD) activity can be enhanced already at 35°C (Ranalli *et al.*, 2001). On the other hand, increase in malaxation time can enhance the synthesis of C6 aldehydes and alcohols (Angerosa *et al.*, 1998). Several works where therefore performed in the last years trying to find the best combination of malaxation temperature and time to ensure a good ratio between oil yield and oil quality. For phenol-rich cultivars such as Cornicabra (Gómez-Rico *et al.*, 2009) it has been reported that malaxation temperatures below 28°C for a total malaxation time of more than 60 minutes can give the best results, decreasing total phenols but enhancing VOCs biosynthesis. According to Angerosa *et al.* (2001) a temperature of 25°C for 30-45 minutes can be the best compromise for cvs. Frantoio and Coratina in order to ensure a good shelf life, a high content of desirable esters and a low content of undesirable VOCs. On the contrary, the effect of changing malaxation time and temperature seemed not to be important in Correggiola olives grown in Australia, where no differences in olive oil VOCs were observed (Tura *et al.*, 2004). Therefore, geographical and cultivar variations have to be taken into account.

As stated before, olive pastes contain also oxidoreductases such as PPO and POD that can lower the overall quality of olive oil, acting both on volatile profile and on phenolic compounds that may be oxidised. Some works have therefore been performed in order to study the effects of oxygen and its removal in the malaxing chamber to prevent oxidative processes, using nitrogen as an inert gas. TEOPAC (time of exposure of olive pastes to air contact) can be consequently controlled to ensure a proper concentration of VOCs and phenols (Servili *et al.*, 2003). Servili *et al.* (2003) noticed that for Frantoio drupes 30 minutes of TEOPAC at 22°C were the best conditions for obtaining oil with optimal phenolic and volatile profiles, whilst for Moraiolo drupes 0 minutes of TEOPAC at 26°C were the best conditions. Servili *et al.* (2008) reported that with the progression of malaxation time the absence of oxygen in the malaxing chamber leads to a higher content in total phenols, and that this effect was stronger in Ogliarola cv. compared to Coratina, but that aroma production was not affected by this condition. The effect of the removal of oxygen on olive oil quality is therefore cultivar-dependent, and the time of exposure to air or an anoxic extraction must be arranged according to variety. Moreover, since enzymes involved in the biosynthesis of volatiles and phenols are known to show a ripening-

dependent pattern, it is of utmost importance also to define whether the removal of oxygen can have an impact on the overall quality of the olive oil extracted from olives at different ripening stages. A detailed study covering this aspect is at present still lacking.

Olive oil quality can be influenced also by the last step of extraction, which is the separation of the oil from olive pastes. The traditional method for separation is the use of pressing mats that are hydraulically pressed, causing liquid percolation followed by decantation or centrifugation. Since pressing mats can be difficult to clean, oils separated in this way can show the sensorial defect of "pressing mats". The modern method uses an industrial decanter, where olive pastes are centrifuged and the different phases can be separated. In three phases decanters, water is added to the pastes and the results of separation are vegetation water, pomace, and oil. This method may anyway decrease phenol content in olive oils (Di Giovacchino *et al.*, 2002b), and water addition can cause a decrease in C6 alcohols (Angerosa *et al.*, 2004). Two phases decanters allow separation between wet pomace and oil, and no water is added in the process.

Oil storage

If extracted olive oil is not intended for immediate consumption, it has to be stored in proper containers. During olive oil storage some new compounds can be synthesised, some of them being responsible for "rancid", "cucumber", "muddy sediment" negative attributes. These compounds may arise from the fragmentation of hydroperoxides, and can be unsaturated or saturated aldehydes, ketones, acids, alcohols, hydrocarbons, lactones, furans, and esters (Angerosa *et al.*, 2004).

Some chemical reactions may take place during oil storage, if storage conditions are not optimal. Major changes that can be observed in stored olive oil are mainly related to the exposition to light, high temperatures, and oxygen. Pristouri *et al.* (2010) stated that these variables carry a different weight on olive oil quality deterioration, light and temperature being the most important factors of deterioration, followed by headspace and packaging material. Light can electronically excite pigments in oils, and energy can be subsequently transferred to oxygen to produce singlet state oxygen, which is highly reactive (Kalua *et al.*, 2007). In Spanish oils stored for up to one year in darkness at 15°C no significant changes in the pigment profile has been observed (Roca *et al.*, 2003), but storage of oils in clear

bottles in presence of light causes loss of chlorophylls and carotenes, as mentioned by Kiritsakis and Dugan (1984).

Temperature control is another critical point, since it has been observed that oils stored at 35°C don't comply with the "extra-virgin olive oil" definition requirements because K_{232} and K_{270} absorbances and the peroxide value/acidity exceeded their limits after respectively 3 and 9 months (Pristouri *et al.*, 2010).

It is acknowledged that a high content of polyunsaturated fatty acids is usually responsible for the reaction of autoxidation, but the high content of monounsaturated fatty acids such as oleic acid and the presence of natural antioxidants such as some phenolic compounds make olive oil less prone to oxidative processes and therefore more stable (Di Giovacchino *et al.*, 2002a). It has been stated that filling the headspace with nitrogen can provide a good strategy against increasing peroxide value and K_{232} observed in oils stored with an usual air headspace (Di Giovacchino *et al.*, 2002a).

For what concerns packaging material, Pristouri *et al.* (2010) stated that dark coloured glass is preferable, followed by dark coloured polyethylene terephthalate (PET). Oils stored in these containers at 22°C may have a shelf life of 6 months.

Fruit ripening

It is widely acknowledged that olive fruit ripening stage at harvest has the strongest effect on olive oil quality. During drupe ripening many physiological processes take place in fruits, which involve texture and colour changes, regulation of enzymatic pathways, oil accumulation, evolution in the phenolic and volatile profiles. All these changes significantly influence oil yield and quality and therefore the correct choice of the harvesting time is of paramount importance. A thorough study of olive ripening process is of utmost importance in order to allow olive fruits to be harvested at the optimal harvest time and ensure the production of superior quality extra-virgin olive oil.

Given that a deep understanding of olive fruit ripening is needed to assess correctly the best harvest time, the major aspects of ripening will be dealt with extensively and separately.

Olive fruit growth and ripening

According to the botanical classification, olive fruit is a drupe, such as cherries, peaches, mangoes and dates. The main structures of olive drupe are the exocarp (skin), the mesocarp (flesh), and the endocarp (better known in olives as stone or pit). Olive fruit growth lasts approximately 4-5 months, during which drupes show biochemical and physiological changes that involve size, colour, texture, flavour and susceptibility to pathogens (Conde *et al.*, 2008). Olive drupe growth and development comprises 5 phases (Connor and Fereres, 2005; Conde *et al.*, 2008) according to a double-sigmoid pattern as shown in Figure 5, which is not observed in irrigated olive trees (Connor and Fereres, 2005):

- fertilisation and fruit set (I), starting with flowering and lasting approximately 30 days; during this period a high cell division rate is observed;
- seed development (II), which consists of cell division and expansion with rapid fruit growth taking place mainly in the endocarp;
- pit hardening (III); during this period the pit becomes sclerified and overall fruit growth slows down;
- mesocarp development (IV), which includes cell expansion and oil accumulation;
- ripening (V), with changes in colour, texture and a continuous increase in dry matter content.

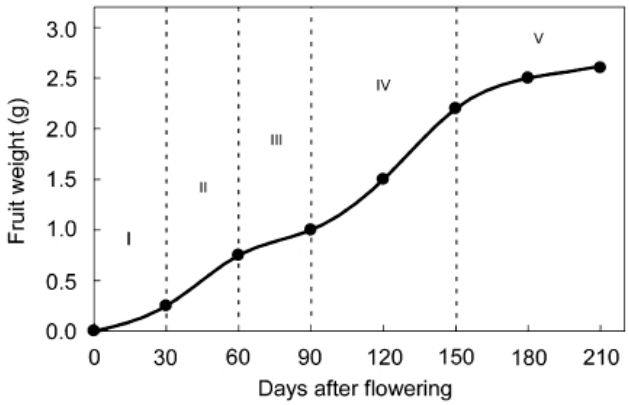

Figure 5. Olive fruit growth, showing the typical double-sigmoid pattern (Conde *et al.*, 2008).

The last stage of olive fruit development, i.e. ripening, is the major factor influencing olive oil quality. According to Cimato *et al.* (2001), the olive ripening process can be divided into four stages classified as: 1) green stage, 2) *véraison* stage, 3) ripening stage and 4) over-ripening stage. In the first phase, fruits are green and firm, are photosynthetically active, their content of soluble sugars reaches its maximum values and tocopherols and phenolics can be found in high concentrations. When fruit colour shades off into yellowish green due to changes in pigment profiles (mainly chlorophyll breakdown) the second phase (*véraison*) starts, photosynthesis slows down and small purplish spots appear on fruits' surface due to the accumulation of anthocyanins in the epicarp; in the third phase, consisting of the actual ripening, drupes reach their maximum size and oil content. In the last phase, which is over-ripening, fruits lose water and chemical reactions that can worsen oil quality take place (e.g. lipolysis, aldehydes and tocopherols degradation).

The ripening process in fruits is characterised by different regulatory mechanisms with respect to the plant hormone ethylene. Ethylene is a gaseous compound that regulates different mechanisms in plants, among which there are flower development, senescence, response to biotic and abiotic processes and above all fruit ripening (Lin *et al.*, 2009). According to their ethylene behaviour during ripening, fruits can be divided into two classes: climacteric and non-climacteric fruits. Climacteric fruits, such as peach, tomato, apple, banana, kiwifruit, plum, pear and many others, show a peak of respiration and an ethylene burst at the onset of ripening, whilst non-climacteric fruits such as cherry, grape, strawberry, and citrus fruits, lack these phenomena. Olive classification in one of the two classes is not unambiguous, since some authors include olive fruits among climacteric fruits (Srivastava, 2002) whilst some other don't (Bouzayen *et al.*, 2010). A work performed with the aim of optimising harvesting to ensure the best oil quality (Ranalli *et al.*, 1998) anyway pointed out that olives' respiration rate reaches a maximum during ripening, and therefore have a climacteric phase, and that extracting oil from drupes in this ripening stage allows to obtain the best quality. The respiratory rate has been therefore proposed as a ripening marker.

Textural changes in ripening olives

Olive ripening, similarly to ripening of other fleshy fruits, is associated with a change of texture that results in drupes softening. This event is the result of enzymatic activities involving degradation of structural and storage polysaccharides (reviewed by Prasanna *et al.*, 2007). Loss of firmness is of utmost importance in olives to be harvested for table use, because these fruits need to be handled carefully to ensure the best quality, whilst for olives intended for milling this softening can represent a marker of ripening.

Fruit cell wall comprises many components, among which the most important are pectic polysaccharides, cellulose, and hemicellulose even if other components also contribute to fruit texture, such as proteins and lignin. Pectic polysaccharides contain methylated galacturonic acid residues, methyl esterified pectins, deesterified pectic acids, pectates and neutral polysaccharides (arabinogalactans, arabinans, galactans) (Prasanna *et al.*, 2007). Cellulose is a polymer made up of β(1→4) linked D-glucose, whilst hemicellulose has an amorphous structure of xyloglucans and glucuronarabinoxylans among others.

In many fruits, among which also olive shows the same behaviour, the major event in textural change is the solubilisation/depolymerisation/deesterification of pectic polysaccharides. The two enzymes mainly involved in these processes are glycanases, such as polygalacturonase (PG) and pectin methyl esterase (PME or PE). PG causes an hydrolysis of polygalacturonic acid, whilst PME removes methoxyl groups from methylated pectin determining a release of methanol. Among the other enzymes involved in cell wall loosening, β-galactosidase, a glycosidase, is responsible for the degradation of both pectic and hemicellulosic components and is likely related to late-ripening events (Prasanna *et al.*, 2007).

In olive it has been demonstrated that the major changes in drupe texture in the transition between green and purple fruits are driven by the solubilisation of homogalacturonans, a fraction of pectic polysaccharides, and the decrease of tightly bound hemicelluloses. In the transition between purple and black stages, cell wall modifications are caused primarily by a decrease of hemicelluloses and cellulose, and only secondly by changes in the pectic fraction consisting of release of rhamnogalacturonan. In this ripening stage an high endoglucanase activity is observed, involved in hemicelluloses and cellulose degradation (Jiménez *et al.*, 2001a). An increase in hemicellulose-related sugars, such as xylose,

mannose, galactose, and glucose, is observed in the last phases of olive ripening (Jiménez et al., 2001b).

Mínguez-Mosquera *et al.* (2002) determined the enzymatic activities of PG and PE in ripening olives of Hojiblanca variety in Spain, and results are shown in Figure 6.

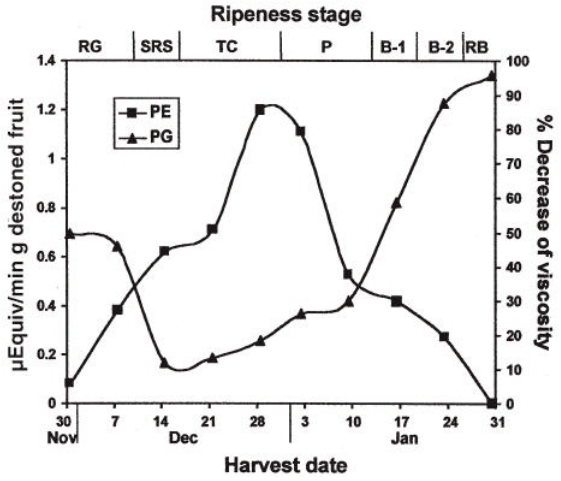

Figure 6. Enzymatic activities of PE (expressed as microequivalents of carboxyl groups hydrolysed per gram per minute) (squares) and PG (expressed as decrease of viscosity) (triangles). RG, ripe-green; SRS, small reddish spots; TC, turning colour; P, purple; B-1, fruits with black surface and white pulp; B-2, fruits with black surface and purple pulp; RB, ripe-black; (from Mínguez-Mosquera *et al.*, 2002).

Pectin methyl esterase showed a gradual increase in enzymatic activity peaking between turning and purple stages, whilst polygalacturonase activity decreased at turning stage to increase gradually until reaching maximum values at B-2 stage.

Drupes firmness has been evaluated as a possible ripening marker for assessing the optimal harvest time, even if it shows a clear cultivar-dependent trend as shown in Figure 7 and can be considered only an approximate idea about olive texture and olive fruits storage potential (García *et al.*, 1996).

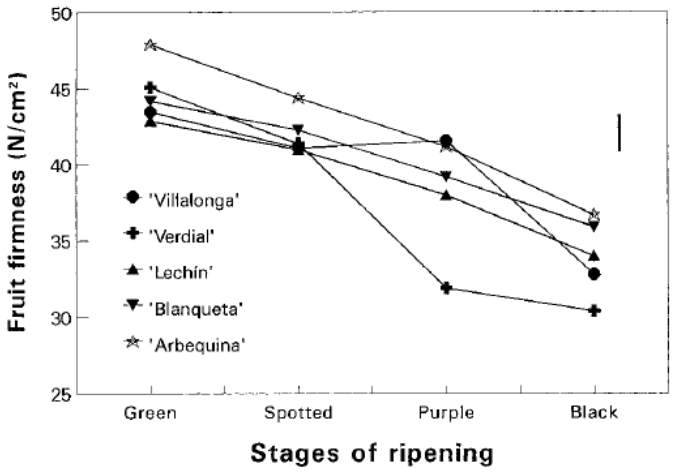

Figure 7. Decrease of fruit firmness with regards to ripening stages on five Spanish cultivars (from García *et al.*, 1996).

Pigments in olive fruits

The quantity and quality of pigments found in fruits depend on different factors, among which developmental and ripening stages are of primary importance. As stated before, olive fruits undergoes a colour shift during ripening, and this variation is due mainly to a change in ratio and quality of pigments found in the drupes. When the ripening process progresses, photosynthetic activity of olive drupes decreases in parallel with the decrease of chlorophyll and carotenoid content, followed by the appearance of anthocyanins that confer the fruits the typical purple and violet colours. The latter are anyway water-soluble pigments, so they don't give any contribution to virgin olive oil colour. Chlorophylls and carotenoids are soluble in lipids and have been studied historically for their contribution to oil colour, but scientific interest is lately raising because these compounds may be considered quality markers since they are present in stoichiometric ratios due to their functions in photosynthetic membranes (Aparicio-Ruiz *et al.*, 2009). Moreover, these compounds can have nutraceutical and functional properties since β-carotene, a carotenoid, is a precursor of vitamin A whilst chlorophylls can protect oils form oxidative processes together with phenolics if oils are stored in darkness, but may work as pro-oxidants if oils are stored in daylight (Cimato *et al.*, 2001).

Changes in chlorophyll and carotenoid content with respect to drupe ripening can be observed in Figure 8.

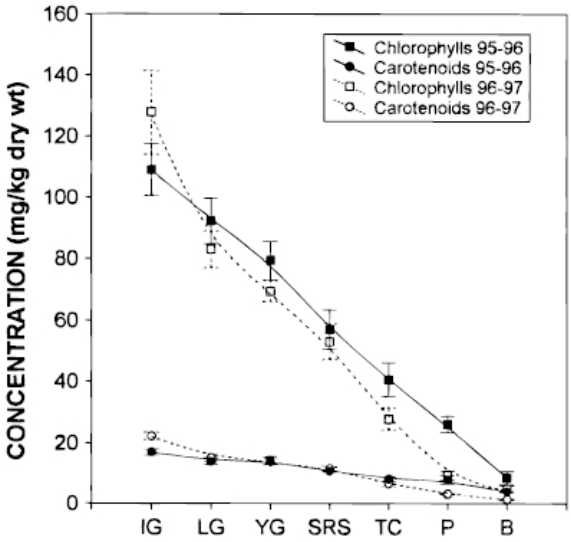

Figure 8. Decrease in total chlorophylls and carotenoids in olives cv. Arbequina in two harvesting seasons. IG: intense green; LG: light green; YG: yellowish green; SRS: small red spots; TC: turning colour; P: purple; B: black (from Gandul-Rojas *et al.*, 1999).

Pigments present in olives and virgin olive oils can not be listed univocally since the profiles change with regards to different factors, as stated before. A study on Italian extra-virgin olive oils from Sicily, Molise and Emilia-Romagna detected the following components: among carotenoids neoxanthin, violaxanthin, luteoxanthin, antheraxanthin, mutatoxanthin, lutein, β-cryptoxanthin, β-carotene, whilst among chlorophyll and its derivatives chlorophyll *a* and *b*, pheophytin *a* and *b*, and pheophorbide *a* can be found (Cerretani *et al.*, 2008).

A study has been carried out on Arbequina and Farga cultivars with the purpose of monitoring chlorophyllase activity and the general pigment profile (Criado *et al.*, 2006). Chlorophyllase is the enzyme involved in the hydrolysis of the ester bond between chlorophillide and phytol, but it still unclear if this enzyme is involved only in catabolism or also in pigments biosynthesis (Criado *et al.*, 2006). This study pointed out that the main pigments in studied samples were chlorophyll *a* and *b*, and the carotenoids neoxanthin, violaxanthin, lutein, antheraxanthin, and trans-β-carotene, with slight differences between cultivars and strong decreases with respect to ripening. In Arbequina olives, chlorophyllase activity decreased during ripening, to increase then slightly in the last stages. Moreover, Arbequina olives had an unusual pigment biochemistry, and it has been observed in this

cultivar that carotenogenesis takes place together with the synthesis of anthocyanins in the last ripening stages (Roca and Mínguez-Mosquera, 2001) and that the synthesis of esterified xanthophylls has been observed in the turning stage (Criado *et al.*, 2007).

Oil biosynthesis – precursors, fatty acids and triglycerides synthesis

Olive mesocarp at the common harvest time contains approximately 60% water, 30% oil, 4% sugars, 3% proteins, and small amounts of fibre and ash.

The precursors for oil biosynthesis in olive fruit cells are sugars. Young mesocarps contain about 20% sugars (dry matter), but this content decreases when oil accumulation begins. The carbon backbone for fatty acids biosynthesis is acetyl-CoA, whose biosynthesis requires pyruvate. Pyruvate is synthesised from carbohydrates via glycolysis, and acetyl-CoA can be synthesised from pyruvate by means of the action of pyruvate dehydrogenase in plastids (Conde *et al.*, 2008). Seed growth seems to rely only on phloem translocation to gain energy sources, whilst mesocarp growth rely also on carbohydrate import. Even if the carbohydrate translocation system has not been fully elucidated in olive yet, it is known that sugars (mainly stachyose and sucrose) are translocated through the phloem from leaves to fruits or can be synthesised directly in fruits by photosynthesis, which therefore may contribute to some extent to lipid biosynthesis in drupes (Connor and Fereres, 2005). Mannitol, a sugar alcohol found in phloem sap and leaves, serves as reserve carbohydrate and osmoprotectant and therefore its content increases during ripening. It has been stated (Conde *et al.*, 2008) that high amounts of mannitol result in more glucose available for oil biosynthesis via acetyl-CoA, and mannitol can be consequently used as an indicator of the potential oil accumulation of different cultivars.

Fruit can sequester respiratory CO_2 and CO_2 generated by cell metabolism and fix it to oxaloacetate (that is then converted to malate) by means of mesocarpic phosphoenol pyruvate carboxylase. As a result of these reactions, it can be summarised that, during the light-dependent phase of photosynthesis, CO_2 is therefore fixed into triose-phosphates.

Fatty acids are synthesised in plastids, starting with the carboxylation of acetyl-CoA to malonyl-CoA (Conde *et al.*, 2008). The malonyl group is then transferred to the acyl carrier protein (ACP), and starting from malonyl-ACP fatty acid synthases add cyclically C_2 units yielding fatty acids. The last step in fatty acids synthesis yields stearoyl-ACP, which is then

desaturated to oleoyl-ACP. The enzyme responsible for this reaction, the stearoyl-ACP Δ9-desaturase, has been identified and its gene expression is developmentally regulated. Desaturase transcript accumulation profile shows an upward trend up to 28 weeks after flowering, and shares an overlapping trend with oleic acid synthesis in olives (Haralampidis *et al.*, 1998). The acyl-ACPs synthesised in plastids are then transferred to the cytosol as acyl-CoAs, and finally enter the endoplasmic reticulum for assembling triglycerides by means of the Kennedy pathway, which involves glycerol-3-phosphate as a backbone and fatty acids synthesised in plastids. Acyltransferases involved in the Kennedy pathway may have different specificity, accounting for the different fatty acid composition of the resulting triglycerides and finally of the oil itself, and their level/activity are ripening-dependent.

It has been pointed out (Connor and Fereres, 2005) that oil accumulation starts approximately in the phase of pit hardening, and persists for more than 100 days. Whilst oil accumulation in the seed reaches a maximum before the onset of ripening, in the mesocarp this process continues after this stage and has therefore been suggested as a possible ripening index and oil quality marker by many authors (e.g. Dag *et al.*, 2011).

In fact, the authors pointed out that oil yield is the major economic factor for growers, but also that the increase in oil accumulation is associated with oil quality improvement only in the first ripening stages. Since the determination of the best harvest time is difficult considering cultivar variability, seasonal changes, crop load and many other factors, studies on oil yield in comparison to other physiological parameters can be help the growers define the best and easiest harvest period.

An example of oil accumulation in cultivar Frantoio with regards to olive ripening cultivated in Spain can be observed in Figure 9, for three successive years (Beltrán *et al.*, 2004).

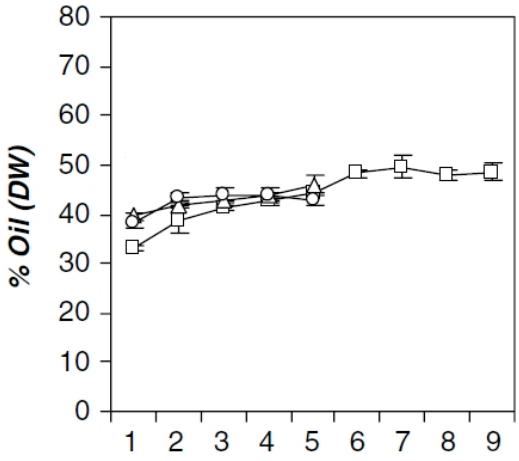

Figure 9. Percentage of oil accumulated in olive drupes (DW, dry weight) for season 96/97 (square), 97/98 (triangle), 98/99 (circle). Considering only season 96/97, for which a complete dataset is provided, samples (1-9) were collected approximately every two weeks starting from September 16th to January 30th (modified from Beltrán *et al*., 2004).

As pointed out by the authors, the oil accumulation profile changes markedly with regards to cultivar and year but after a defined date (depending on climatic and genotypic factors) no significant changes can be observed in oil content. The authors concluded therefore that even it was widely acknowledged that the best harvest period is when drupe skin is black, olives could be harvested earlier in order to ensure a better oil quality.

Changes in the phenolic profile

It is well known that the ripening process causes a decrease in the phenolic content in olive fruits. It has been stated that oleuropein content increases steadily during early stages of olive fruit growth in cultivars Lucques, Salonenque and Picholine (Amiot *et al.*, 1986), reaching a maximum between the end of August and the beginning of September (depending on cultivar) and then showing a sharp decrease in the following stages. In young fruits, oleuropein can reach 14% of the dry matter, decreasing to 3-6% in all the studied cultivars before the purple stage. It has been also pointed out that this decrease is not caused by a dilution effect during fruit growth. The authors then deduced that oleuropein could be reused during ripening biochemistry, since no degradation compounds could be found in the studied samples.

A clear decrease in phenolics has been observed by Rotondi *et al.* (2004) in Nostrana di Brisighella cultivar. Dividing olive samples in four classes of ripening according to skin and flesh colour the authors pointed out that significant changes can be seen in both total phenols content (from 441.43 mg gallic acid/kg of October 22nd to 209.57 mg gallic acid/kg of December 3rd) and *o*-diphenols (hydroxytyrosol, 3.4-DHPEA-EDA, 3,4-DHPEA-EA; from 212.19 mg gallic acid/kg to 127.47 mg gallic acid/kg, considering the same timespan). Changes in phenolic content have been observed also in virgin olive oils (Yousfi *et al.*, 2006). Total *o*-diphenols, total secoiridoids and hydroxytyrosol showed a significant decrease throughout all considered ripening stages in olive oils extracted at eight harvest dates for cultivar Picual and seven harvest dates for cultivar Arbequina.

Changes in the volatile profile

Volatile compounds are produced in detectable amounts specifically during drupes ripening in a phase corresponding to the respiration rate increase demonstrated by Ranalli *et al.* (1998). Angerosa et al. (2004) emphasised that the amount of VOCs reaches a maximum when fruits are at the turning stage, and that after this stage volatile compounds decrease resulting in a loss of some "green" sensory notes.

Compounds that can be responsible for virgin olive oils "green" notes, and more precisely hexanal, 3-*E*-hexenal, 3-*Z*-hexenal, 2-*E*-hexenal, hexyl acetate, 3-*Z*-hexenyl acetate, hexan-1-ol, 3-*Z*-hexen-1-ol, 3-*E*-hexen-1-ol, and 2-*E*-hexen-1-ol, have been studied with respect to fruit ripening in oils by milling green, medium-ripe and black over-ripe drupes (Aparicio and Morales, 1998). A decrease in the concentration in extracted oils of the compounds listed above was observed by means of GC-MS, with the exception of 3-*E*-hexenal that showed a peak in oils extracted from black olives.

Since genes encoding enzymes involved in the LOX pathway have been isolated in olive, for some of them gene expression profiles throughout drupe ripening are known.

Lipoxygenases can be divided into two classes (LOX1 and LOX2) according to the structural features of the proteins (Shibata and Axelrod, 1995). A LOX1 has been isolated in olive mesocarp, cv. Leccino, and mRNA expression showed an increase in ripe olives reaching a maximum in black samples and being not detectable in green ones (Palmieri-Thiers *et al.*, 2009). The authors conclude that this LOX can be related to late ripening and

senescence events, and that also in kiwifruit six LOX were found to be differentially expressed during ripening and senescence. Padilla *et al.* (2009) isolated two olive LOX2 in cv. Picual mesocarp, of which one showed a peak in transcript accumulation in correspondence to the onset of ripening. This form may be therefore involved in the synthesis of C5 and C6 flavour compounds.

A full length HPL has been isolated in cv. Picual mesocarp (Padilla *et al.*, 2010) showing a similar trend in transcript accumulation with regards to the previously cited LOX2 and suggesting a comparable role in volatile compounds biosynthesis.

Evaluation of fruit ripening

As pointed out before, the choice of the best harvesting period must take into consideration quantitative parameters, such as oil accumulation, content of phenolics, pleasant volatile compounds and compounds related to olive oil health, but also qualitative parameters, such as the global quality of extracted oils, which is not only a sum of parameters but a delicate balance among different variables. Drupe ripening has a major effect on these features, and as highlighted before is one of the steps, together with oil extraction process, which can and should be carefully monitored by the grower or the oil producer.

Therefore the evaluation of olive fruit ripening is a very important point, and must be evaluated in an objective, easy, and repeatable way. The scientific community has tried to evaluate ripening stages in many different ways, designing ripening indices with the purpose of finding the best balance between oil quantity, for its obvious economic importance, and oil quality. Cimato *et al.* (2001) listed some of the parameters studied in the past to evaluate drupe ripening: fruit respiration, drupe growing measurements (dry and fresh weight, volume), fruit drop, fatty acids content and ratio, role of carbohydrates or proteins in the seed, ratio between anthocyanins and some phenols, ossalic acid/citric acid ratio in drupes, evolution of sterols, phenols and anthocyanins, and drupe colour.

One of the most known and used ripening indices is the so-called Jaén or Ripening Index (RI), proposed by Uceda and Frías (1975). This index can be calculated picking 100 olives and dividing them into classes with respect to exocarp and mesocarp colour. Drupes are therefore divided in eight classes, and each number of drupes must be multiplied by a coefficient from 0 to 7. The values are then added up and divided by 100, yielding a

number corresponding to the index. The authors established that a RI of 3.5 is the best harvesting period, even if this value has a geographical dependent meaning and cannot therefore be easily applied everywhere. Moreover, a strong dependence on fruit yield and cultivar has been demonstrated by Dag *et al.* (2011), highlighting that this index can be useful for a quick and easy evaluation of ripening, but also that many other factors have to be taken into account if the best results in production and quality must be achieved.

Drupes colour has been used as a ripening marker also by Yousfi *et al.* (2006), not only as suggested for the calculation of RI but also considering the colorimetric spectra of fruit according to the CIE *L*a*b** system. These parameters have been used by the authors to calculate a colour index (CI) that has been also useful to evaluate table drupe degreening. The authors moreover point out that the calculation of RI can be sometimes misleading, since the grouping of drupes can be subjective.

Cherubini *et al.* (2009) used sugar concentration in drupes as a ripening marker, and it gave interesting results since it was reproducible, objective, and correlated to oil content. Nevertheless, it gave poor results in predicting harvest time, since pedoclimatic and varietal aspects were responsible for variations in sugar kinetics.

Aims of the thesis

The aim of this work was a thorough study of ripening related aspects in two olive varieties grown in Veneto region, Frantoio and Grignano, with respect to qualitative characteristics of extracted extra-virgin olive oils. To this end, different approaches were followed taking into account technological and molecular aspects:

a) The importance of oil extraction technology has been studied, extracting oil in a modified anoxic atmosphere. Considering that olive fruit ripening is a primary factor influencing the expression of enzymes responsible for olive oil flavour development, the adoption of the controlled atmosphere (CA) during extraction was applied to olive batches at four successive ripening stages. This approach was adopted to evaluate which factor (CA and ripening) has a prevalent role in defining the global aromatic profile of olive oil.

b) Subsequently, since the ripening stage of olives had a pre-eminent role on the definition of olive oil sensory notes, a study has been carried out on the major physiological changes occurring in ripening olives, evaluating differences in terms of firmness, chlorophyll content, oil accumulation, colour evolution and expression profiles of genes putatively involved in the development of volatile components.

c) Considering the peculiar flavour profile of oils obtained in Veneto region, and especially of Grignano oil, and the fact that terpenes are important players in defining some varietal specific aspects of virgin olive oil aroma, a study of the terpenic metabolism of olive fruits has been performed by isolating and characterising for the first time three olive terpene synthases with enzymatic assays and gene expression analyses.

d) Finally, the previously conducted gene expression analyses highlighted the lack of validated reference genes for *Olea europaea*. For this purpose, a detailed literature and EST database search and qPCR validation of selected reference genes in olive fruit and leaf tissues were performed and allowed the identification of 12 candidate reference genes for gene expression studies in olive.

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Chapter II

Influence of olive (*cv* Grignano) fruit ripening and oil extraction under different nitrogen regimes on volatile organic compounds emissions studied by PTR-MS technique

Influence of olive (cv Grignano) fruit ripening and oil extraction under different nitrogen regimes on volatile organic compounds emissions studied by PTR-MS technique

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Abstract

Volatile organic compounds of extra-virgin olive oils obtained from the local Italian cultivar Grignano were measured by proton transfer reaction-mass spectrometry. Oils were extracted by olives harvested at different ripening stages across *veraison*, performing each extraction step and the whole extraction process in nitrogen atmosphere to observe the changes in the volatile profiles of the oils. Principal component analysis carried out on the full spectral signature of the PTR-MS measurements showed that the stage of ripening has a stronger effect on the global definition of volatile profiles than the use of nitrogen during oil extraction. The fingerprint-like chemical information provided by the spectra were used to construct a heat map, which allowed to dynamically represent the multivariate nature of mass evolution during the ripening process. This provided the first evidence that some groups of volatile organic compounds displayed a time-course of regulation with co-ordinated increasing or decreasing trends in association with specific stages of fruit ripening.

Introduction

Extra-virgin olive oil (EVOO) may be considered a functional food, because of its demonstrated anti-thrombotic, anti-inflammatory, anti-hypertensive, and anti-atherogenic properties [1]. A regular consumption of olive oil can provide a good protection against oxidative damage due to the presence of oleic acid and phenolic compounds [2]. EVOO is a major component of the Mediterranean diet, and especially in Italy, Greece, and Spain, the consumption of this vegetable fat is significant [3].

Besides its healthful properties it is well known that olive oil is often preferred by consumers for its palatable taste and pleasant aroma, attributes which are given by minor components like phenols and volatiles [4, 5]. As concerns phenolic compounds, they are released during olive oil extraction [6] and their concentration can decrease during olive fruit processing due to oxidation [7]. The biogenesis of volatile compounds in olive oil and their contribution to its quality and flavour have been described in detail by some recent reviews [5, 8]. Olive oil volatile compounds are synthesized through the lipoxygenase (LOX) pathway, starting from linoleic and linolenic acid and requiring oxygen. Among these components the most important for virgin olive oil aroma are C_6 and C_5 compounds [8].

Olive oil extraction consists of three operations: in the first step, called milling, olive fruits are crushed to obtain a paste. In the second step, called malaxation, the paste is kneaded to form large drops of oil; while during the final step, the centrifugation, a decanter is used to separate the phases formed in the previous steps. A considerable amount of research has been devoted to address the effects of oxygen control during the different steps of olive oil production by evaluating its impact on the development of aroma and on phenolics. In [9], olive oil volatile compounds have been shown to be produced when fruit tissues are disrupted during the milling step, and oxygen has been described as crucial for these chemical reactions to take place. Several studies have attempted to identify the effects of reducing the oxidation processes during olive oil extraction, by using an inert gas such as nitrogen (N_2) or by changing the malaxation conditions, e.g. by applying different regimes of temperature and duration time [6, 9-12]. Even though these studies have shed light on the role played by oxygen on the development of oil aroma components during milling and

malaxation, systematic studies are lacking investigating the effects of oxygen removal during each olive oil extraction step and specifically relating these effects to the different ripening stages of the olive fruits.

There are, on the other hand, contributions dealing exclusively with the influence of different ripening stages on volatile and phenolics content in olive oils. These studies, however, only have evaluated the differences between aroma profiles of EVOO obtained from olives at very far apart stages of ripening (e.g. green/immature versus purple/black - ripe/over-ripe fruits) [13-15], and have not attempted a systematic comparison of temporally close successive ripening phases across the *veraison* (used by agronomists to describe the change in color, marking the onset of the maturation and of the harvesting period). In addition, these and other studies, dealing more in detail with the ripening process, have adopted targeted approaches (by GC-MS) mostly focusing on specific groups of volatiles [13, 16, 17], while no study has tried to depict a "global" profile of VOCs in virgin olive oils in relation to ripening and, specifically, in relation to different extraction procedures.

In this paper we discuss the results of a preliminary study about the characterization of the role of different ripening stages of the olives on EVOO aroma profiles, taking simultaneously into account the effects of extractions in different oxygen regimes at different steps of olive oil processing. To the best of our knowledge, this combined investigation has never been reported before.

The volatile profiles of the oil samples have been measured by Proton Transfer Reaction Mass Spectrometry (PTR-MS). The full spectral signature provided by PTR-MS has been exploited as a complex fingerprint able to capture sample global patterns relating the effects of using nitrogen at different steps of olive processing to ripening stages of the olives. The exploratory nature of this study, which has not been focused *a priori* on specific groups of volatiles, has required the application of unsupervised multivariate techniques, that is to say Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA). As a new visualization approach, we have introduced the use of heat maps (HMs), extensively applied in DNA microarray studies [18], to represent and explore at a glance the multivariate nature of the PTR-MS dataset. To our knowledge, heat maps have never been used in studies involving data obtained by means of spectrometric techniques. This

graphical representation has allowed to capture characteristics and relationships that would be not otherwise evident by considering either PCA or HCA carried out separately on the sample dataset and on the peak (mass) dataset.

Materials and methods

Olive Samples and Olive Oil Extraction

Olive fruits (*Olea europaea* L cultivar Grignano) were harvested in an orchard at Mezzane di Sotto (Verona, Veneto Region, Italy) during the 2008 season. Cultivar Grignano, despite of being only locally spread [19], is well known for its resistance to cold and pathogens and, most importantly, for its peculiar lemon peel aromatic notes [20]. The time-course experiment spanned a period from the onset of *veraison* until late ripening. Picking was performed at four ripening stages defined on the basis of the percentage of green and black olive fruits within the whole fruit population of the trees: 1) 3rd November: 15% green olives, 5% black olives; 2) 10th November: 5% green olives, 10% black olives; 3) 17th November: 30% black olives; 4) 25th November: 45% black olives.

Extra-virgin olive oil was extracted in an OLIOMIO 200 plant (Toscana Enologica Mori, Tavarnelle Val di Pesa, FI, Italy) using a batch of 100 kg of olives for each sample, collected randomly from trees of the same age (at least five trees for each batch), to ensure the biological representativeness of the samples. All experimental conditions for EVOOs extraction (temperature, duration of extraction steps, nitrogen application) have been chosen to reproduce as closely as possible those normally used in the common olive milling practice and those adopted in other studies with a similar experimental set up [9-12], in order to ensure the highest possible comparability of results. The experimental conditions are given in detail as follows. Extraction was performed within 4 h from picking. Olive fruits were milled in a hammer crusher with a power output of 12.5 kW, and then the olive pastes obtained were malaxed in a two-chamber malaxing machine. Malaxation was performed for 20 min at 25°C. Then, the kneaded olive pastes were processed in a two-phase decanter for centrifugation and separation and oils were finally filtered by means of

paper filters. Oils were subsequently stored in stainless steel containers filled with carbon dioxide to obtain a modified atmosphere and then analyzed within two months.

The effect of oxygen removal has been studied by using nitrogen as an inert gas. For processing in N_2 atmosphere, the oil extraction plant was sealed to allow the flush of nitrogen, and two nitrogen cylinders were plumbed to the equipment with a pressure regulator to keep a constant pressure of 0.6 atm inside the plant. Oil extraction was then carried out with the following operating conditions: extraction fully performed in nitrogen atmosphere ("AAA" samples), olive milling in nitrogen ("ASS" samples), pastes kneading in nitrogen ("SAS" samples), centrifugation and separation in nitrogen ("SSA" samples), and control sample fully extracted in air ("SSS" sample). For olive oils extracted from olives harvested at the last date (25th November) only SSA, SSS and AAA samples were analysed. The list of samples analysed at the different dates is summarized in Table 1.

	Regimes of Extraction in N_2 atmosphere				
Date of Harvest	No steps in N_2	Milling in N_2	Malaxation in N_2	Centrifugation in N_2	All steps in N_2
3rd November	SSS_03	ASS_03	SAS_03	SSA_03	AAA_03
10th November	SSS_10	ASS_10	SAS_10	SSA_10	AAA_10
17th November	SSS_17	ASS_17	SAS_17	SSA_17	AAA_17
25th November	SSS_25	NA	NA	SSA_25	AAA_25

Table 1. Sample abbreviations according to the date of harvest and the different nitrogen regimes applied during extra-virgin olive oil extraction. NA= sample not available.

The described oil sample collection manifestly implied a worth considering olive availability (1800 kg) and also the collaborative decision of the cultivator not to assign this quantity to the market production. These aspects have *de facto* hindered the possibility of biological replica. Therefore, this study has to be considered as a preliminary though informative exploration of the complexity of the EVOO aromatic profile development along the progression of olive fruit ripening and under different oil processing conditions.

PTR-MS Measurements

Proton transfer reaction-mass spectrometry was developed about 15 years ago as a "real-time" technique to measure volatile organic compounds in air [21-23]. It has been exploited in applications related to atmospheric and environmental science, flavor and food chemistry, life science and medicine (see e.g. [24] and reference therein, [25]). This technique has been also recently used in studies dealing with the oxidative alteration of olive oil [26] and the geographic characterization of European olive oils [27].

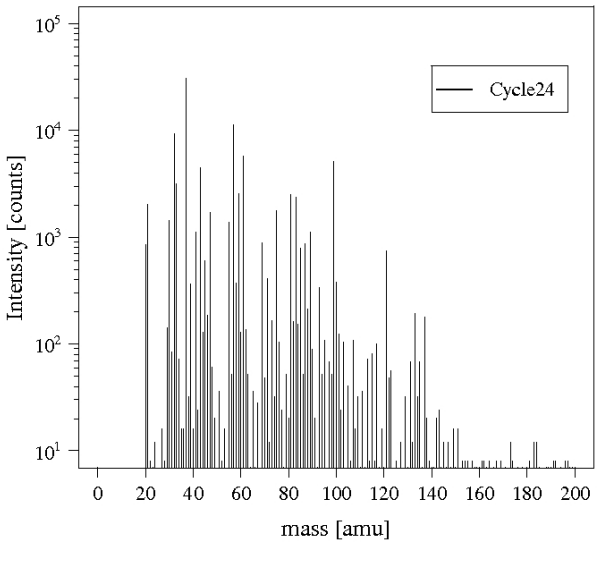
The PTR-MS measuring approach is based on the chemical ionization of VOCs molecules by proton transfer inside a drift tube, which is continuously flushed with ambient air. At this aim, a plasma discharge ion source produces an intense and pure flux of hydronium (H_3O^+) ions. For measurements in air, these are suitable proton donors, as they do not react with any of common atmosphere components (N_2, O_2, Ar, CO_2, etc); H_3O^+ otherwise reacts with VOCs having proton affinity greater than that of water, which is the case of a variety of organic species (e.g. alcohols, ketones, nitriles, aldehydes, aromatics, etc) in air. The primary and product ions are then selectively detected by a mass spectrometer in a conventional manner. Olive oil samples of this study have been analysed by a PTR-MS equipment, using a quadrupole mass spectrometer, available at the Center for the Physics of Aggregated States (CeFSA) of the Fondazione Bruno Kessler (Trento, Italy). If both proton donor and protonated acceptor ($VOCH^+$) concentration obey the relation $[VOC]>>[H_3O^+]>>[VOCH^+]$, then, by a non-destructive analysis and without concentration or preparatory steps, it is possible to obtain the absolute molecule concentrations on a short time scale (within seconds) and with a detection limit of a few pptV. Therefore, PTR-MS combines the desirable attributes of high sensitivity and short integration times with good precision and accuracy [24]. In addition, PTR-MS ensures a reduction of the fragmentation of the analyte molecules with respect to more traditional MS techniques based on electron impact ionization, allowing for an easier spectra interpretation (and quantification). A detailed review of the PTR-MS methodology, of its historical development and of its diverse applications can be found in [28] (see also reference therein).

The best available approach for the detection and quantification of very low concentrations of VOCs is probably GC-MS. This widely used technique, though highly sensitive and

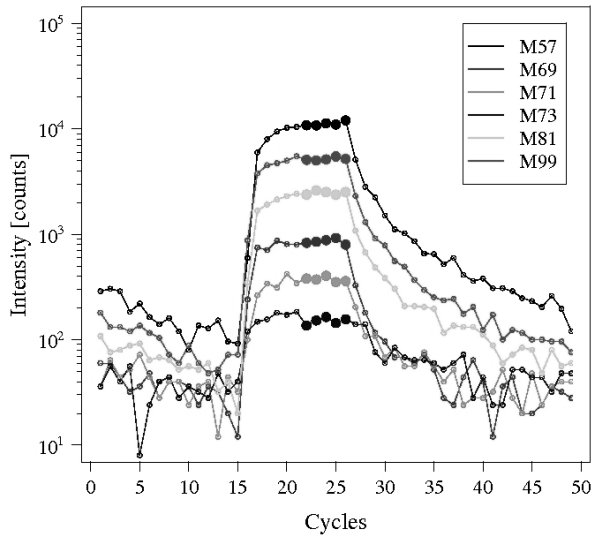
reliable, is however time-consuming (from few to tens of minutes). The complete separation of the molecules of a gas mixture often needs multiple extractions, different capillary columns and experimental conditions. In addition, to carry out VOC detection in air a pre-concentration step is often necessary. The sample preparation, concentration and analysis might also introduce experimental artefacts. Unlike PTR-MS, GC-MS is therefore inherently slow and it is not advantageous to use it as a “real-time” technique, especially when the determination of complete aroma profiles of more samples is required.

Besides ion fragmentation, clustering and secondary ion molecule reactions in the drift tube that can influence the measurement, the principal weakness of PTR-MS technique is that it provides fragmentation patterns that do not always allow the unambiguous discrimination between isomeric or isobaric compounds. However, this limitation can be overcome with the aid of data found in the literature (as described by [29-30]), thus allowing the univocal identification of many molecular masses.

In our study, 20 ml of every oil sample has been placed in a 100-ml glass bottle (Kavalier, Sázava, CZ) and topped with Teflon® caps. The bottles have been kept in a water bath at 30°C for thermal conditioning: in this way the VOCs present in the headspace of different samples have been sampled at a fixed temperature and sent to the drift tube of the PTR-MS system through a gas inlet maintained at 20°C, with an air flux of 15 sccm/min (standard cubic centimetre per minute). Mass data have been collected in a range from 20 to 200 amu (m/z) with a dwell time of 0,5 s per mass under drift tube conditions of 120 Td (Townsend, where 1 Td = 10-17 V cm2 mol-1). A typical mass spectrum is shown in Figure 1A. A representative spectrum for each sample has been obtained by averaging the last five acquired spectra after that the stationary conditions have been reached. As an example, Figure 1B illustrates the time evolution during measurements for some selected masses. For each oil sample a single representative spectrum has been considered. Then, blank subtraction has been carried out and values have been converted to a part per billion concentration (ppb).



A



B

Figure 1. A Example of a mass spectrum collected in a range 20–200 amu (m/z) with a dwell time of 0.5 s per mass. **B** Time evolution of six selected masses during measurements shows the stationary condition. Each cycle represents a spectrum. The representative mass spectrum of the sample is obtained by averaging the last five spectra (bold points) acquired when stationary conditions are reached.

It is worth noting that the choice of PTR-MS technique also allowed us to discuss the development of a new methodological approach, combining the complex and multivariate information arising from the complete PTR-MS spectral signature of EVOO aroma profiles with unsupervised machine learning analyses and HM data representation.

Multivariate Data Analysis

PCA-, HCA- and HM-based data representations have been applied on the preprocessed spectra using the available statistical packages (*stats, ChemoSpec, amap, gplots*) [31-33] of the R software environment [34].

Results and discussion

Principal component analysis

PCA [35] produces score and loading plots in an unsupervised way. Given the space of spectral masses considered in this work, once all PCs have been calculated using eigenvalue/eigenvector matrix operations, only the first three PCs have been retained and the original high-dimensional space has been projected onto these axes. The exploratory graphical visualization of relationships among PTR-MS spectra was made easier in this compressed space, which retained most of the original information (score-score plots). In addition, the loading plots summarized the relationship among the original spectral masses and helped to understand the patterns highlighted in the score plots. In this way, PCA allowed to explore in a multivariate way the relationship among spectra and spectral masses. The plots have been obtained by mean-centering and scaling to unit variance the descriptor vectors, each one obtained by considering the values of a single spectral mass across all the samples.

PCA has been used to identify natural groups of data in the complete set of PTR-MS spectra and to explain the related global profile of volatile emissions in relation to the different stages of olive fruit ripening and/or the processing under different nitrogen regimes during the main phases of oil extraction. At this aim, both the described multivariate analyses (PCA, HM) have been carried out on the whole spectral range, which provided fingerprint-like chemical information. Therefore, to obtain an unbiased perspective on possible chemical differences we did not consider any *a priori* selection of the spectral features (masses). Rather, by PCA we have automatically obtained a

preliminary indication of those masses principally responsible for the differences among spectra groups illustrated by the score-score plots.

In Figure 2A and 2B, the scores of PC1 versus PC2 and of PC2 versus PC3 are reported. The first three PCs explain an overall variance of 78.5%. In Figure 2A, along the PC2 direction, describing 23.8% of the total data information, samples collected on November 17th and 25th formed two distinguishable groups closely similar and clearly separated from the two groups formed by spectra related to November 3rd and 10th: the first two groups lied on the positive side of the PC2 axis and the last two ones on the negative side of the same axis.

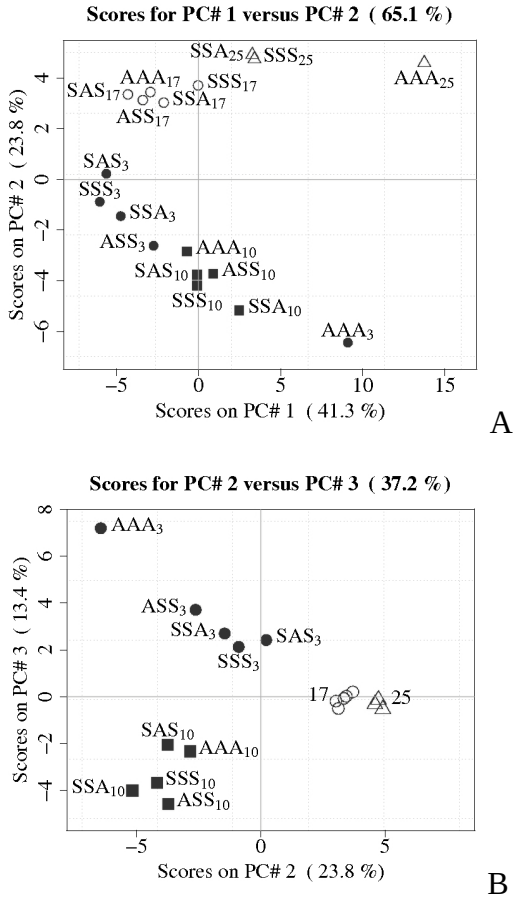


Figure 2. PC1/PC2 (A) and PC2/PC3 (B) score–score plots from PCA performed on the PTR-MS set of spectra outlined in Table 1.

These results clearly point out that the effect of the harvesting date is predominant over that of the extraction procedure on defining the global pattern of olive oil volatile emissions. In addition, groups 17 and 25 appeared more compact than groups 3 and 10. This evidence

was confirmed by the patterns emerging in the PC2 vs. PC3 score/score plot showed in Figure 2B. As the PCs are sorted by the descending amount of variance they accounted for in the original dataset, the different information obtainable by the different PCs can here be interpreted as the amplification of dataset patterns having an increasing fine-structure. Consistently, Figure 2B confirmed that the effect exerted by the removal of oxygen during different steps of oil extraction on the volatile profile of EVOOs appeared to some extent different at the four ripening stages considered and, in particular, the spectra intra-variability was higher in the November 3rd and 10th groups with respect to the November 17th and 25th ones. In the same Figure, the 13.4% variance captured by PC3 was mainly associated with the distance (dissimilarity) between groups of November 3rd and 10th samples, while the two remaining groups were still distinguishable but closer, therefore more similar. Consequently, the time-dynamics of the global profile of volatiles emissions, following the time-course experiment, suggest a saturation effect in parallel with the progression of olive fruit ripening, consistently with the general evolution of other parameters of fruit ripening, as previously observed only for grapes [36].

In the PC1 versus PC2 score/score plot, showed in Figure 2A, when PC1 was considered, especially AAA_25, but also AAA_03, could be separated from the rest of samples and therefore could be identified as possible outliers. To investigate this hypothesis, the diagnostic plot [37] is reported in Figure 3, which highlights outliers by means of two different distance definitions, and classifies them into four different types [38].

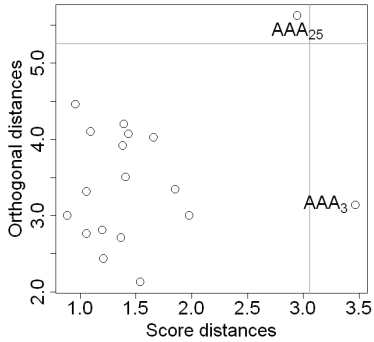


Figure 3. Diagnostic plot providing the outlier map of the PTR-MS set of spectra based on the three first PCs retained in the PCA model.

The orthogonal distance (OD) expresses the distance of an original spectrum to the reduced space, spanned by the retained PCs. The score distance (SD) is used to compute the

distance of a projected observation (i.e. of a projected spectrum) from the centre of the data within this PCA subspace. The diagnostic plot classifies the observations, by displaying the ODs versus the SDs and drawing two lines in correspondence to the respective cut-off values that allow to identify outlying spectra. We used the same critical values described in [37]. In Figure 3 all the spectra but two of them had SD and OD lower than the respective cut-off values; these objects did not have outlying distances and therefore form the regular part of the data. Spectrum AAA_03 was a good leverage point, that is it had a large SD, but a small OD. This observation lied close to the space spanned by the principal components but far from the regular data, causing a stabilization of the PCA space, as there was only a little loss of information when it was replaced by its projection in the PCA-subspace. Spectrum AAA_25 was an orthogonal outlier, as the OD was larger than its critical value while SD was still in its low range; it lay far from the PCA subspace. It is worth noting that bad leverage points (both SD and OD higher than the critical values) were not present. In fact, points of this type can have severe leverage to a classical PCA by tilting the subspace towards them, and are hence able to completely distort the results [38]. Our analysis therefore confirmed that AAA_25 and AAA_03 did not obey, even if in different ways, the pattern of the majority of the data and seemed to suggest that extraction in an anoxic environment may have significant effects only on VOCs profiles of oils obtained from the most distant stages of olive fruit ripening. However, in our experimental design, the hereinbefore described practical constrains have hindered the possibility of collecting sample replica for every combination of harvesting date and extraction procedure. Therefore, as long as this result will not be verified on a novel independent dataset, it is not possible to rule out the occurrence of artifacts while preparing or measuring samples AAA_25 and AAA_03.

In Tables 2, 3 and 4 the loadings of each spectral mass, in PC1, PC2 and PC3 respectively, are reported. The spectra of loadings for PC1, PC2 and PC3 are provided as Supplementary Figures S1, S2 and S3. The loadings allowed relating the existence of different spectra groups, described by the corresponding PC, in terms of increase or decrease of mass counts. After inspecting the loading plots and detecting the masses of VOC emissions that drove the group separations in the score-score plots, we assigned them to specific molecules,

exclusively on the basis of published data. A full list of masses detected in this work along with corresponding aromatic qualities and references to the literature are given in Table 5.

>= 0.15	m33(5), m35(6), m43(10), m47(12), m49(13), m57(16), m61(18), m63(19), m65(20), m71(24), m73(25), m75(26), m77(27), m79(28), m83(31), m91(35), m93(36), m99(40), m100(41), m101(42), m103(43)
[0.10, 0.15[	m29(2), m45(11), m51(14), m53(15), m89(34), m95(37), m97(38), m105(44), m107(45), m109(46), m111(47), m113(48), m115(49), m117(50), m121(51), m123(52), m133(57), m135(58), m139(60)
[0.05, 0.10[	m27(1), m39(7), m41(8), m42(9), m67(21), m69(22), m70(23), m85(32), m125(53), m127(54), m129(55), m131(56), m145(62), m155(63)
[0.0, 0.05[	m30(3), m31(4), m59(17), m82(30), m98(39), m137(59), m143(61)
< 0	m81(29), m87(33)

Table 2. The spectral masses listed according to their loadings in PC1 and grouped in distinct loading ranges. In each range, the masses are listed by reading the corresponding loading plot (Supplementary Figure S1) from left to right. For every mass, the variable number is given in parentheses.

>= 0.15	m59(17), m111(47), m117(50), m125(53), m127(54), m129(55), m131(56), m145(62), m155(63)
[0.10, 0.15[	m61(18), m103(43), m135(58), m137(59), m143(61)
[0.05, 0.10[	m43(10), m63(19), m75(26), m77(27), m83(31), m87(33), m89(34), m101(42)
[0.0, 0.05[	m71(24), m73(25), m79(28), m91(35), m123(52)
[−0.05, 0.0[	m45(11), m47(12), m51(14), m65(20), m93(36), m105(44), m107(45), m109(46), m115(49), m121(51), m133(57), m139(60)
[−0.10,−0.05[	m29(2), m33(5), m35(6), m49(13), m57(16), m95(37), m99(40), m100(41)
[−0.15,−0.10[	m27(1), m41(8), m53(15)
< −0.15	m30(3), m31(4), m39(7), m42(9), m67(21), m69(22), m70(23), m81(29), m82(30), m85(32), m97(38), m98(39), m113(48)

Table 3. The spectral masses listed according to their loadings in PC2 and grouped in distinct loading ranges. In each range, the masses are listed by reading the corresponding loading plot (Supplementary Figure S2) from left to right. For every mass, the variable number is given in parentheses.

>= 0.15	m39(7), m51(14), m53(15), m59(17), m107(45), m115(49)
[0.10, 0.15[	m33(5), m35(6), m71(24), m73(25), m95(37), m101(42), m113(48)
[0.05, 0.10[	m57(16), m65(20), m69(22), m81(29), m85(32), m87(33), m99(40), m100(41), m109(46), m135(58)
[0.0, 0.05[	m63(19), m70(23), m79(28), m82(30), m103(43), m111(47), m117(50)
[−0.05, 0.0[	m43(10), m61(18), m75(26), m77(27), m89(34), m125(53), m127(54)
[−0.10,−0.05[	m45(11), m47(12), m67(21), m91(35), m97(38), m131(56), m145(62), m155(63)
[−0.15,−0.10[	m49(13), m83(31), m93(36), m98(39), m105(44), m123(52), m129(55)
< −0.15	m27(1), m29(2), m30(3), m31(4), m41(8), m42(9), m121(51), m133(57), m137(59), m139(60), m143(61)

Table 4. The spectral masses listed according to their loadings in PC3 and grouped in distinct loading ranges. In each range, the masses are listed by reading the corresponding loading plot (Supplementary Figure S3) from left to right. For every mass, the variable number is given in parentheses.

When loadings of PC2 were considered, the grouping of EVOOs obtained on November 17th and 25th (having positive scores on PC2) appeared to rely, among other masses, on m59, which can be referred to acetone and is responsible for some sensorial defects [39] and on a number of molecules that could not be univocally identified (m111, m117, m125, m127, m131, m145, m156) except for m129 (2-octanone) [30], associated with mould, green sensory notes [5]. Among masses leading to the grouping of November 3rd and, more effectively, of November 10th samples (negative scores on PC2), m69-m70 (octanal/nonanal: citrus-like aroma, peculiar in oils obtained from Grignano olives), m81-82 (*cis*-3-hexenal: green, leaf-like), m85 (hexanol: fruity, aromatic, soft, cut grass), m97-m98 (heptanal: oily, fatty, woody) could be identified [8, 26, 30, 40].

When loadings of PC3 are considered, among the positive loadings underlying the separate grouping of November 3rd samples (having positive scores on PC3), only m59 (acetone) could be identified, while no masses with negative loadings have been univocally assigned.

From Table 2, it appears that the separation of AAA_03 and AAA_25 from remaining spectra was driven by positive loadings of a wider group of univocally identified masses (this result is also confirmed by inspecting the AAA25 and AAA3 rows in the HM figure): m33 (methanol), m47 (ethanol) [30], and m65 (ethanol-H_2O cluster), m57 (*trans*-2-hexenal [26], green, almond-like, apple like odour qualities [5]), m61 (acetic acid and acetyl esters

amu	Tentative identification	References	Attributes/Notes	References
33	Methanol	[30]	Associated with ripening	[45]
43	Esters	[30]	Ester, green, pungent, sweet, fruity	[8]
45	Acetaldehyde	[29]	Associated with ripening	[46]
47	Ethanol	[30]	Associated with ripening	[46]
57	*trans*-2-Hexenal	[26]	Green, almond-like, apple-like	[5]
59	Acetone	[29]	Sensorial defects	[39]
61	Acetic acid	[29]	"Vinegary" defect	[5]
63	Dimethyl sulphide	[21]	Wet earth, organic, beetroot	[8, 42]
65	Ethanol+H_2O cluster	-	-	-
69	Octanal/Nonanal	[30]	Citrus-like	[8]
73	2-Butanone	[30]	Ethereal, fruity	[43]
75	Esters	[30]	Ester, green, pungent, sweet, fruity	[8]
81	*cis*-3-hexenal	[40]	Green, leaf-like	[8]
82	Isotope of m81	-	-	-
83	Hexanal	[30]	Green, apple, cut grass	[8]
85	Hexanol	[30]	Fruity, aromatic, soft, cut grass	[8]
87	3-Pentanone	[29]	Sweet, green	[4, 5]
89	Ethyl acetate/Other esters	[29]	"Vinegary" defect	[5]
93	Toluene	[47]	Gasoline vapours	[48]
97	Heptanal	[30]	Oily, fatty, woody	[26]
98	Isotope of m97	[30]	-	-
99	*trans*-2-Hexenal	[26]	Green, almond-like, apple-like	[5]
101	2-Hexanone	[30]	-	-
103	Esters	[30]	Ester, green, pungent, sweet, fruity	[8]
105	Styrene	[47]	Gasoline vapours	[48]
129	2-Octanone	[30]	Mould, green	[5]
137	Monoterpenes fragment	[49]	-	-

Table 5. List of the predominant masses found in Grignano extra-virgin olive oils. The list is provided in ascending order according to the ion mass. A tentative assignment of the masses and some interesting notes about the aromatic qualities or other peculiarity are given.

[29], “vinegary” defects in olive oils [5]), m63 (dimethyl sulphide [21], a sulphur compounds found also in capers [41], responsible for the unpleasant odour of “beetroot” and “organic” [8, 42]), m73 (2-butanone [30], ethereal fruity sensory notes [43]), m101 (2-hexanone [30]) and m43, m75, m89, m103 (globally referred to acetate, butyrate and propionate esters, [30]). In the groups of the November 3rd and 17th samples, PCA showed

also a relative increase of mass m87. Interestingly, m87 is a ketone (3-pentanone) [29] associated with sweet and green sensory notes [4, 5].

Heat Map data representation

HMs are false color images used to give a graphical representation of a set of measurements. For their easy readability, HMs are tools often used in bioinformatics and molecular biology [18]. In Figure 4, HM has been used to describe all acquired PTR-MS measurements: each row corresponds to the spectrum of a different sample, while each column to the counts of a single mass across the samples.

HM allowed therefore to get at a glance a highly informative representation of the multivariate nature of the PTR-MS dataset. HM also allowed to evaluate the dynamics of the spectral masses in relation to the progression of the olive fruit ripening process and to oxygen removal at different steps of extraction. The ordering of samples and masses in the HM depends on the dendrograms added to the left side and to the top, which have been obtained by HCA exploiting the full spectral pattern, used as a fingerprint for an overall chemical classification. By grouping the PTR-MS measurements into clusters of similar spectra and masses, HCA allowed to explore in an unsupervised way the existence of structure and relationships in the dataset. HCA requires the definition of a measure of dissimilarity (distance) between objects and one of distance between clusters (linkage method). To reduce the computational load and to perform the best association between HM and PCA results, the dendrogram on samples (reported along the HM rows) has been calculated on the first three PCs of the PTR-MS data space. We have chosen for it the average linkage method and the Euclidean metric. The mass clustering was performed on the peak counts after individually mean-centering and scaling them to unit variance across the samples. The resulting increasingly positive (negative) peak intensities are colored with reds (greens) of increasing intensities. The obtained dendrogram has been calculated choosing a distance based on the Pearson correlation coefficient and the average linkage method.

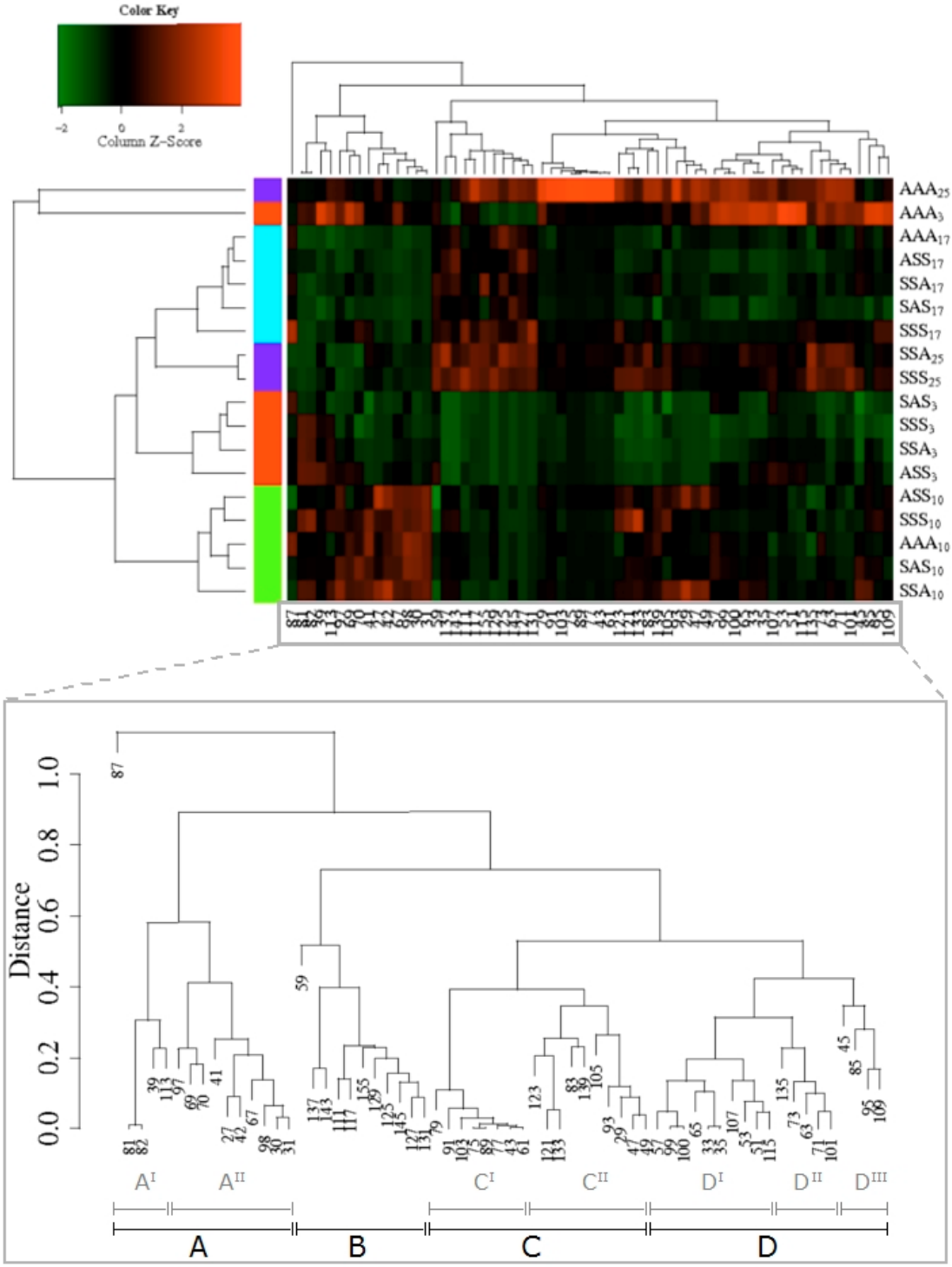


Figure 4. Heat map (HM) representation of the data resumed in Table 1. The hierarchical clustering reported on the rows groups the spectra, whilst the clustering of the spectral masses is reported on the columns. Also, to enhance the visual representation, the algorithm for HM drawing has scaled the colours for each column, i.e. each peak intensity has been individually mean-centred and scaled to unit variance across the samples. The clustering of the spectral masses is blown up to highlight the A–D clusters and sub-clusters discussed in the text. Colour bars on the left of the HM indicate the sampling dates: November 3rd (red); November 10th (green); November 17th (blue); November 25th (violet)

Taking a closer look on Figure 4, a higher level of volatiles included in the A^I cluster could be evidenced in oils obtained from olives collected on November 3rd. The levels of these VOCs remained steady on November 10th, with a slight decreasing trend, before returning to basal levels from November 17th onwards. Generally higher emissions of VOCs belonging to the entire A^{II} cluster and, to a lesser extent, to the C^{II} cluster, were evident in November 10th samples before decreasing to basal levels on November 17th, suggesting a transient up-regulation for these VOCs. Interestingly, augmented levels of masses corresponding to the B group were evidenced on samples collected on November 17th with a steady increase up to November 25th. Finally, masses included in the C^{II} and D^{II} clusters showed increased levels in samples of November 25th.

These data taken together point out, to our knowledge for the first time, that some clusters of peaks display a time-course of regulation with co-ordinated increasing or decreasing trends in association with specific stages of fruit ripening. The analysis also confirmed the result, already pointed out by PCA, that the ripening stage is prevalent over the extraction procedure on defining the global profile of VOCs of the EVOOs. Despite the discussed limited number of samples in our dataset, this allows *de facto* to consider in the HM analysis the existence of four measurement replica for each ripening stage, represented by the four independent measurements on EVOOs obtained by different extraction procedures. Remarkably, masses included in the clusters associated with early ripening stages (A^I), can be generally identified with grassy-green flavours: m81, m82 (*cis*-3-hexenal) and m87 (3-pentanone, typical determinant of sweet-green aromas; see Table 5). Unfortunately, masses included in the A^{II} sub-group could not be identified unequivocally as specific molecules. The 11 masses between m59 and m131, clustering in group B, related to late ripening events, appeared to be all over 111 amu with the exception of m59. In this group, some molecules have been identified: m59 (acetone, which contributes to negative sensory attributes in olive oils), m137 (it can be referred to a fragment of terpenes), m129 (2-octanone, responsible for mould and green notes; see Table 5). In the C^{II} group, m47 (ethanol) and m83 (hexanal) [30] are associated with floral, green, grassy notes [8]. Within the group of masses included between m135 and m101 (D^{II}), present at high levels in November 25th oils obtained from fully ripe olives, m63 and m73 were identifiable. We

already pointed out that the former is associated with negative attributes of wet earth/organic, the latter can give ethereal and fruity notes.

When the effect of olive oils extraction in the presence of nitrogen is considered, major changes in the VOCs profiles took place only in samples obtained from early (November 3rd) and late (November 25th) harvesting dates. From the HM representation it is evident that AAA_03 and AAA_25 samples appeared different from all other samples in that they shared a comparable increasing trend of sub-groups D^{I} and D^{II}. The actual separation between these two samples and the remaining ones should, however, be verified on a new independent EVOO sample set. This would in case allow confirming, along with the up-regulation of masses included in the D^{I} and D^{II} sub-groups, a concomitant significant increase of molecules belonging to the entire cluster C, observed in AAA_25 olive oil, that appeared dramatic for masses included in whole sub-cluster C^{I}, present at negligible levels in all the other samples. Among the eight masses included in the sub-cluster C^{I}, five (m103, m75, m89, m43, m61) are usually referred to acetate, butyrate and propionate esters. These volatiles are overall associated to several aspects of olive oil aroma conferring ester, green, pungent, sweet, and fruity attributes [5, 8, 29, 30]. Therefore, the combination of anoxic oil extraction conditions and over-ripe olive status would enhance esters biosynthesis. Esters are produced through the activity of AAT, an enzyme that has been reported to be up-regulated by ethylene in apple [49]. These data suggest that a similar pathway may be enhanced by anoxia, most probably in olive pastes during malaxation.

Most of the masses, which PCA has pointed out as being related to differences among some of the detected spectra groups, also belonged to those clusters (Figure 4), able to illustrate the onset and progression of ripening. In the HM, the cluster memberships of masses having positive or negative loadings on a certain PC could in some cases provide a better explanation of the role of those masses in driving the separation among groups of spectra, as described by the score plots. For example, as previously discussed, m81, m82, m39, and m113 of the A^{I} cluster displayed higher level of intensity in oil samples obtained from olives harvested on November 3rd and 10th. These masses also showed high negative loadings on PC2, which have been associated with the grouping of those two harvesting dates mainly on the negative side of PC2 axis. The masses in A^{II} and some masses in C^{II} clusters showed a transient up-regulation on day 10th and, in fact, the loading with negative

values on PC3, describing the separation of November 10^{th} on the negative side of PC3 axis, belonged to the same masses. Interestingly, only masses m69 and m70 of A^{II} sub-group have shown positive loadings on PC3 and, in fact, they were not up-regulated in the whole November 10^{th} group. Masses m137 and m143, having high negative loadings on PC3 but not belonging to A^{II} or C^{II} sub-groups, clearly showed on HM peculiar intensity values (very close to the corresponding average values) that surely did not directly characterize the ripening progression but allowed to distinguish November 10^{th} from all the other harvesting dates. Similar considerations on all other spectra groups may be drawn by cross-comparing loading plots, score plots and HM peak clusters.

Conclusions

As concluding remarks, in our research we have compared for the first time the global profiles of VOCs emissions from EVOOs obtained from olives at four gradually progressive stages of ripening by sampling at closely successive time points separated by a maximum gap of a week, spanning a period of four weeks across the *veraison* stage, and taking simultaneously into account the effects of extractions in different oxygen regimes at different steps of olive oil processing. Multivariate analyses of VOC emissions (PCA and HM) have provided a preliminary demonstration in this dataset that: (1) the ripening stage is prevalent over the extraction procedure in defining the global aroma of *cv* Grignano EVOOs; (2) the time-course of the global profile of VOCs suggests a saturation effect in parallel with the progression of olive fruit ripening; and (3) different groups of VOCs are biosynthesised in a coordinated manner in coincidence with specific stages of fruit ripening, some of them being "expressed" only transiently and then returning to basal levels already within a period of a few days. Even though a further assessment of these results on a larger independent dataset should be in future considered, these data taken together suggest that the determinants of olive oil aroma are highly dynamic, as it should be expected, significantly influenced by the ripening stage of olives, and transiently present depending on the cultivar being considered. As a consequence, studies focusing on EVOOs aroma development in response to different processing procedures must take careful consideration of the ripening stage of the olive fruits used. Current work in our laboratory is in progress towards the elucidation of the physiological and molecular mechanisms underpinning these aspects.

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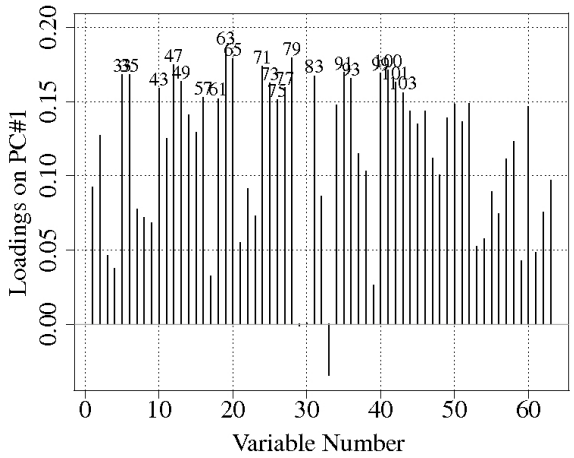
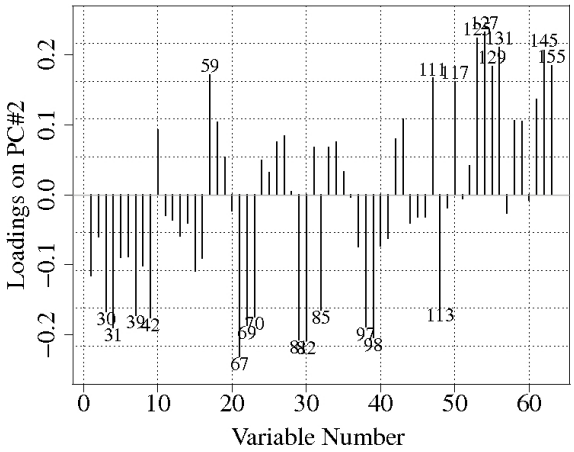
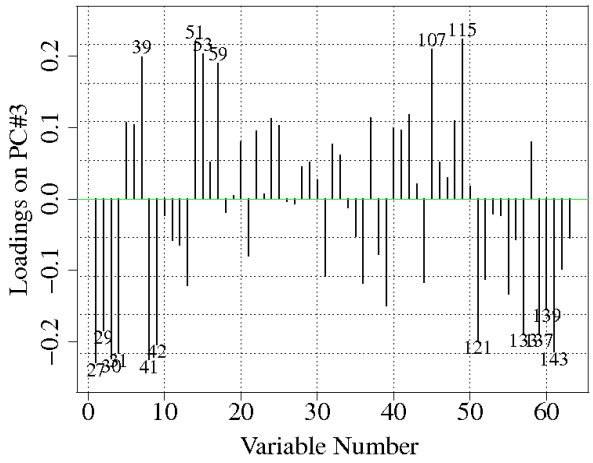
Supplementary figures



Supplementary Figure S1. Loading plot for PC1 from PCA carried out on the PTR-MS set of spectra outlined in Table 2. For visibility, only the labels of masses having positive loadings higher than 0.15 are reported in the plot.



Supplementary Figure S2. Loading plot for PC2 from PCA carried out on the PTR-MS set of spectra outlined in Table 3. For visibility, only the labels of masses having loadings in absolute value higher than 0.15 are reported in the plot.



Supplementary Figure S3. Loading plot for PC3 from PCA carried out on the PTR-MS set of spectra outlined in Table 4. For visibility, only the labels of masses having loadings in absolute value higher than 0.15 are reported in the plot.

Chapter III

Preliminary analysis on destructive and non-destructive ripening-related parameters in olive (*Olea europaea*) fruits in Veneto

Introduction

Olive oil quality is influenced by many factors, among which fruit ripening has a major effect and must be taken carefully into consideration. The choice of the best harvesting period is, besides technical factors adopted during milling, a crucial step to obtain olive oil of the best quality. This poses the need for a deep knowledge of the olive ripening process, since the optimal harvesting period represents a careful balance between positive features (that are desirable in olive oil) and negative characteristics that may arise from olives harvested at a late picking period. Moreover, in some areas such as Northern Italy, which are considered the northern limit for olive cultivation, olive growers need to harvest olives earlier than in warmer zones, due to the risk of frosts, and therefore in these areas a thorough knowledge of the ripening process is necessary in order to ensure the best compromise between the maximum yield and good quality oils.

Olive fruits undergo many changes during the ripening process. These changes involve modifications in pigment profile, fruit firmness, oil content, activity of enzymes involved in the biosynthesis of flavour compounds and phenolics (Dag *et al.*, 2011). Therefore, many of these parameters have been used in previous works to design indices that can be suitable for the evaluation of the best ripening stage for harvest. Among these indices, Uceda and Frías (1975) proposed an index, still widely used, which takes into account the number of olives belonging to seven different colour classes to yield a number that can be used to "mark" a specific ripening stage. However, this index needs to be validated in every specific growing area, and is not therefore suitable everywhere. Colour changes in ripening olives have been studied by many authors, such as Roca *et al.* (2001), resulting in a decreasing trend in chlorophylls and carotenoids concentration as a consequence of olive fruit ripening. Yousfi *et al.* (2006) used a colour index, obtained by mathematical calculations starting from spectrophotometric data, to monitor olive fruit degreening during ripening. Loss of fruit firmness, a phenomenon that occurs in ripening fleshy fruits due to structural changes in cell wall polysaccharides, has been evaluated throughout olive ripening by some authors, such as García *et al.* (1996), showing a ripening-dependent trend. Many of these studies anyway pointed out that variety has a strong effect on the global ripening profile of olive fruits, and also that the growing season can influence some

ripening-related parameters. Therefore, studies that help define the best harvest period must be performed taking into account differences that can occur between different cultivars and must be carried out in every growing area, especially in marginal areas for olive growing such as Northern Italy that may have unfavourable climatic conditions but are anyway acknowledged for producing top quality olive oils, e.g. the areas surrounding Garda Lake, in Veneto region.

In this study an evaluation of physiological parameters in ripening olives has been performed in samples picked weekly from Frantoio and Grignano cultivars in two seasons, 2009 and 2010, in Verona province (Veneto region, Northern Italy). Fruit firmness, oil accumulation, chlorophyll *a* content and the colorimetric profile have been evaluated and statistical analyses have been carried out in order to understand what variables are more important in the definition of changes that occur in ripening olive drupes. Gene expression analyses were also performed on olive samples with the purpose of evaluating changes in transcript accumulation of genes encoding enzymes that have an important role in the definition of olive oil quality. These genes are lipoxygenase (LOX) and hydroperoxide lyase (HPL), two enzymes participating in the so-called lipoxygenase pathway, which is involved in the synthesis of the major volatile compounds responsible for olive oil flavour (Sánchez and Harwood, 2002), and microsomal oleate desaturase (FAD), responsible for the synthesis of linoleic acid (Hernández *et al.*, 2009).

Materials and methods

Plant material

Olive fruits (*Olea europaea* L.) of cultivars Frantoio and Grignano were picked in an orchard at Mezzane di Sotto (Verona, Italy) during the 2009 and 2010 seasons according to the sampling calendar shown in Table 1. Sampling began when the pit hardening process was completed and went on until olives were overripe. Only healthy fruits were collected and used for subsequent analyses.

	Season	
	2009	2010
Sampling dates	02-sep	20-sep
	14-sep	27-sep
	21-sep	04-oct
	28-sep	11-oct
	05-oct	18-oct
	12-oct	25-oct
	19-oct	02-nov
	26-oct	08-nov
	02-nov	15-nov
	09-nov	22-nov

Table 1. Overview of olive fruits' sampling in 2009 and 2010 seasons.

The two cultivars were chosen on the basis of their peculiar features: cv. Frantoio is well spread throughout all olive oil producing Italian regions and abroad, while cv. Grignano, typically grown in a limited area around Verona province, has recently re-gained interest for the highly appreciated sensory attributes of its oil.

Fruit firmness measurement

Fruit firmness measurements were performed by means of a hand digital penetrometer (UKCA, Fairlight, UK). Groups of 20 olives per sampling date per cultivar were used for firmness determination, puncturing the fruits in the equatorial zone on two opposite sides with a 2 mm diameter disk. Results were expressed in newtons (N).

Chlorophyll *a* (Chl *a*) extraction and quantification

Chlorophyll *a* concentration was determined in olives picked during 2009 season as described by Moran and Porath (1980), with slight modifications. Briefly, extraction of total chlorophylls was performed by incubating 200 mg of olive flesh in *N*,*N*-dimethylformamide (Sigma-Aldrich, Milano, Italy), using a 1:10 volume/weight ratio, for 24 hours at –20°C. The liquid phase was then filtered and absorbance values were obtained at 664.5 and 647 nm by means of a spectrophotometer (Thermo Scientific, Milano, Italy) in 1 cm plastic cuvettes. Chl *a* concentration in samples was determined by means of the equation calculated by Inskeep and Bloom (1985): Chl $a = 12.70*A_{664.5} - 2.79*A_{647}$ and then expressed as mg/g fresh tissue. Analyses were performed in triplicate.

Oil accumulation determination

Oil accumulation percentage was determined in triplicate for olives picked in 2009 (only seven harvest dates starting from September 21st and ending up on November 2nd) and 2010 seasons (all sampling dates) from olive paste by means of hexane extraction in a Soxtec apparatus (Soxtec System HT 6, Tecator, Saskatoon, Canada). Briefly, 5 g of olive paste were added with 5 g anhydrous sodium sulfate (Na_2SO_4) and oven dried at 105°C for 60 min. Samples were then weighted again so that both fresh and dry weight were recorded and put in thimbles that were dipped in petroleum ether at 40-60°C for 30 min and then washed for 60 min. Then, solvent was recovered and the oil in the thimbles was weighted to calculate fresh/dry weight-based oil content.

Colorimetric analysis

Colorimetric analyses were performed on the same drupes used for firmness measurement with a portable spectrophotometer (Minolta CM-2500d) both for 2009 and 2010 samples. Colour space of olive fruits was determined by capturing the CIE $L^*a^*b^*$ coordinates of the basal zone of the drupe with an 8 mm target mask, a viewing angle of 10° and the standard illuminant D65. L^* represents the lightness, a^* is the position between magenta and green whilst b^* is the position between yellow and blue. C^*, the chroma, and H, the hue, were also calculated. These parameters were used to calculate colour indices retrieved in literature and previously used for fruit colour spectra determination for evaluation of quality and ripening progression. Formulae of the indices that were used for this work are shown in Table 2.

Color index	Description	Formula	Reference
CI	Colour Index (olives)	$L^* (b^* - a^*) / 100$	Castellano *et al.* (1993)
CIRG	Colour Index for Red Grapes	$(180 - H) / (L^* + C)$	Carreño *et al.* (1995)
CIRG2	Colour Index for Red Grapes 2	$(180 - H) / (L^* \times C)$	Rolle *et al.* (2011)
CIRWG	Colour Index for Red Wine Grapes	$ARCTAN (b^* / a^*) / (L^* \times b^*) \times 100$	Rolle and Guidoni (2007)
CCI	Citrus Colour Index	$(1000 \times a^*) / (L^* \times b^*)$	Jiménez-Cuesta *et al.* (1981)
COL	Tomato Colour Index	$(2000 \times a^*) / (L^* \times C)$	Hobson (1987)

Table 2. Formulae for colour indices found in literature and used to evaluate quality or the ripening stage of olive fruits.

Multivariate Data Analysis

Principal Component Analysis (PCA; Jolliffe, 1986) was applied on oil accumulation and colour indices data using the available statistical package *stats* of the R software environment (R Development Core Team, 2009).

Ripening related genes expression analyses

Olives from season 2009 were then pitted and mesocarp used for RNA extraction according to the method described in Conde *et al.* (2007). DNAse treatment and subsequent cDNA synthesis were performed as reported by Nonis *et al.* (2007). A thorough search of the literature for genes that show a clear ripening-related trend in olive drupe was carried out, and four of them were chosen and subsequently used for gene expression analyses and are listed in Table 3 together with primer sequences used by the authors.

Gene name	Description	Primer sequences	Reference
1LOX2	Lipoxygenase 2	FW - GAGAATTGGGTGCGTTCATAC REV - TCCTCTGGTGTGGCTAATGTC	Padilla *et al.* (2009)
2LOX2	Lipoxygenase 2	FW - TCGCTGGGAAAGTGAAAGAG REV - TCAAATGGAAACGCTGTTAGG	Padilla *et al.* (2009)
HPL	Hydroperoxide lyase	FW - TCTCATACCCTTTCTGGCTTG REV - ATCGTAAACCCACCAAATGC	Padilla *et al.* (2010)
FAD2-2	Microsomal oleate desaturase	FW - CTTGTGGGCTTTACCGTCTC REV - AGGGAGGGATGTGTATGCTG	Hernández *et al.* (2009)

Table 3. Ripening-related genes used in this study for a better characterisation of the overall ripening profile of 2009 olive samples. Primers used by the authors are given (FW: forward primer; REV: reverse primer, 5'-3' direction).

Quantitative real-time PCR (qPCR) was performed as described by Nonis *et al.* (2007) on a StepOnePlus instrument (Applied Biosystems, Foster City, CA, USA). Total reaction volume was 10 µL: 2.5 µL cDNA, deriving from 1 ng/µL of theoretically retrotranscribed RNA, 0.2 µL forward and reverse primers (10 µM each), 5 µL Power SYBR® Green PCR Master Mix (Applied Biosystems) and 2.1 µL RNAse-free water. Experiments were performed in triplicate on three independent biological replicas. The thermal cycling conditions were as follows: denaturation step at 95° C for 10 min, and 40 cycles (95° C 15 s, 60° C 1 min). The absence of multiple products or primer dimers was checked by means of a melting curve analysis. Assays included no-template controls and normalisation to polyubiquitin OUB2 (accession no. AF429430; forward primer sequence GCTGGAGGATGGAAGGACTC, reverse primer sequence CCACGACTCAACAGAGACGA) was performed. StepOne Software ver. 2.1 (Applied Biosystems) was used for Cq (quantification cycle) data export, and data were subsequently analysed according to Livak and Schmittgen (2001).

Results and discussion

Destructive analyses on ripening olives

Some physiological parameters that show an evolution during the olive ripening process have been evaluated in this study in the cultivars Frantoio and Grignano grown in Veneto region, Italy.

Fruit firmness. Firmness in olive fruits decreases with the progression of ripening, as expected for fleshy fruits. The loss of firmness profile is genetically determined and therefore changes depend on cultivar, as shown for example for Spanish olive varieties by García *et al.*, 1996, and for fleshy fruits in general. The same authors have evaluated the potential of olive fruit firmness as a ripening marker. However, whilst García *et al.* (1996) reported the gradual softening of five Spanish olive cultivars in four consecutive ripening stages (green, spotted, purple, and black), Yousfi *et al.* (2006) stated that fruit firmness can be used to discriminate olives picked at early ripening stages only. We have measured the decrease of firmness on Frantoio and Grignano olives with respect to fruit ripening and the data obtained are shown in Figure 1.

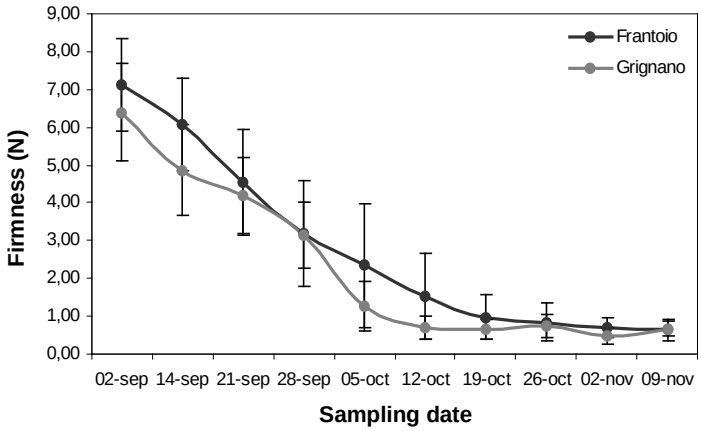


Figure 1. Fruit firmness decrease (N) of drupes picked at ten successive ripening stages during the 2009 season. Bars represent the standard deviation (SD; N = 40).

Firmness values for both cultivars were similar in the first and last sampling date, while some differences could be observed in the overall trend of firmness loss. Frantoio olives

showed a gradual decrease, with a hyperbola-shaped curve, starting already from early stages, when olives were still green, and proceeding until October 19th. After this date fruit firmness values remained stable. For what concerns Grignano olives, the overall loss of firmness appeared faster, to some extent, even if without evidencing significant differences in comparison with Frantoio, until October 12th, coinciding with the onset of ripening (*véraison*). After *véraison*, firmness values became stable, two weeks earlier than for cultivar Frantoio.

As a concluding remark, fruit firmness seemed in this study unsuitable for the discrimination between olives at successive ripening stages after the *véraison* stage, since both Frantoio and Grignano olives showed only slight differences in firmness from that ripening stage onwards. In fact, the gradual decrease in firmness from green to black olives observed by García *et al.* (1996) was not noticed for these two Italian cultivars that, on the contrary, confirmed the data reported by Yousfi *et al.* (2006).

Chlorophyll a content. The chlorophyll *a* profile of olives picked in 2009 season was determined for all the ten sampling dates and expressed as mg/g fresh weight, as shown in Figure 2.

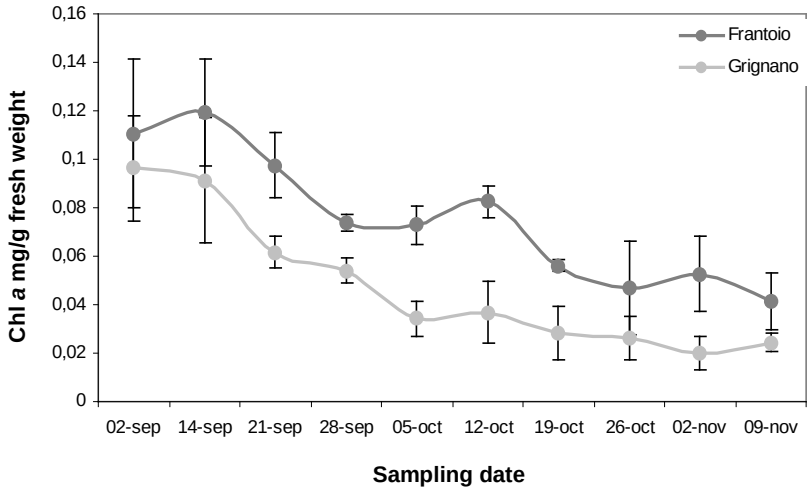


Figure 2. Evolution of Chl *a* content in cvs. Frantoio and Grignano samples picked at ten consecutive ripening stages during 2009 season. Bars represent the standard deviation.

Notwithstanding chloroplastic pigments in olive fruits are made up of different components, among which chlorophyll *a* and *b*, and many compounds deriving from the

catabolism of these pigments (such as pheophytins and chlorophyllide), it has been demonstrated that the most significant changes in pigment concentration with respect to fruit ripening occur in Chl *a*, since Chl *a* alone may account for up to 80% of the total chlorophyll content in olive fruits (Roca *et al.*, 2001). Figure 2 shows that Chl *a* content of ripening Frantoio and Grignano olives was similar in the first sampling date, decreasing in parallel in the two cultivars along with the progression of ripening, even if Grignano samples showed a generally lower Chl *a* content. As for the softening process also the chlorophyll profile is strongly dependent on variety (Roca *et al.*, 2001), together with the overall evolution profile of pigments throughout ripening. Different olive cultivars, as pointed out by Roca *et al.*, show a different slope in chlorophyll decrease and chlorophyll retention, and the appearance of anthocyanins associated with the pigmentation of ripening fruits can be observed as a sharp change in the chlorophyll retention slope. In Frantoio and Grignano samples the decrease of Chl *a* content appeared quite gradual with respect to the ripening of drupes, and therefore it can not be considered a suitable ripening marker since no sharp changes were observed when olive fruit changed skin colour.

Oil accumulation. Oil content in olive fruits is a key factor for olive growers, since it determines the final yield. In past times it was a common practice to leave olive fruits as long as possible on the tree in order to reach a higher oil yield. However, while improving yield, this practice may have major negative effects on the quality of olive oil due to the physiological deterioration events that occur in overripe olives (García *et al.*, 1996). García *et al.* (1996) reported that no changes in oil content was observed in Villalonga, Verdial, Lechín, Blanqueta, and Arbequina cultivars in four consecutive ripening stages (green, spotted, purple, black), suggesting that oil biosynthesis in olive fruits reach a maximum at the “green” stage and therefore from that moment on oil yield is constant. For this reason milling black olives is not advisable since oil content is not higher and oil quality can be negatively affected. Oil accumulation profiles throughout ripening are anyway dependent on fruit load, growing season characteristics and, above all, variety (Dag *et al.*, 2011). In this study, the evaluation of oil accumulation in ripening olives of Frantoio and Grignano cultivars in two consecutive seasons (2009 and 2010) confirmed these findings only partially (Figure 3).

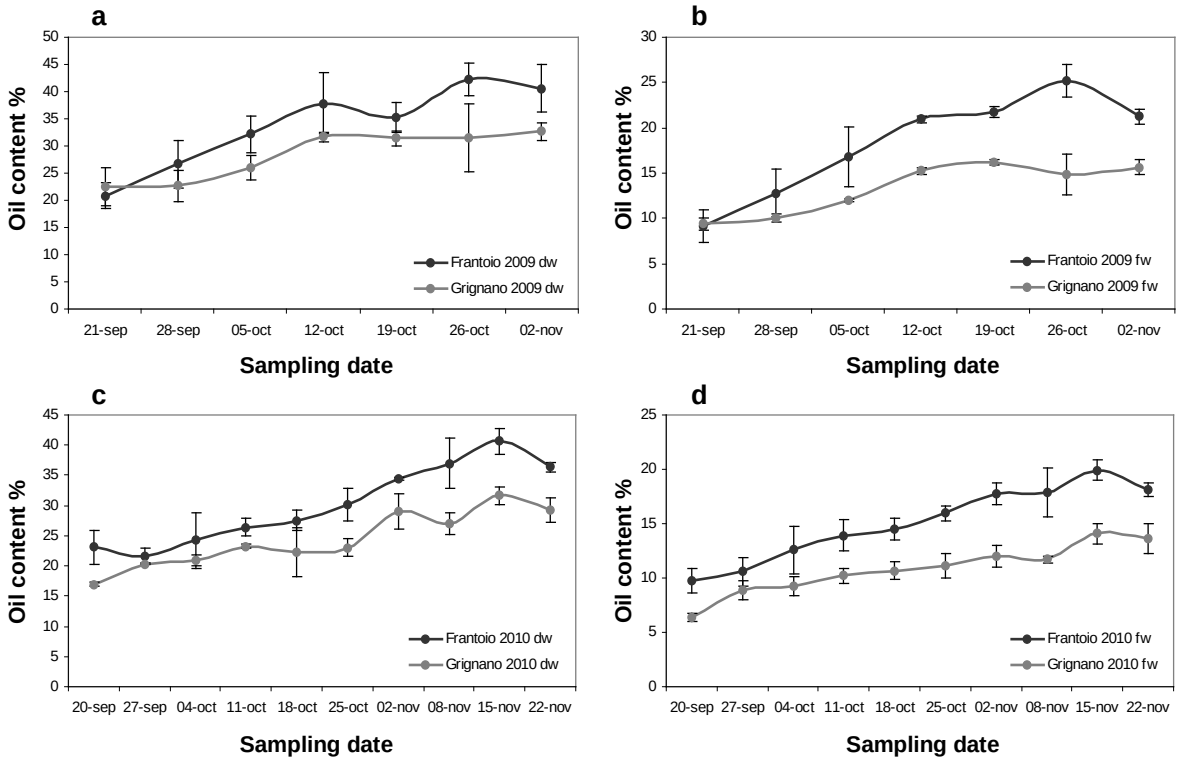


Figure 3. Oil accumulation in Frantoio and Grignano olives for season 2009 (**a**, expressed on dry weight; **b**, expressed on fresh weight) and season 2010 (**c**, expressed on dry weight; **d**, expressed on fresh weight). Bars represent the standard deviation.

In fact, in 2009 season oil content increased in both cultivars until a week after *véraison*, reaching its maximum levels with a rapid trend and remaining almost constant at later stages. This appeared evident mainly for cv. Grignano, for which no changes in oil accumulation could be observed after October 12th. In this case, harvesting olives in close proximity to *véraison* could ensure the best compromise between oil content and the absence of negative attributes due to overripeness. For what concerns 2010 season, the oil accumulation trend resulted to be different, since oil content increased gradually and more slowly until the end of olive ripening. These differences were mainly due to a different amount of water in the analysed samples, since water content sharply decreased in 2009 samples until October 19th, and then remained almost constant, whilst in 2010 water content showed a slow decline but generally had higher values with respect to 2009 season (data not shown). It can also be noticed that oil content in 2009 season was generally higher than oil content in 2010. Therefore, the general assumption that oil accumulation may stop when

olives are green or spotted is not valid for all varieties and, most importantly, for the same varieties in different growing seasons due to the evident season dependent changes in the oil accumulation pattern. As a consequence, a more detailed analysis of physiological parameters needs to be carried out with the help of statistical tools to evaluate the ripening progression of olives with regard to oil accumulation.

Colorimetric data

Colorimetric parameters obtained by means of spectrophotometric analyses were processed calculating colour indices found in literature. More in detail, CI (Colour Index) has been proposed by Castellano *et al.* (1993) as a tool to evaluate changes in Picual olives stored under controlled atmospheres, and has been claimed by the authors to be useful in the discrimination between green and purple/black olives. CIRG (Colour Index for Red Grapes) has been conceived by Carreño *et al.* (1995) and has shown a good linearity with berry colour and a good ability to discriminate berries. CIRG2 (Colour Index for Red Grapes 2) and CIRWG (Colour Index for Red Wine Grape) have been successfully used in grapes (Rolle and Guidoni, 2007; Rolle *et al.*, 2011), whilst CCI (Citrus Colour Index) has been proposed by Jiménez-Cuesta *et al.* (1981) for citrus fruit, and has been successfully used to study the chromatic evolution of clementines treated with Ethephon to enhance fruit colour (Pons *et al.*, 1992). COL index, proposed by Hobson in tomato (1987), has been used to evaluate the effects of cold storage on tomato colour. These indices were chosen even if some of them, such as CCI and COL, have been conceived for fruits that have a different pigment profile in comparison to ripening olives. Pigment changes throughout olive ripening have been evaluated by means of statistical analyses, similarly to oil accumulation data, performing a Principal Component Analysis.

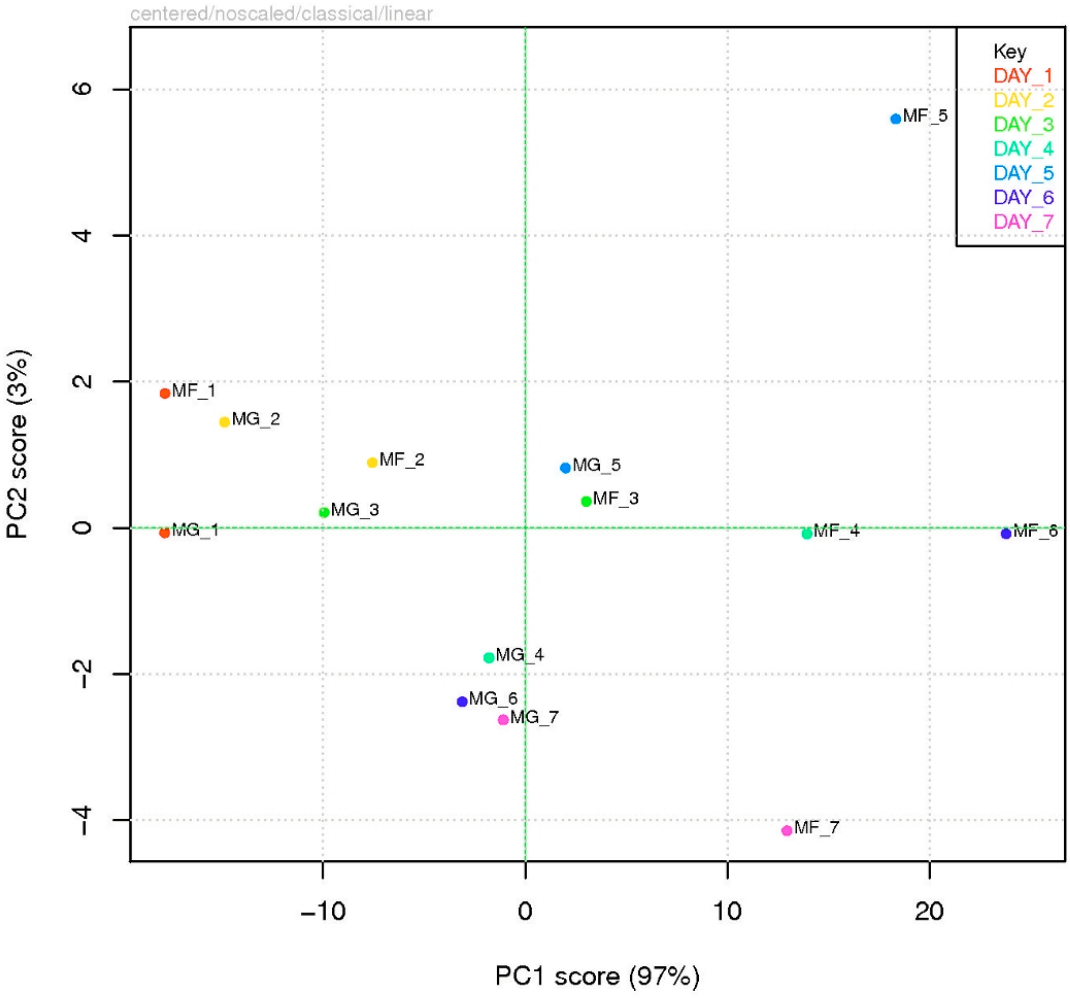
Principal component analysis

PCA is a powerful technique frequently used in biological sciences, in which multivariate data analysis is common and a simplification of the global data pattern is required. This procedure produces new Cartesian coordinates, called principal components. The first

principal component accounts for most of the variability of data. In this study, only the first two principal components were kept, as they always retained a highly significant percentage of the initial data information. For each PC score/score plot, loading plots were also designed and are shown. Loading plots can help understand the score plots' structure and represent the "weight" carried by the starting variables on the definition of dataset variability. Principal component analysis was used in this work to identify potential patterns in both oil accumulation and colorimetric data with regards to the evolution of the ripening process.

Oil accumulation data. Every olive sample considered in this analysis, marked in plots as a dot, was defined by three variables: water percentage, oil content on fresh weight (%) and oil content on dry weight (%). The PCA is therefore responsible for grouping of samples on the basis of the different "strength" of the single variable to statistically discriminate the samples.

Score plot and loading plot of PCA performed on oil accumulation data in both cultivars for season 2009 are shown in Figure 4.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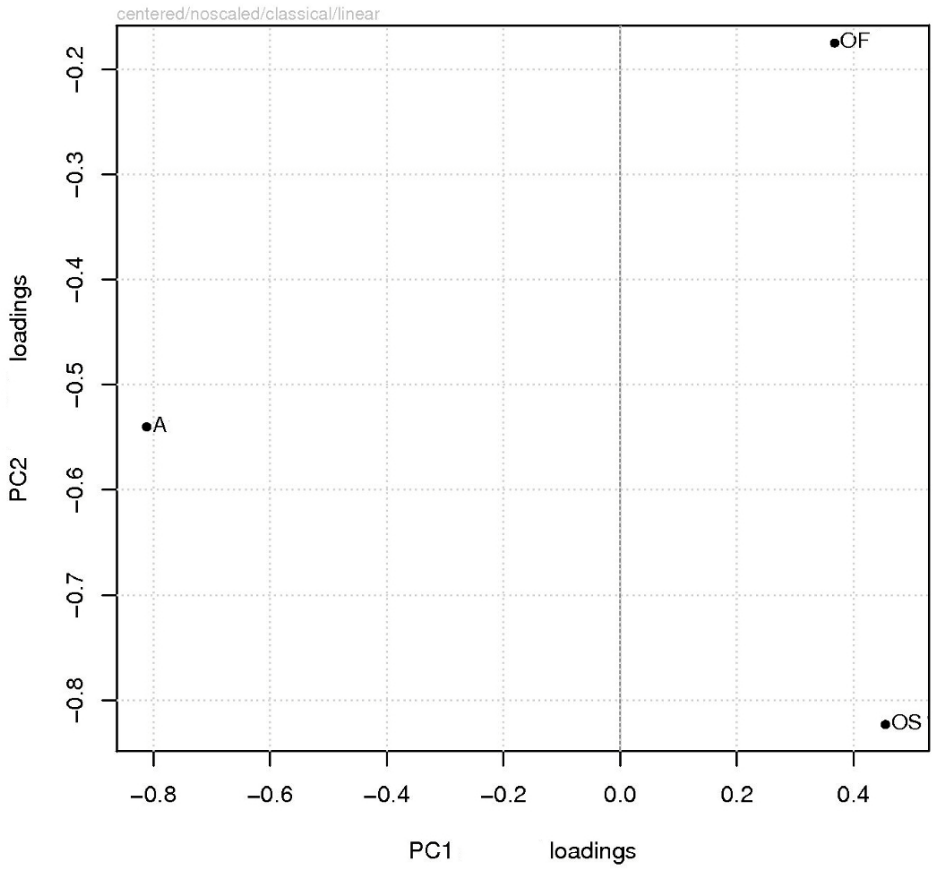


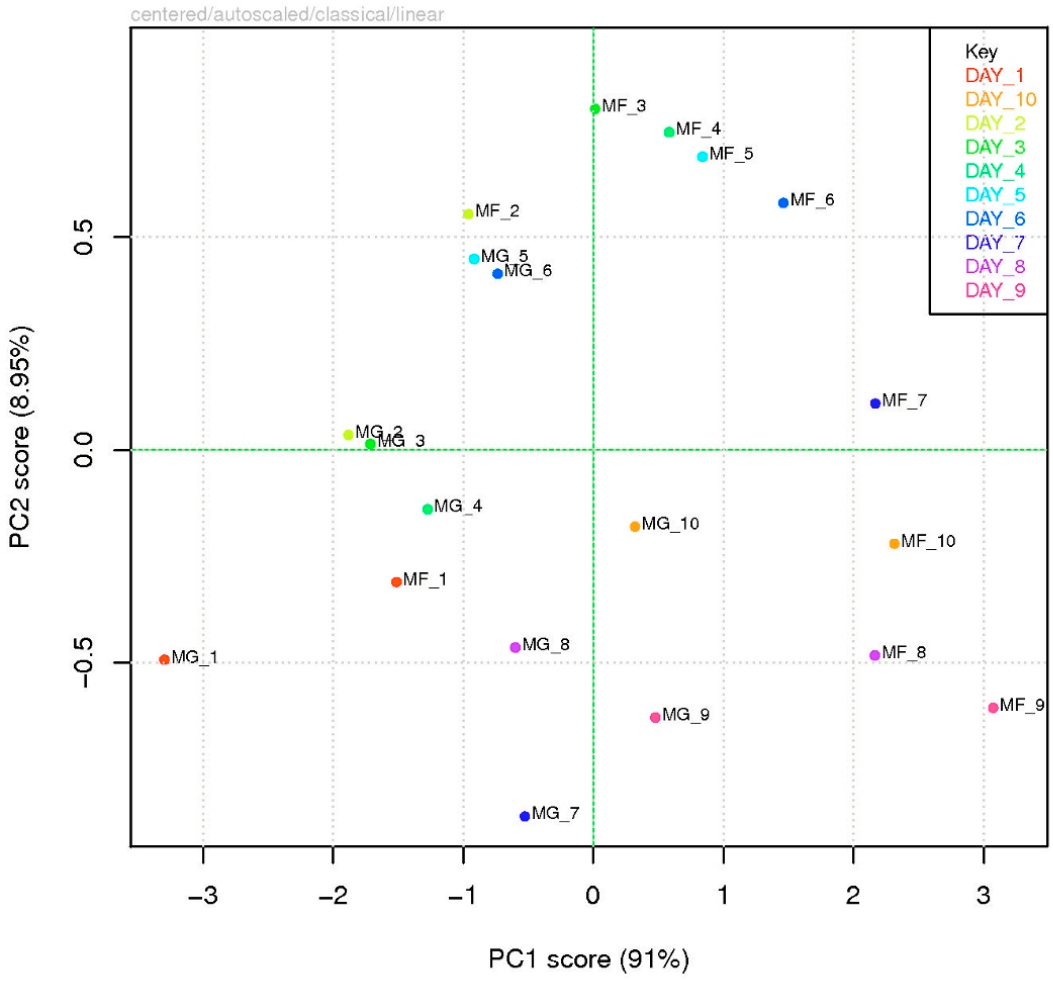
b

Figure 4. PCA score plot (a) and loading plot (b) of Frantoio and Grignano samples oil accumulation data for 2009 season, in seven weekly sampling dates from September 21st to November 2nd. MF: Frantoio samples; MG: Grignano samples; A: water content; OF: oil content on fresh weight; OS: oil content on dry weight.

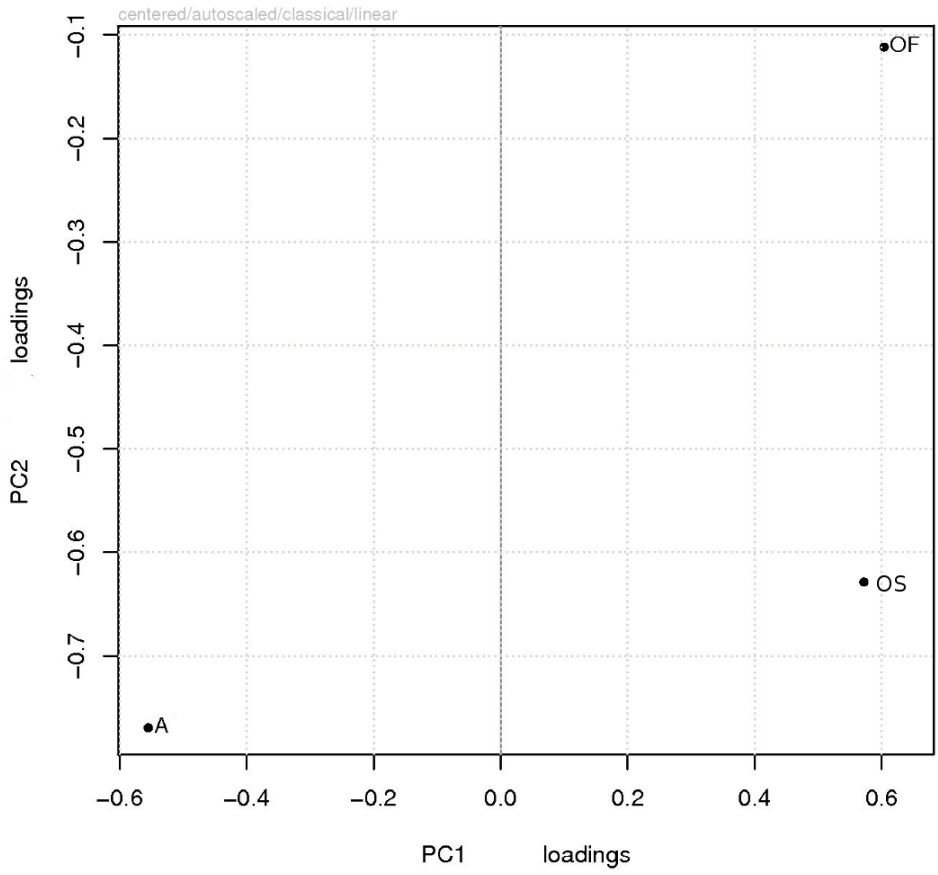
A first consideration on these graphs is the greater variability of cv. Frantoio dataset in comparison to the Grignano one. Moreover it could be noticed that the first principal component (PC1) could be used to describe the temporal distribution of oil accumulation data, and that this trend could be equally described by any of the three studied parameters, i.e. water, oil accumulation on fresh weight, and oil accumulation on dry weight as highlighted in the loading plot. The variance captured by PC2 highlighted the different behaviour of samples MF_5 and MF_7, which despite having an equal dependence on water content, were greatly influenced by a high (MF_7) and low (MF_5) oil accumulation/dry weight content (OS). Considering the two cultivars separately, it was evident that for Frantoio samples a clear temporal trend could be observed according to PC1 for the first four sampling dates, whilst for the other dates a kind of saturation effect

could be noticed since it became difficult to see the temporal evolution according to PC1. This saturation phenomenon could be ascribed to a lower variability in oil content among samples, which reflected a steady trend in oil accumulation from the fifth sampling date. This kind of saturation effect could be observed also in cv. Grignano samples, in which a temporal sequence was noticed for the first four sampling dates, suggesting an earlier saturation effect and a subsequent greater difficulty to define olive ripening progression according to the oil accumulation profile.

The greater variability in cv. Frantoio dataset was clear also for the ten sampling dates in season 2010, in which samples were more stretched than the cv. Grignano ones along PC1. This phenomenon can be observed in Figure 5.



a



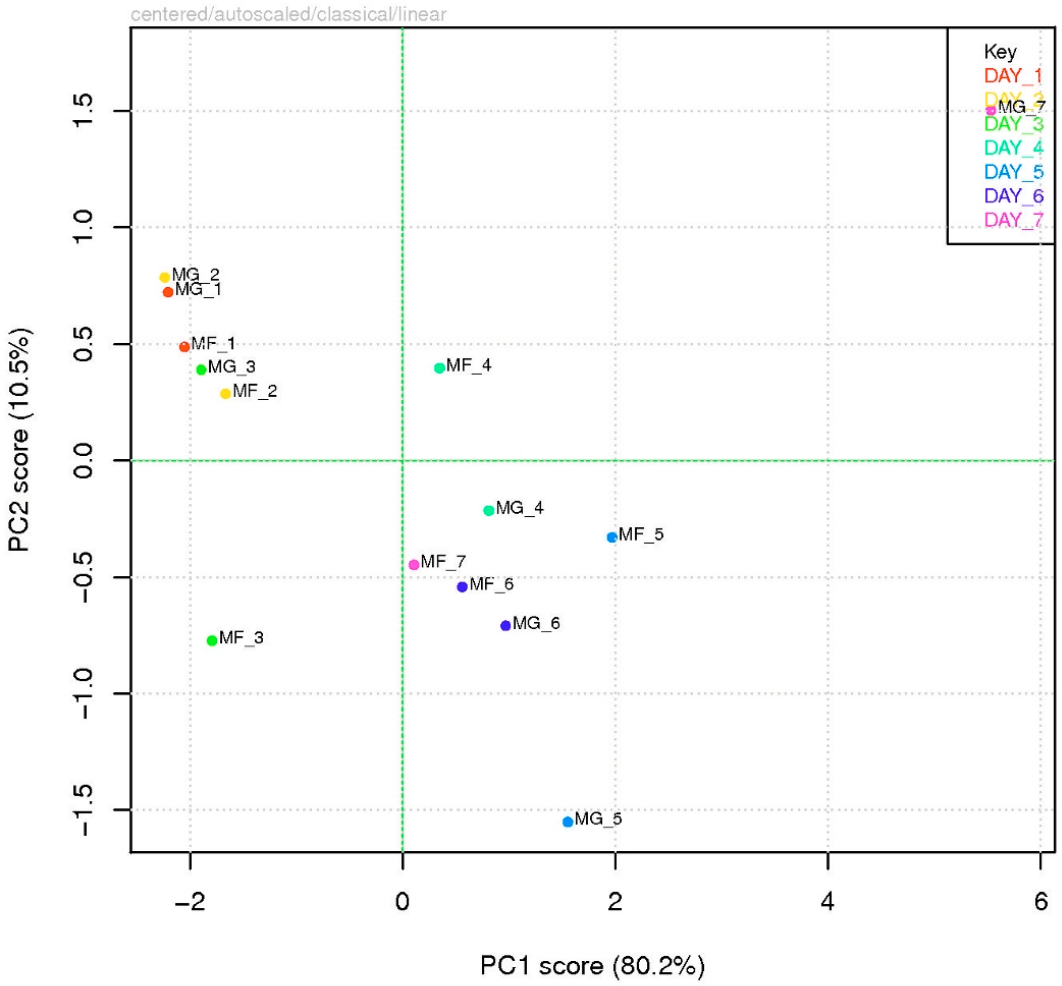
b

Figure 5. PCA score plot (a) and loading plot (b) of Frantoio and Grignano samples oil accumulation data for 2010 season, in ten weekly sampling dates. Abbreviations are as in Figure 4.

It could also be noticed that the score plot variance along PC2 was greater in 2010 than in 2009. For what concerns Frantoio samples, a clear temporal progression of samples could be observed with respect to PC1 until the sixth/seventh date in the second sampling season, similarly to what observed in 2009, then showing the already described saturation effect. The main difference between the two seasons was the already mentioned greater variance in PC2, which reflected a higher variation in oil content in fresh/dry weight. Cultivar Grignano showed, as seen in 2009 season, a more complex behaviour mainly due to the fragmentation of samples into five groups: MG_1, MG_2-4, MG_5-6, MG_7-8, and MG_9-10, where MG_10 could be included in the last group due to similar values along PC1 in comparison to MG_9. The temporal sequence of ripening olive samples could be

noticed along PC1, but only until MG_5 or MG_6 since for the following samples changes in oil accumulation were evident therefore not on a weekly basis but on a larger scale.

Colorimetric data. For what concerns colorimetric data, values calculated for the different ripening indices of the 20-olive groups were averaged out. Every sample was therefore described by six variables in the plots, corresponding to the six colour indices calculated as mentioned above, and marked as a dot in the score plots. The PCA allowed to group samples according to the different statistical weight carried by the variables, similarly to what was performed for oil accumulation analyses. In order to make possible a comparison between oil and colorimetric data, even if the latter were calculated for every sample in 2009 season, only the seven dates for which oil data were available are discussed here. Results of PCA are shown in Figure 6 (2009 season) and Figure 7 (2010 season).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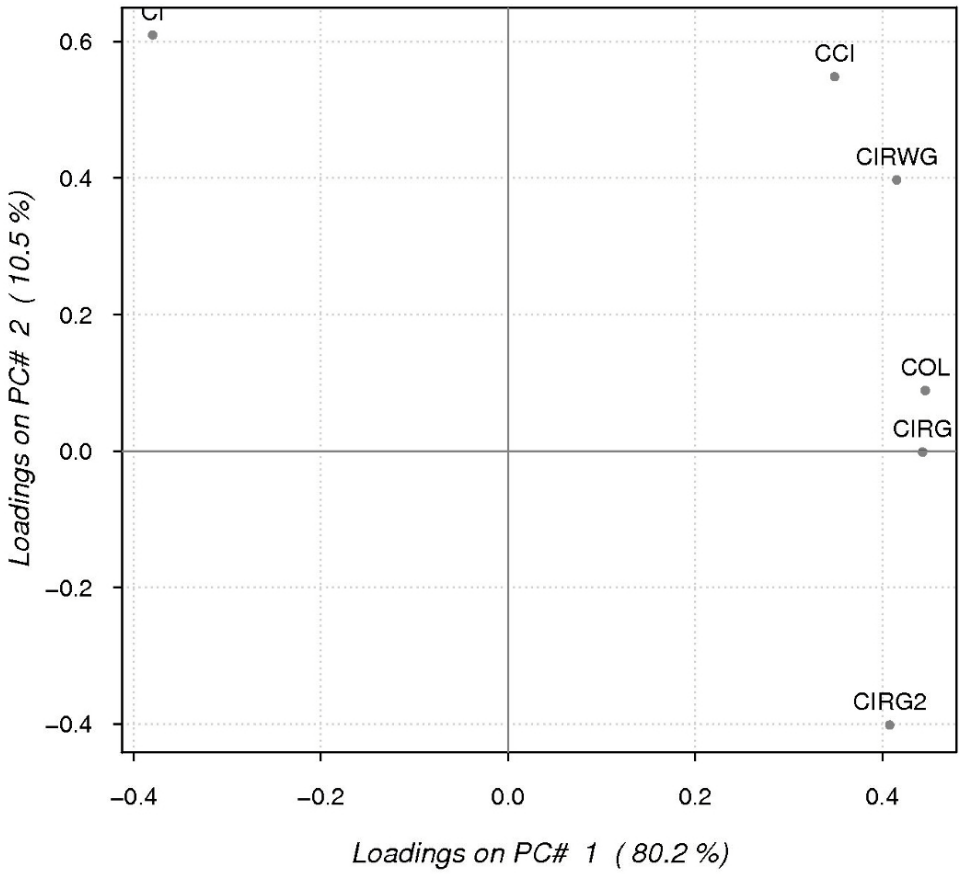
MF_6
MG_6
MF_3
MG_5
PC2 score (10.5%)
PC1 score (80.2%)
1.5
1.0
0.5
0.0
−0.5
−1.0
−1.5
−2
0
2
4
6

a



b

Figure 6. PCA score plot (a) and loading plot (b) of colorimetric data for cultivars Frantoio and Grignano picked in 2009 season. Abbreviations are as in Figure 4 and Table 2.

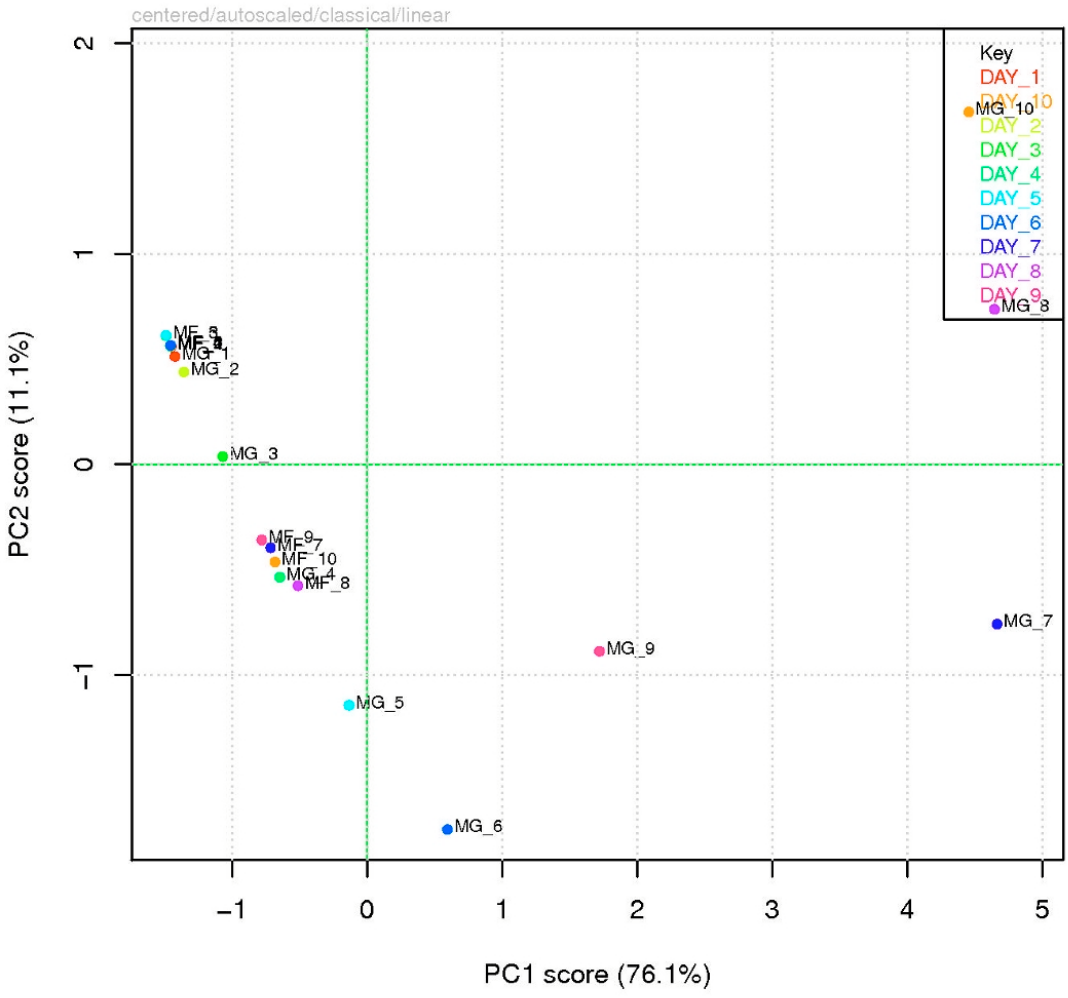
During the 2009 season both cv. Frantoio and Grignano showed a similar behaviour, since two clear groups of data could be noticed along PC1, therefore related to the major part of data variability: a first group including samples of the first three sampling weeks, which had similar colour profiles since olives were mainly green or greenish-yellowish, and a second one including the remaining samples. Considering the loading plots, it was evident that the mentioned grouping was driven mainly by CI, which had high values in the first group and low values in the second group, but also by CCI (that could discriminate MG_7 as an outlier) and CIRG2.

Cultivar Frantoio samples appeared to have a complex distribution in the score plot, as shown in Figure 6. A closer look on PC1 allowed grouping Frantoio samples in two clusters, the first one including sampling dates 1-3 (PC1 scores < 0) and the second one

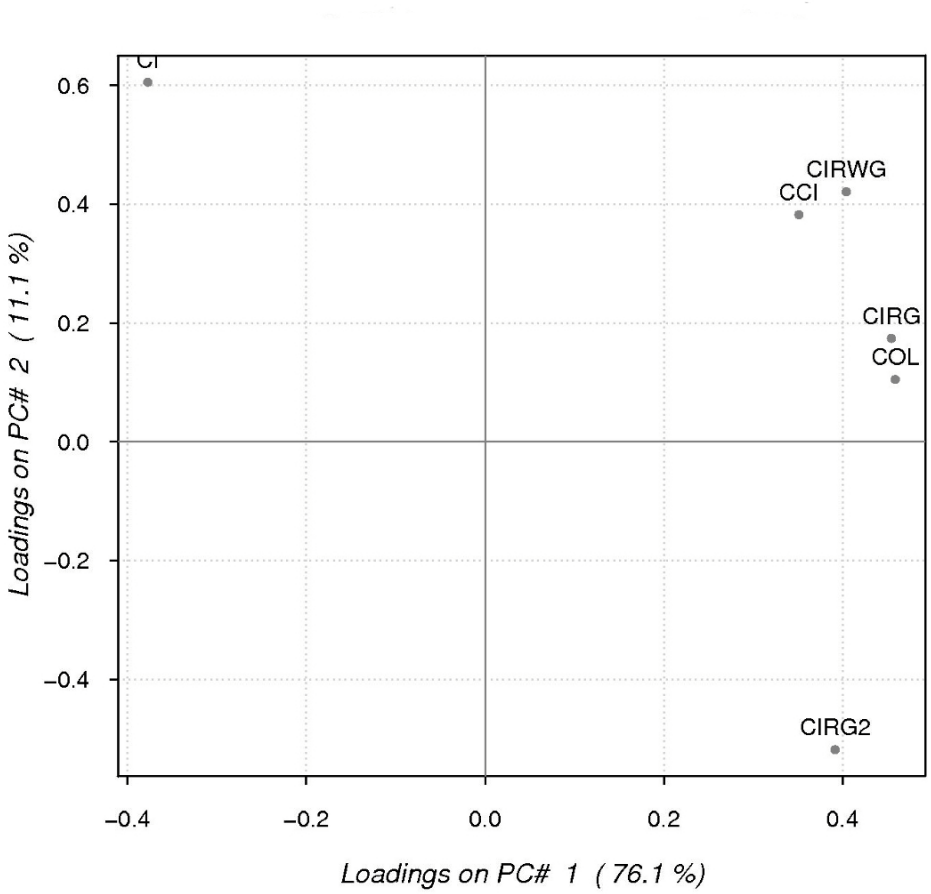
including the remaining samples (PC1 > 0). This grouping could be similarly observed also in cv. Grignano samples. For cv. Frantoio these two groups showed a temporal progression if considered separately, according to decreasing PC2 scores, with only a turnaround of sample MF_7. Looking at the loading plot, it seemed that these trends in Frantoio samples were driven mainly by changes in CI values, to a lesser degree in CCI values.

For what concerns Grignano samples, a grouping along PC1 was distinguishable, since groups MG_1-3, MG_4-6 and MG_7 were discernible. MG_7, as previously noticed, appeared to be an outlier due to higher CCI or CIRWG values, as shown in the loading plot.

It can be summarised that for both cultivars in 2009 season no saturation effect was seen in colorimetric data, CI seemed the best index for the discrimination of ripening samples, together with other indices that provided a fine tuning, and that the first two samples, i.e. green/greenish olives, were not discernible using these colour indices.


centered/autoscaled/classical/linear
Key
DAY_1
DAY_10
DAY_2
DAY_3
DAY_4
DAY_5
DAY_6
DAY_7
DAY_8
DAY_9
MG_10
MG_8
MG_1
MG_2
MG_3
MF_9
MF_7
MF_10
MG_4
MF_8
MG_7
MG_9
MG_5
MG_6
PC2 score (11.1%)
PC1 score (76.1%)
2
1
0
−1
−1
0
1
2
3
4
5

a



b

Figure 7. PCA score plot (a) and loading plot (b) of colorimetric data for cultivars Frantoio and Grignano samples, picked in 2010 season. Abbreviations are as in Figure 4 and Table 2.

Colorimetric data for 2010 season consisted of 10 sampling dates, similarly to what discussed for oil accumulation. As shown in Figure 7, the PCA score plot highlighted a greater variability in Grignano samples in comparison to Frantoio ones, that could be read along PC1. Whilst in 2009 season sample MG_7 appeared to be an outlier, it seemed clear from this plot that the whole group MG_6-10 had a different behaviour, showing PC1 scores > 0. In the first three sampling dates, dots were squeezed in the upper left quadrant and were not therefore well discernible, which implied a poor ability of any of the studied colour indices to monitor earlier ripening stages.

Frantoio samples, as pointed out before, showed a low variability in colorimetric data since all the relative dots could be found in the area of the plot described by negative PC1 scores. From sampling week 7 onward it was possible to notice some differences among the

samples, but to a lesser extent compared to Grignano samples. Grignano samples, on the other way, showed an impressing temporal progression according to PC1 and partly according also to PC2, with the exception of MG_9. The major responsible for this ripening-related trend was CI, whose decrease described samples MG_1-6 and increase described samples MG_7-8 and MG_10. However, samples picked in the last harvest dates seemed to show a saturation effect considering only CI, and CIRG2 appeared therefore to be more useful for the discrimination.

Altogether, 2010 data supported the trend seen in 2009, at least partially, since it was evident that the colour index CI was responsible for a clear grouping of samples and that a fine tuning could be provided by other indices, among which CCI and CIRG2 stood out.

Ripening-related gene expression analysis

Gene expression analysis on genes responsible for important biochemical reactions in ripening olives picked in season 2009 was performed by means of qPCR. The transcript accumulation patterns can be observed in Figure 8.

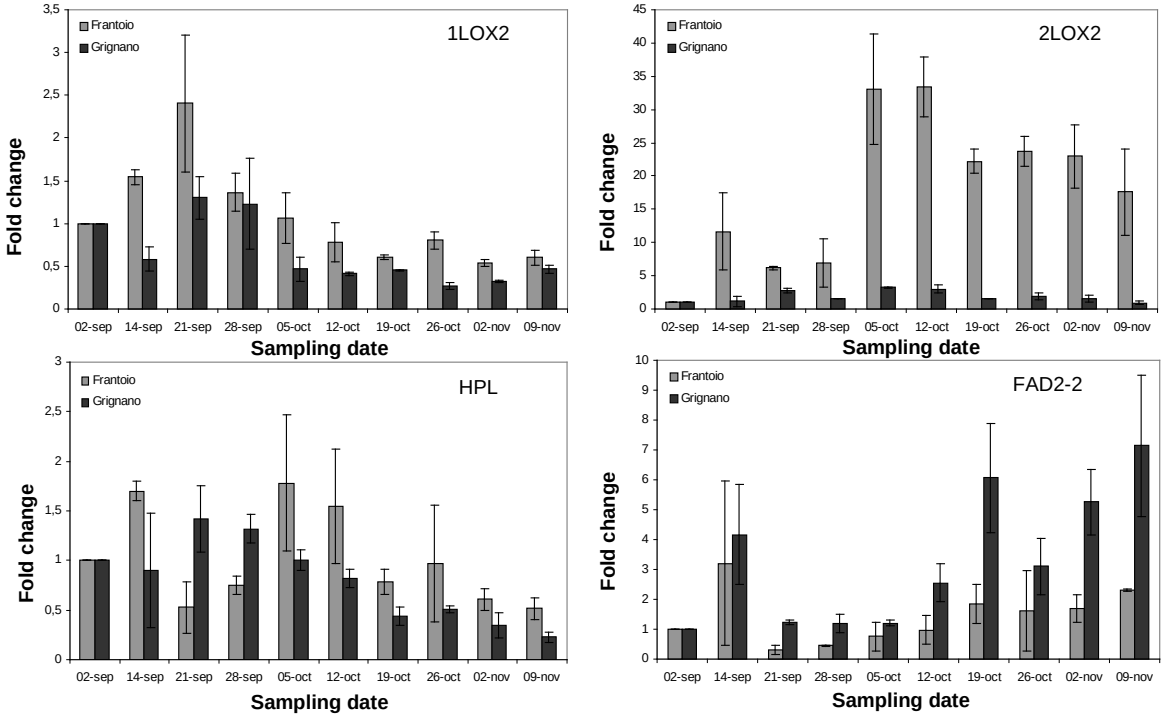


Figure 8. Fold change values in gene expression of ripening olives. 1LOX2: 1-lipoxygenase-2; 2LOX2: 2-lipoxygenase-2; HPL: hydroperoxide lyase; FAD2-2: fatty acid desaturase 2-2. Bars represent the SD.

More in detail, two components of the LOX family were chosen for their known ripening-related expression pattern, according to Padilla *et al.* (2009) who isolated these genes for the first time from Picual and Arbequina olives. 1LOX2 and 2LOX2 are chloroplastic enzymes that synthesise 13-hydroperoxide derivatives from polyunsaturated fatty acids. Padilla *et al.* noticed that whilst 1LOX2 showed no changes in gene expression with respect to fruit ripening, 2LOX2 had a clear ripening-dependent trend since a peak in transcript accumulation was observed soon after the onset of ripening. As seen in Figure 8, these patterns were found also in Frantoio and Grignano samples, where 2LOX showed a clear peak at *véraison* whilst 1LOX2 gene expression, even if a small peak was visible, remained more stable throughout ripening. Hydroperoxide lyase is the enzyme responsible for the cleavage of polyunsaturated fatty acids hydroperoxides to yield aldehydes and oxoacids. According to Padilla *et al.* (2010), HPL shares a similar trend in transcript accumulation with respect to the two isolated LOXs, showing a peak next to the onset of ripening. qPCR on Frantoio and Grignano samples confirmed this finding, with a small peak in both cultivars in *véraison* samples even if the increase in fold change was little.

Finally FAD2-2, an oleate desaturase isolated by Hernández *et al.* (2009) and defined as the major gene responsible for the linoleic acid content in olive mesocarp, showed a decrease in transcript accumulation next to the onset of ripening, and then an increase in the final stages of ripening. This pattern in gene expression was noticed also for Frantoio and Grignano samples, in which an increase in fold change values could be observed after *véraison* in comparison to green stages.

Altogether these gene expression analyses point out that some features of olive ripening process are retained among different cultivars grown in different areas, but that these characteristics are not enough to define the overall ripening process since many parameters were demonstrated to have a clear variety-dependent behaviour.

Conclusions

This preliminary analyses on physiological parameters and above all on oil accumulation and colorimetric data on a thorough weekly sampling and a subsequent analysis to define

potential correlations between oil content and colour indices may lay the foundations of an easy way to predict the best ripening stage. It can be hypothesised that this may help the olive growers to define the best combination between oil content and ripening stage to maximise oil quality.

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Chapter IV

Isolation and characterisation of terpene synthases potentially involved in flavour development of ripening olive (*Olea europaea*) fruits

Isolation and characterization of terpene synthases potentially involved in flavour development of ripening olive (Olea europaea) fruits

(article being submitted)

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Abstract

The flavour and taste of fruits are often determined by terpenes, a large and structurally diverse class of plant natural compounds. Most of the structural diversity of terpenes is generated by terpene synthases which are responsible for the conversion of prenyl diphosphates into a vast diversity of terpene carbon skeletons. We identified three cDNAs encoding putative terpene synthases in olive fruit of cv. Frantoio and Grignano. Heterologous expression in a bacterial system demonstrated that one of the terpene synthases, OeGES1, was an active monoterpene synthase that converted geranyl diphosphate to the monoterpene alcohol geraniol. The transcript accumulation pattern of this gene showed a peak at 109 DAF in cultivar Frantoio fruits, and an earlier peak in cv. Grignano, indicating that the enzyme may be involved in the production of monoterpene flavour compounds during olive fruit ripening. Although the putative terpene synthases OeTPS2 and OeTPS3 clustered with α-farnesene synthases and a group of angiosperm monoterpene synthases, no detectable *in vitro* activity was found after expression in a bacterial system. Nevertheless, their transcript accumulation was quite similar during fruit ripening, with sharp increases in transcript levels starting from *véraison*.

Introduction

Terpenes are the largest family of plant natural compounds, with more than 40,000 known molecules. Many of these terpenes are volatile compounds that contribute to the flavour and taste of plants. Much of the structural variety of terpenes is formed by the enzyme family of terpene synthases which are responsible for the conversion of prenyl diphosphates into a vast diversity of terpene carbon skeletons (Degenhardt et al., 2009). Studies of terpene synthases have often been focused on plant families that produce terpene-rich essential oils, for example the Labiatae family with perilla (Masumoto et al., 2010) and oregano (Crocoll et al., 2010). Expression of terpene synthases can be associated with fruit ripening (Sharon-Asa et al., 2003; Luecker et al., 2004) and with herbivore defence where terpenes are released in response to leaf damage (Pichersky and Gershenzon, 2002). A recent review has given a wide and exhaustive overview of plant terpene synthases by focusing on seven model plant genomes (Chen et al., 2011). Among crop trees, only grapevine (*Vitis vinifera* L.) terpene synthases have been extensively studied, due to the fact that terpenes are some of the most important volatile components of berries, wine and flowers (Chen et al., 2011). The number of TPS gene models isolated to date and the amount of putative full length TPSs is quite different among model plants. For instance, in *Arabidopsis thaliana* there are 40 TPS gene models, of which 32 are putative full length TPSs, whilst in *V. vinifera* this ratio is 152:69 (Chen et al., 2011).

Olive (*Olea europaea* L.) fruits are the source of olive oil, a vegetable oil that is known for its typical and pleasant flavour, which is given by major C6 and C5 compounds (Kalua et al., 2007). Terpenes, even if present at very low concentrations, can affect olive oil flavour profiles (Cavalli et al., 2004) and their presence is thought not to be influenced by technological factors but only by the genotypic (cultivar) and environmental (growing area) factors (Vichi et al., 2006). Terpenoids have been in fact used to characterise and trace the geographical origin of olive oils (e.g. Zunin et al., 2005; Vichi et al., 2003; Vichi et al., 2006).

In spite of the economical and social importance of olive oil, available genetic information resources on *Olea europaea* and on olive fruit ripening are still quite poor. Recently, two studies have tried to close this gap by performing a computational annotation of genes

(Galla et al., 2009) and an identification of ESTs (Alagna et al., 2009) expressed during olive fruit development.

To understand the molecular aspects of terpene biosynthesis in olive fruits during development and ripening we isolated and characterised three terpene synthase genes from olive. The expression patterns of these genes have been studied during olive fruit ripening and in response to mechanical damage mimicking herbivore attack to olive leaves. Since ethylene is a plant hormone involved in a plethora of processes during plant life cycle including fruit ripening and response to biotic and abiotic stresses (Lin et al., 2009), we have tested whether expression of these terpene synthases was controlled by ethylene.

Materials and methods

Plant material and treatments

Olive fruits (*Olea europaea* L. cultivars Frantoio and Grignano) were harvested in an orchard at Mezzane di Sotto (Verona, Italy) during the 2009 season at ten sampling dates, expressed in days after flowering (DAF): 90 DAF, 102 DAF, 109 DAF, 116 DAF, 123 DAF, 130 DAF, 137 DAF, 144 DAF, 151 DAF, 158 DAF. Sampling began after pit hardening was completed (September) (green and unripe olives), and went on until completion of the ripening process (mid-November) (black and overripe olives). Olive fruits used as a starting material to isolate TPSs were picked when the average fruit population on trees was 50% at *véraison* (defined as change of colour of the drupes) and 50% purple, that is 130 DAF. Olives were pitted, flash frozen in liquid nitrogen and finally stored at –80°C until the following step. Cultivar Frantoio was chosen as it is well spread throughout Italy, whilst cultivar Grignano was chosen because of its traditional regional value and its pleasant oil aroma.

Lateral branches of olive (*Olea europaea* L. cultivar Frantoio) were collected in an orchard at Valsanzibio (Padova, Italy) during the 2010 season and leaves were mechanically wounded by exerting a pressure with the teeth of a meat mallet. The branches were then divided into four groups: the first group was put in a glass jar flushed with 500 ppm propylene, the second one was treated with 1 ppm of 1-methylcyclopropene (1-MCP) in a

closed vessel supplied with potassium hydroxide as carbon dioxide scrubber to prevent its build up, and the third group was kept in a jar flushed with air. A fourth group of branches with non-wounded leaves was kept in a jar flushed with air to have a negative control of the wounding effect. All treatments were performed in large volume glass containers to avoid build up of carbon dioxide and/or ethylene. Treatments were performed for 4 h to detect early response wounding events. At the end of treatment, leaves were flash frozen in liquid nitrogen and then stored at –80°C until the following analyses were performed.

Determination of cDNA sequences of terpene synthases from olives

Putative sequences for genes involved in terpene biosynthesis were identified by data mining on available sequences obtained by Alagna et al. (2009). Three ESTs were selected using the Search tool provided by the authors and available at http://140.164.45.140/oleaestdb/utbr.php and choosing Secondary Metabolism in the list of available KEGG (Kyoto Encyclopedia of Genes and Genomes) maps. ESTs were named after the cluster name found in the database, as follows: 011546, 066222, 025907. ESTs were then amplified from cDNA using the following primers: 5'-ACAGCGGCTACAAAACTGGT-3' (forward) and 5'-TGCAACTTTGGTGATGGCTA-3' (reverse) for 011546; 5'-AGAGAAGAATTCCCACCTTGTG-3' (forward) and 5'-TGGAAAGGCGAACTGCTAAT-3' (reverse) for 066222; 5'-CGCGGGGACAATAAACATAG-3' (forward) and 5'- TCCGCATCTTCATCATCAAA-3' (reverse) for 025907. Fragments were cloned for sequencing into the sequencing vectors pJET1.2 (Fermentas, St. Leon-Rot, Germany) or pCR®4-TOPO® (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. Full-length genes were obtained by means of the SMARTer™ RACE method (Clontech, Mountain View, CA, USA). 5'- and 3'-RACE-Ready cDNA synthesis and 5'-/3'-RACE-PCRs were performed following the manufacturer's instructions. To achieve a better RACE PCR result, a nested PCR with new primers was performed for 066222 EST. RACE primers for the three putative terpene synthases and nested primers (NGSPs) were: 5'-GGTTCAACGGTGGACATGGACCAAA-3' (gene specific primer 2) and 5'-CTCCCAGCAGCGGAAAAGAGTTTCG-3' (gene specific primer 1) for 011546; 5'-

CGGTCAAATGAACGATCCAGTGGCTA-3' (gene specific primer 2) and 5'-GCGGTGTGGTAGAGGTTCTCTTTCATGC-3' (gene specific primer 1) for 066222; 5'-TTGGAAACACCGAACGAAGGTGGAG-3' (gene specific primer 2) and 5'-GGAACCAAGGGGTAGGAGGAAGATCG-3' (gene specific primer 1) for 025907; 5'-TGCATTAGCAAGTTAGCTCTTTC-3' (nested gene specific primer 2) and 5'-GCAGGAAACAAAGTTGTTCATATTC-3' (nested gene specific primer 1) for 066222.
PCRs (Polymerase Chain Reactions) were performed in a peqSTAR 96 Universal thermal cycler (PEQLAB Ltd., Erlangen, Germany). DNA sequencing reactions were performed by GATC Biotech (Konstanz, Germany).
Sequences were deposited in GenBank with the accession numbers JN408072 (*OeTPS1*), JN408073 (*OeTPS2*), and JN408074 (*OeTPS3*).

Expression of terpene synthases in *E. coli* and His-tagged protein purification

Primers for subcloning of the putative TPS genes into the expression vector pASK IBA37+ (IBA, Göttingen, Germany) were designed by means of PrimerDSigner (IBA). Primers for targeting peptide-truncated and full-length clones were designed. The amplified fragments were digested and ligated into the pASK-IBA37+ vector according to the manufacturer's instructions. Vectors were then introduced into TOP10 competent cells (Invitrogen). The transformed cells were grown at 37°C in a flask with LB medium containing 100 μg/mL ampicillin until they reached an optical density of 0.3÷0.8 and protein expression was induced by adding anhydrotetracycline (final concentration: 200 μg/L). Cell density measurements were performed in an Ultrospec 10 Cell Density Meter (Amersham Biosciences, Uppsala, Sweden). Induced cells were cultured at 18°C overnight and harvested by centrifugation after 20 h. Cells were then resuspended in extraction buffer (50 mM Tris-Cl, 10% glycerol, 5 mM dithiothreitol, 5 mM magnesium chloride, 5 mM sodium ascorbate, 0.5 mM phenylmethanesulfonyl fluoride) and sonicated (3x30 s at 50% power, Branson Sonifier 250, Dietzenbach, Germany). After centrifugation, the supernatant was flowed through an Econo-Pac 10DG column (Bio-Rad, Hercules, CA, USA) and was desalted with assay buffer (10 mM Tris-Cl, 10% glycerol, 1 mM dithiothreitol). His-tagged proteins were purified with Profinity IMAC resin (Bio-Rad). Briefly, cells were centrifuged

and resuspended in lysis buffer (50 mM Tris-Cl, 500 mM sodium chloride, 20 mM imidazole, 10% glycerol, 1% Tween 20). After sonication, the nickel charged resin was equilibrated with lysis buffer and supernatant was incubated with the resin at 10°C for 1 h. The protein-resin complex was then flowed through a Econo-Pac 10DG column and washed with wash buffer (50 mM Tris-Cl, 500 mM sodium chloride, 20 mM imidazole, 10% glycerol). The His-tagged purified protein was then eluted with elution buffer (50 mM Tris-Cl, 500 mM sodium chloride, 150 mM imidazole, 10% glycerol). Purified protein fraction of OeTPS1 was analysed by SDS-PAGE. Therefore a 10% polyacrilamide gel was run at 100 V for approximately 2.5 h and then stained by means of Coomassie blue.

Enzyme assays and GC-MS analysis

Enzyme activity assays were performed in glass vials, with caps provided of a silicone septum, using 30 µL of purified protein, 5 µL of 440 ng/µL geranyl pyrophosphate or farnesyl pyrophosphate, 10 µL of 100 mM $MgCl_2$ and assay buffer (10 mM Tris-Cl, 10% glycerol, 1 mM dithiothreitol) to a total volume of 100 µL. Samples were incubated for 40 min at 40°C and the product volatiles were collected by a polydimethylsiloxane coated SPME fibre (SUPELCO, Bellefonte, PA, USA) which was exposed to the headspace of the assay. In order to analyse terpene products, the fibre was then introduced into the injector of a GC-MS QP2010S system (Shimadzu, Duisburg, Germany). Compounds were separated by means of an EC-5 column (Grace, Deerfield, IL, USA). For monoterpenes the temperature program was as follows: 50°C for 3 min, first ramp 7°C/min to 170°C, second ramp 100°C/min to 300°C, final 2 min hold, for sesquiterpenes: 80°C for 2 min, first ramp 7°C/min to 200°C, second ramp 100°C/min to 300°C, final 2 min hold. GC-MS carrier gas: hydrogen (1 mL/min). Pure geraniol was purchased from Sigma (St. Louis, MO, USA). All terpene products were identified by using the Shimadzu software “GCMS Postrun Analysis” with the mass spectra libraries “Wiley8” (Hewlett-Packard, Palo Alto, CA, USA) and “Adams” (Adams, 2007).

Phosphatase activity assay

Phosphatase activity assay was performed as described by Iijima et al. (2004). The result of the colorimetric reaction was measured at 420 nm in a spectrophotometer (Ultrospec III, Pharmacia LKB Biotechnology, Uppsala, Sweden).

RNA extraction, cDNA synthesis and Quantitative real-time PCR

RNA extraction from olives and olive leaves was performed as described by Conde et al. (2007). DNAse treatment and cDNA synthesis were performed as described by Nonis et al. (2007).

Quantitative real-time PCR experiments were performed on a StepOnePlus instrument (Applied Biosystems, Foster City, CA, USA) following the procedure described by Nonis et al. (2007). Reactions were carried on a total volume of 10 µL containing 2.5 µL cDNA (1 ng/µL of theoretically retrotranscribed RNA), 0.2 µL of each primer (10 µM), 5 µL Power SYBR® Green PCR Master Mix (Applied Biosystems) and 2.1 µL RNAse-free water. The full list of primer sequences is given in Table 1.

Primer name	Sequence (5'- 3')
OUB2_FW	GCTGGAGGATGGAAGGACTC
OUB2_REV	CCACGACTCAACAGAGACGA
OeGES1_FW	TGTGAAAACGAAGGCAGAAA
OeGES1_REV	CGACGCTGGAAAAGTAGGAG
OeTPS2_FW	GCAAATGTTACAGAACAGGAAGC
OeTPS2_REV	AAATTAGCAACTCGCGCTATG
OeTPS3_FW	AGGAAGTTGCTCGTCGATACA
OeTPS3_REV	TGTGAGCATTTCCATGTTGG

Table 1. List of primers used for quantitative real-time PCR, including the housekeeping gene polyubiquitin OUB2.

All experiments were performed in triplicate on two independent biological replicas with the following thermal cycling conditions: a first denaturation step at 95° C for 10 min, followed by 40 cycles (95° C 15 s, 60° C 1 min). A melting curve analysis was performed to confirm the absence of multiple products or primer dimers formation. All assays included no-template controls (NTC). All quantifications were normalized to polyubiquitin OUB2 (accession no. AF429430) (Table 1). Cq data were exported using the software of the instrument (StepOne Software, ver. 2.1, Applied Biosystems), and analyzed according to the method described by Livak and Schmittgen (2001).

Sequence analysis

Alignments of amino acid sequences and phylogenetic tree construction were performed using the MegAlign software (DNASTAR, Lasergene, Madison, WI, USA). The phylogenetic tree was obtained by means of an alignment of the three translated open reading frames (ORFs) isolated herein with protein sequences of annotated TPSs found in GenBank. Additional protein sequences retrieved in a previously published study (Danner et al., 2011) were also included to cover all the known TPSs subfamilies (Bohlmann et al., 1998; Dudareva et al., 2003). Alignment was performed using ClustalW method and the resulting tree was bootstrapped with 1000 replicates. The presence of putative signal peptides was evaluated by means of the bioinformatic tools ChloroP (http://www.cbs.dtu.dk/services/ChloroP/), SLP-Local (http://sunflower.kuicr.kyoto-u.ac.jp/~smatsuda/slplocal.html), TargetP (http://www.cbs.dtu.dk/services/TargetP/), WoLF PSORT (http://wolfpsort.org/), and BaCelLo (http://gpcr2.biocomp.unibo.it/bacello/pred.htm).

Results

Isolation of cDNAs encoding terpene synthases from olive fruit and sequence analysis

To identify putative terpene synthases encoding genes from olive the OLEA EST database was searched using the provided Search tool and selecting the suitable KEGG map

"Monoterpenoid biosynthesis". The three longest contigs retrieved with the Search tool were selected and the corresponding sequences encoding partial terpene synthases were amplified using specific primers and cDNAs generated from olive fruits. The complete coding sequences were obtained by 3' and 5' RACE (Rapid Amplification of cDNA Ends) PCR. The three putative full length TPS genes have been named *OeTPS1*, with an ORF of 1,749 nucleotides encoding a predicted protein of 583 amino acids (calculated molecular weight 67 kDa and p*I*=4.926), *OeTPS2*, with an ORF of 1,644 nucleotides encoding 548 amino acids (calculated molecular weight 64 kDa and p*I*=5.255), and *OeTPS3*, with an ORF of 1,827 nucleotides encoding a predicted protein of 609 amino acids (calculated molecular weight 71 kDa and p*I*=6.888).

BLASTp queries of the three deduced amino acid sequences have been performed against the non-redundant protein database at NCBI (National Center for Biotechnology Information, http://blast.ncbi.nlm.nih.gov/Blast.cgi). OeTPS1 shared high similarity with several acyclic monoterpene synthases, including two geraniol synthases from *Phyla dulcis* (ADK62524) (68% sequence identity) and *Ocimum basilicum* (AAR11765) (62% sequence identity), and a R-linalool synthase (*O. basilicum*, Q5SBP3) (58% sequence identity). OeTPS2 was similar to some generic terpene synthases of *Solanum lycopersicum* (AEP82776.1; 53% sequence identity) and *Populus trichocarpa* (AEI52902.1; 51% sequence identity) but also with an α-farnesene synthase of *Ricinus communis* (AEQ27768.1) (49% sequence identity). OeTPS3 revealed high similarity levels with many hypothetical or unknown proteins of *P. trichocarpa* and *Vitis vinifera* but also with a (E)-β-ocimene synthase (ADR74205) of *V. vinifera* (56% sequence identity).

The amino acid sequences were compared and aligned to check for conserved regions and domains (Figure 1).

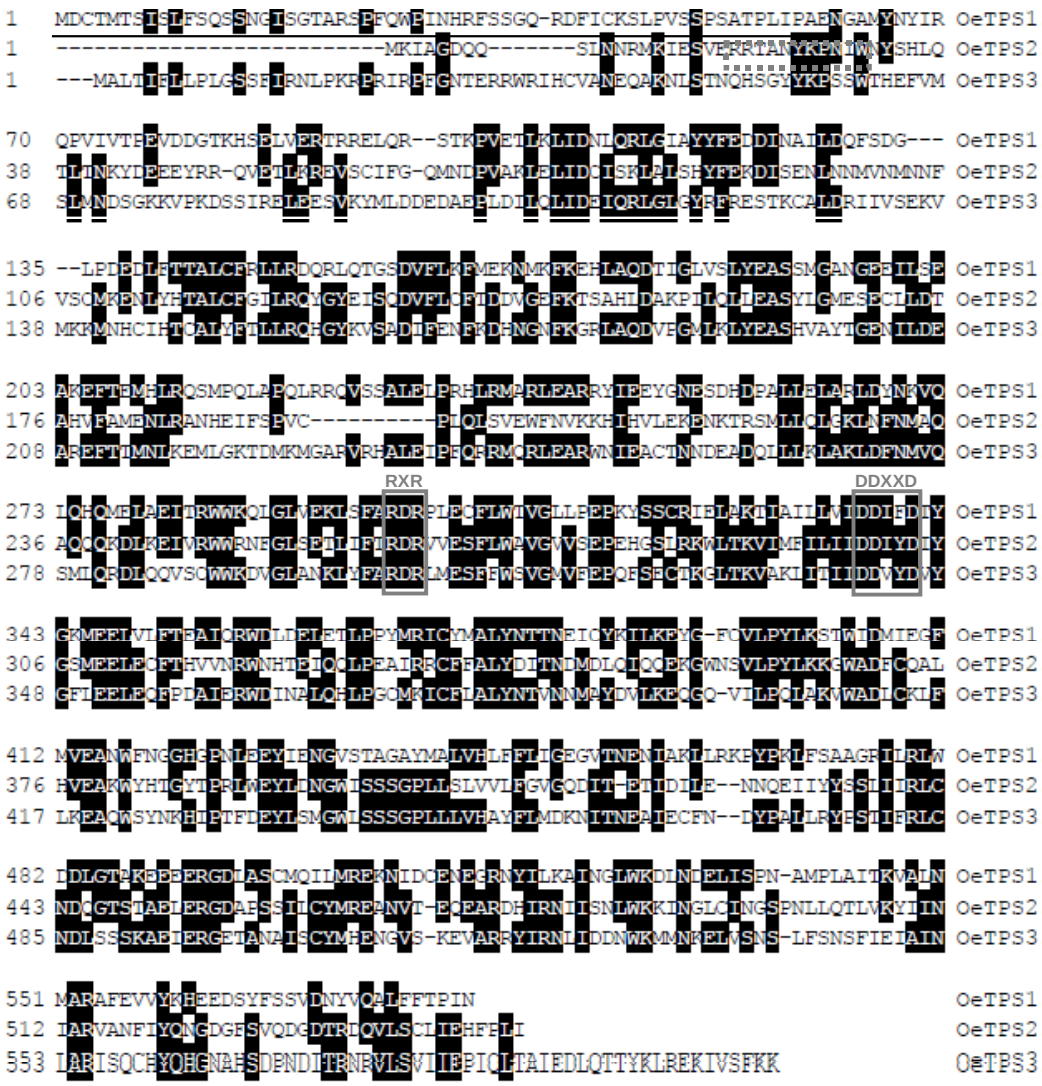

Figure 1. Alignment report of the deduced amino acidic sequences of *OeTPS1*, *OeTPS2*, and *OeTPS3*. DDXXD and RXR conserved motifs are labelled and boxed. The solid line shows the predicted signal peptide of OeTPS1. The dotted box shows the RRX_8W motif in OeTPS2.

The three encoded proteins showed some typical features of terpene synthases, such as the highly conserved aspartate-rich motif DDXXD, which is known to be involved in binding divalent metal ions (Degenhardt et al., 2009). The RXR motif, involved in the complexation of the diphosphate group after substrate ionization (Starks et al., 1997) was also present in the three isolated TPSs about 35 amino acids upstream of the DDXXD motif, as expected. OeTPS2 showed a RRX_8W motif in the N-terminal region, which is usually found 60 amino acids from the N-terminus of many monoterpene synthases (Degenhardt et al., 2009).

Monoterpene and diterpene synthases usually show an N-terminal signal peptide that targets these enzymes to the plastids, whilst sesquiterpene synthases are cytosolic enzymes. We therefore checked the amino acid sequences to search for predicted signal peptides by means of the protein localisation prediction tools listed above. For what concerns OeTPS1, a chloroplast targeting signal peptide was predicted by all the used tools, consisting of the first 58 amino acids starting from the N-terminal region. For OeTPS2 and OeTPS3 no specific signal peptides were predicted, suggesting that they may be putative sesquiterpene synthases and be localised in the cytosol.

Expression of olive TPS in *E. coli* and product analysis

To determine the activity of the olive TPSs, we have expressed the three OeTPSs in *E. coli*. Since signal peptide predictions for OeTPS1 showed a possible plastid localisation, we expressed a truncated version of the TPS, removing the predicted signal peptide as suggested from the prediction tools, but also a full-length version, since predictions give only a probability of the presence of targeting peptides. The truncation is necessary considering that this signal peptide is often shown to cause the formation of inclusion bodies in bacterial cells (Bohlmann et al., 1998, Crowell et al., 2002). For what concerns OeTPS2 and OeTPS3, only expression of the full-length protein was performed.

After protein expression, extracts of *E. coli* were assayed for monoterpene and sesquiterpene activity using geranyl pyrophosphate (GPP) and farnesyl pyrophosphate (FPP) as substrates, respectively.

In case of OeTPS1, enzymatic activity was detected when the truncated protein was incubated with GPP, whilst administration of FPP to both the whole sequence or the truncated version gave no results. The resulting GC-MS chromatogram is shown in Figure 2A. The peak present in the spectrum was identified as geraniol by comparison with an authentic geraniol standard (Figure 2B). An extract of *E. coli* with an empty pASK-IBA37+ vector was used as a control, giving no detectable products.

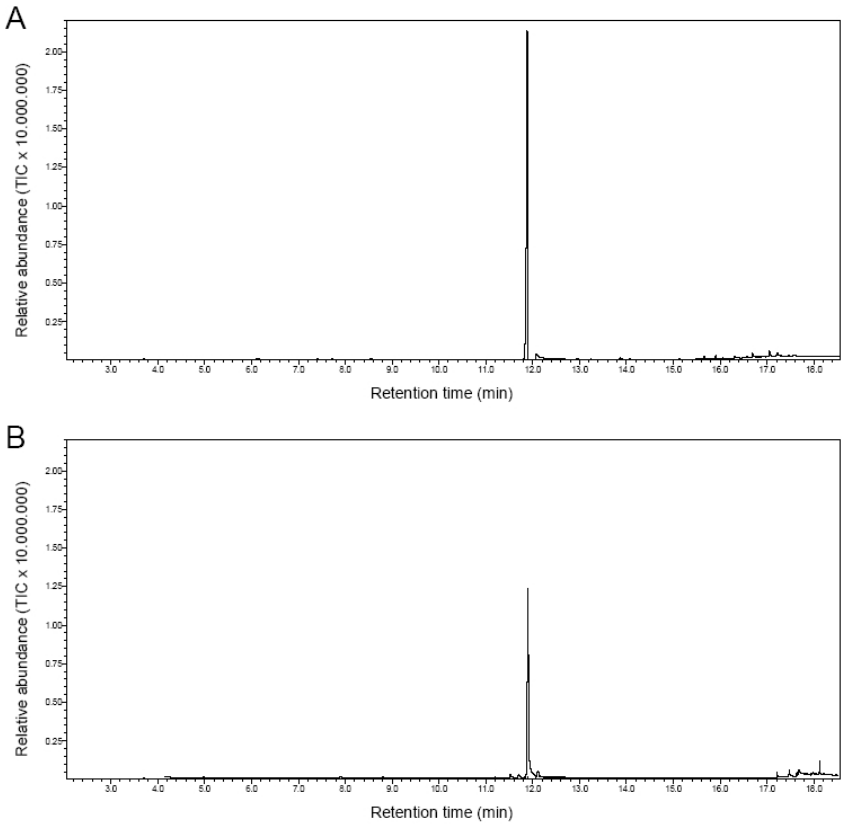

Figure 2. (A) GC-MS total ion chromatogram of the purified enzyme extract of the truncated OeTPS1 protein after administration of GPP, incubation and SPME extraction. A clear peak of geraniol is visible. (B) GC-MS total ion chromatogram of pure geraniol.

The full-length proteins of OeTPS2 and OeTPS3 gave no detectable products with neither GPP nor FPP. OeTPS1 was used for further analyses and characterisation. After purification of the His tagged protein on a nickel-agarose column, the protein formed a strong band, after SDS-PAGE. The band migrated at the predicted size of 67 KDa, and only weak bands were observed in the background (Figure 3).

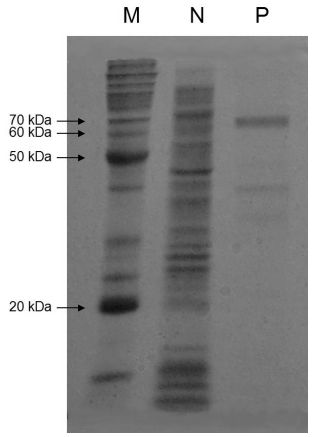

Figure 3. Non-purified (N) and His-tagged purified (P) extract of OeTPS1 analysed by means of SDS-PAGE on 10% polyacrilamide gel and Coomassie staining. After purification a major band of the expected size of 67 KDa is visible on the purified extract. Benchmark protein ladder (Invitrogen) has been loaded in (M) lane.

To verify that geraniol was formed by the putative terpene synthase OeTPS1 rather than a generic phosphatase in the bacterial extract, a phosphatase assay was carried out by means of the colorimetric reaction between *p*-nitrophenylphosphate and *p*-nitrophenol. The purified OeTPS1 enzyme did not show any detectable phosphatase activity (data not shown). In addition, the assays for terpene synthase activity were performed with the phosphatase inhibitors sodium tungstate (Na_2WO_4, 2mM) and sodium fluoride (NaF, 1mM). The activity of OeGES1 was still present, indicating that the reaction is not catalysed by a phosphatase in the background. Since monoterpene synthases need a metal cofactor for enzymatic activity, such as Mg^{2+} or Mn^{2+} (Bohlmann et al., 1998), a control assay using ethylenediaminetetraacetic acid (EDTA, 10mM) as a chelating agent was performed. In the absence of a metal cofactor, no terpene products were detected (data not shown). Based on these results, OeTPS1 was considered to be a *bona fide* geraniol synthase (GES) and therefore renamed and designated OeGES1.

Relationship with known terpene synthases

Phylogenetic analyses performed by means of alignments of the three olive TPSs enabled a further characterisation of the isolated enzymes as shown in Figure 4. Terpene synthases selected to draw the phylogram clearly grouped in eight clusters or subfamilies on the basis

of sequence identity, as described previously (Bohlmann et al., 1998; Dudareva et al., 2003).

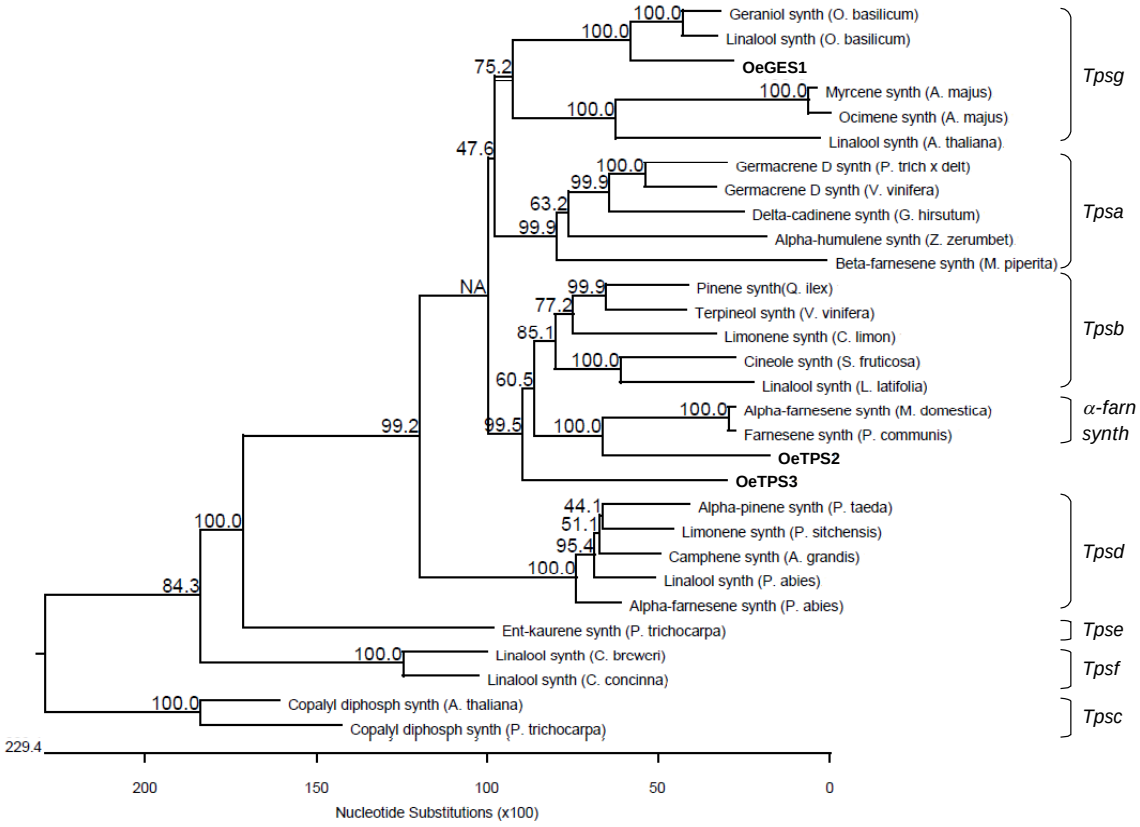

Figure 4. Phylogenetic tree of the deduced amino acid sequences of the isolated *OeTPSs* with selected terpene synthase sequences aligned and designed using the ClustalW method. Terpene synthases group in seven branches (*Tpsa* to *Tpsg*) on the basis of sequence identity as found in literature. An eighth branch is made up of α-farnesene synthases.

Briefly, *Tpsa* cluster consists of angiosperm sesquiterpene synthases, *Tpsb* branch contains angiosperm monoterpene synthases, *Tpsc* subfamily represents copalyl diphosphate synthases, *Tpsd* includes gymnosperm terpene synthases, *Tpse* group comprises kaurene synthases, *Tpsf* branch contains linalool synthases and *Tpsg* comprises angiosperm acyclic monoterpene synthases. An additional branch is made up by α-farnesene synthases. OeGES1 merged with the *Tpsg* cluster, together with a geraniol and a linalool synthases both from *O. basilicum*, giving an additional strong hint that OeGES1 can synthesise an acyclic compound such as geraniol. While OeTPS2 fell in the branch of α-farnesene synthases, OeTPS3 assignment was not unambiguous and this enzyme could group either within the α-farnesene cluster or the *Tpsb* subfamily.

Expression of TPSs-encoding genes during olive fruit development and in response to wounding

Gene expression patterns of the three isolated putative olive TPSs were evaluated by means of real-time PCR during the last phases of development and ripening of olive fruits from two varieties (cv. Frantoio and Grignano) and in olive leaves (cv. Frantoio) in response to wounding stress.

Expression dynamics for the three genes throughout fruit ripening for cv. Frantoio are shown in Figure 5A,B,C.

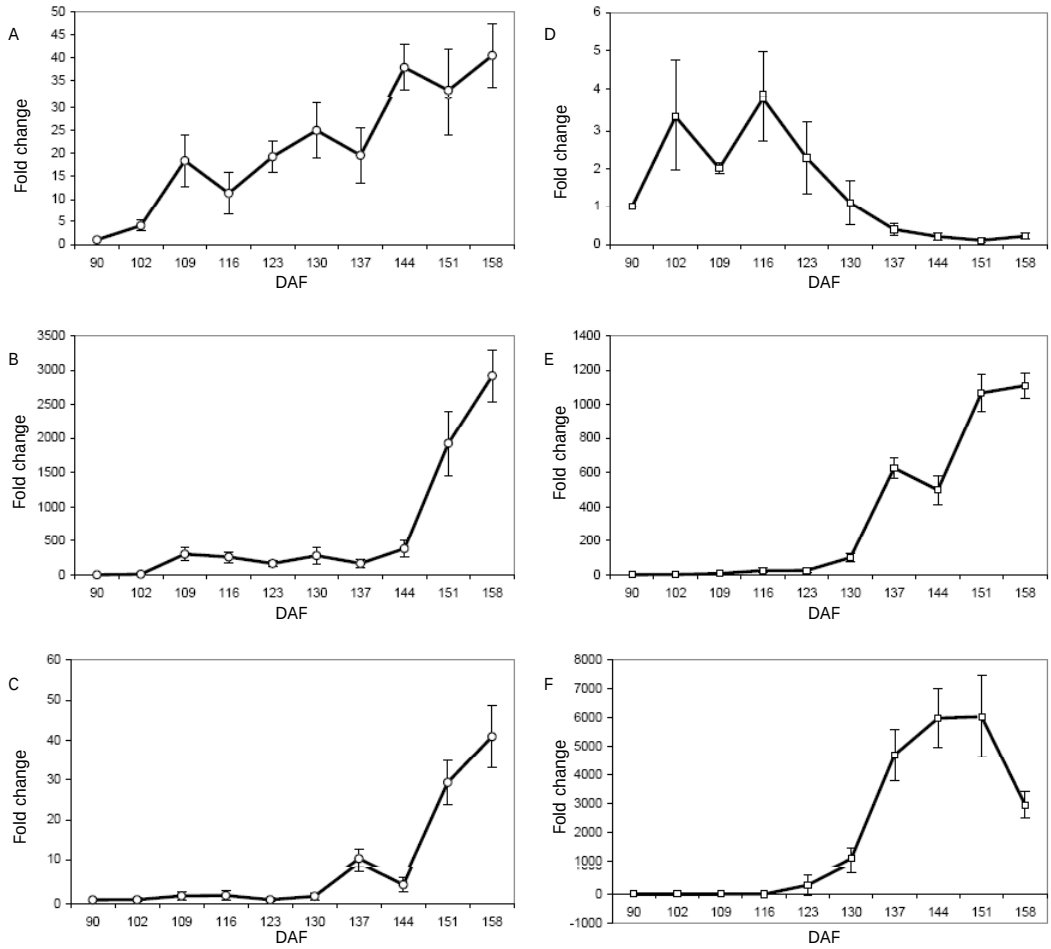

Figure 5. Relative gene expression levels of the three *OeTPSs* along olive fruit development and ripening for cv. Frantoio (A, *OeGES1*; B, *OeTPS2*; C, *OeTPS3*) and Grignano (D, *OeGES1*, E, *OeTPS2*, F, *OeTPS3*). Developmental stage is expressed in days after flowering (DAF). The onset of ripening, usually defined as *véraison*, (corresponding to at least 50% of fruits with visible changes of colour) occurred at 123 DAF.

OeGES1 showed a consistently increasing relative expression throughout fruit ripening, starting from very early stages. An 18-fold increase was evident already from 109 DAF. The increase in relative expression appeared constant until the end of the sampling in mid-November with a maximum of a 40-fold increase. *OeTPS2* showed a dramatic increase in relative expression levels, displaying a 300-fold increase 109 DAF and an upward trend until the end of samplings, with a final 3000-fold rise. *OeTPS3* expression shared a common trend with *OeTPS2*, although with a much lower relative increase in transcript levels (a maximum of a 40-fold increase was observed) and with a first noticeable transient peak (10-factor increment) 137 DAF.

Expression data for cv. Grignano are shown in Figure 5D,E,F; *OeGES1* expression dynamic in ripening fruits appeared very different with respect to cv. Frantoio, since transcript accumulation levels showed an increase in the first sampling dates with a transient peak 116 DAF, followed by a gradual decrease until the end of the ripening process. Also *OeGES1* relative expression values were much lower with respect to cv. Frantoio, as a 4-fold increase was the highest peak observed in Grignano. *OeTPS2* and *OeTPS3* showed both an upward trend in transcript accumulation, that was evident from 130 DAF for *OeTPS2* and reached a 1200-fold increase in the last sampling date. Expression pattern was somehow different for *OeTPS3* since a first dramatic change in transcript accumulation was visible 123 DAF, then reaching a 6000-fold peak 144 and 151 DAF. Transcript accumulation showed then a sharp decrease 158 DAF. *OeTPS3* expression dynamics appeared clearly more dramatic for cv. Grignano than in cv. Frantoio.

The wounding responses of the three isolated TPS genes were studied in cv. Frantoio leaves, in relation to treatment with ethylene or to the inhibitor of its action 1-MCP. In this case, transcript accumulation patterns revealed divergent regulatory pathways in response to wounding for the three genes (Figure 6A,B,C). Wounding exerted a negative effect on *OeGES1* transcript accumulation, by a factor of 7, already after 4 hours. This effect seemed to be ethylene-independent, since administration of 1-MCP or of propylene did not lead to significant changes in the expression profile.

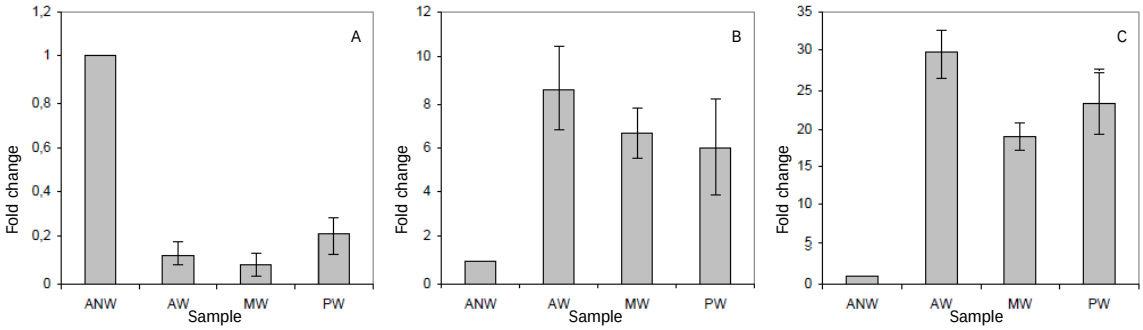

Figure 6. Relative gene expression levels of the three OeTPSs in olive leaves with respect to wounding stress (A, *OeGES1*; B, *OeTPS2*; C, *OeTPS3*). ANW: non-wounded leaves flushed with air; AW: wounded leaves flushed with air; MW: wounded leaves treated with 1-MCP; PW: wounded leaves flushed with propylene.

Wounding induced the expression of *OeTPS2* and *OeTPS3* (ca 10 fold and 30-fold increase, respectively) after 4 hours of treatment, in a manner that appeared ethylene independent since 1-MCP or propylene treatments had small effects and did not lead to significant changes of transcripts accumulation.

Discussion

Isolation olive terpene synthases: sequence analysis and biochemical characterisation

In this work we identified three genes encoding putative terpene synthases (OeTPS1, OeTPS2, OeTPS3) from olive fruit. Full length clones were obtained by 3' and 5' RACE PCR for the three genes and the deduced amino acid sequences were shown to share significant similarity and conserved elements (the DDXXD and the RXR motifs) typical of known terpene synthases from other species. Phylogenetic analysis on a representative number of terpene synthases, including the olive TPSs, pointed out a close similarity of OeTPS1 with terpene synthases of the Tpsg family, while OeTPS2 and OeTPS3 appeared more similar to Tpsb family and/or α-farnesene synthases. For OeTPS1 the presence of a signal peptide could be predicted suggesting that this enzyme may likely represent a monoterpene synthase. This hypothesis was further confirmed by its biochemical characterisation. A truncated version of OeTPS1, from which the signal peptide had been

removed, was expressed in a bacterial heterologous system and resulted to be able to convert GDP into geraniol and was therefore interpreted as a bona fide geraniol synthase and renamed OeGES1. For what concerns OeTPS2 and OeTPS3, no targeting signal peptide could be predicted using different tools and we assumed that these proteins may be putative sesquiterpene synthases. Predictions can however be sometimes inaccurate and not able to always detect the presence of targeting peptides. In fact, although signal peptides are usually rich in serine and threonine residues, no common sequences have been identified yet (Bohlmann et al., 1998). This possible inaccuracy may also explain why, in spite of the length of OeTPS3 amino acidic sequence (609 aa), that makes it more similar to a generic monoterpene synthase rather than to a sesquiterpene synthase, no enzymatic activity was shown. Therefore the lack of activity of heterologously expressed OeTPS2 and OeTPS3 may be due to the presence of an inhibitory signal peptide that could not be recognised, and later removed, using the current bioinformatic tools. Enzymatic assays on the two extracted enzymes gave no detectable products neither supplying them with GPP nor with FPP. However, many putative terpene synthases in plants appear to be non functional. Studies on maize TPSs show that functional and non-functional alleles do coexist, also depending on genotype (cultivar), the latter ones being inactive due to frame-shift mutations or amino acid substitutions, and yet transcribed as previously shown (Köllner et al., 2004). The lack of activity of OeTPS2 and OeTPS3 may therefore be due also to alterations in nucleotide sequences. For what concerns tree species, 152 terpene synthase-like genes have been recently identified in grapevine, of which only 69 are at present considered putative full-length TPSs (Martin et al., 2010).

To the best of our knowledge, geraniol has never been previously reported in the headspace of neither olive fruits nor extra-virgin olive oil. Geraniol metabolism has been studied in berries of V. vinifera cv. Scheurebe showing that stereoselective reduction, isomerisation, and oxidation reactions take place in grape mesocarp, some of them being dependent on the ripening stage (Luan et al., 2005). Since these reactions may presumably also take place in the olive fruit, the absence of geraniol among terpenes usually found in the headspace of olive oil could be ascribed to the conversion of this alcohol to derivative compounds that may yet contribute to the overall oil flavour profile.

Isolated OeTPSs may be involved in fruit ripening and wounding responses

The transcript accumulation patterns of the three OeTPS encoding genes may support their involvement in fruit ripening and in wounding responses. *OeGES1* followed a ripening-dependent gradual increase of transcription for Frantoio olives, whilst in cv. Grignano its pattern appeared significantly different and displayed a transient peak of expression before ripening. Considering these expression dynamics, we can hypothesise that the *OeGES1* transcript accumulation during different phases of olive fruit ripening in cv. Frantoio and cv. Grignano, may be related to some specific events of flavour development, that seem to undergo a clearly different temporal regulation in the two genotypes. Notwithstanding the absence of detected activity of OeTPS2 and OeTPS3, their common pattern of transcript accumulation, and their dramatic (up to thousands fold) up-regulation towards the last stages of olive ripening, may suggest a role of these proteins in ripening fruits. This aspect will need further attention to unravel the biological role of genes encoding putative TPS proteins with no assigned biochemical activity but that do display a dramatic transcriptional regulation.

Terpenoid synthases are known to have essential roles in plant interactions with the environment and other organisms, being involved in defence, chemical communication with pathogens and insects and in wounding response. Terpene synthases are transcriptionally activated by these events, and transcript accumulation occurs with a different timing as seen in grand fir. Some monoterpene synthases were seen to be in fact up-regulated starting from 2 h after the wounding event (Steele et al., 1998). Our data show that also the three putative TPS isolated from olive respond to wounding within a short time frame (4 hours) and independently from ethylene action, as shown by their insensitivity to blocking ethylene perception (by treating with 1-MCP) or to its exogenous supply (propylene). Interestingly opposite behaviours could be evidenced since *OeGES1* transcripts appeared to be downregulated by wounding while those of *OeTPS2* and *OeTPS3* resulted to be up-regulated. Overall our results show that the thee putative olive TPS not only share sequence features typical of terpene synthases but also display a transcriptional regulation that resembles that of TPS encoding genes previously isolated from other species, being early-regulated by wounding. Interestingly all three genes appeared to be

highly regulated at the transcriptional level during the ripening process in two different genotypes suggesting a conserved putative role in flavour development during ripening of olives for these genes.

Acknowledgements

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Chapter V

Optimized RNA extraction and identification of putative reference genes for improved real-time quantitative polymerase chain reaction expression studies on olive (*Olea europaea* L.) fruits

Optimized RNA extraction and identification of putative reference genes for improved real-time quantitative polymerase chain reaction expression studies on olive (Olea europaea L.) fruits

(article being submitted)

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Abstract

Olea europaea L. is an emerging species of interest for molecular biology studies. Genome wide transcriptomic surveys are recently uncovering differentially expressed genes for biologically and agriculturally relevant processes in olive. Differential expression data need to be backed up by independent quantitative approaches performed on selected genes of interest. Being qPCR the most widely adopted technique for mRNA quantification, preliminary work needs to be done to set up robust methods for extraction of fully functional RNA and for the identification of the best reference genes to enable the most reliable quantification of transcripts by qPCR in developmental studies on olive samples. In this work, we have optimised a method for RNA extraction from olive fruits and leaves allowing for highly reproducible qPCR results. Besides, to establish pre-validated assays, thirteen potential candidate reference genes have been evaluated on 21 samples belonging to fruit developmental series and leaf tissues subjected to wounding, propylene treatments or to ethylene inhibition. By using two different algorithms, the best reference genes were defined both tissue by tissue and in the whole dataset. For fruit development, GAPDH2 and PP2A1 were identified as the best reference genes and were adopted to evaluate the expression of two ripening marker genes demonstrating their effectiveness for normalisation of qPCR.

Abbreviations

14-3-3	14-3-3 protein
1-MCP	1-methyl-cyclo-propene
ACT	Actin
bp	base pairs
DAF	Days After Flowering
EF1	Elongation factor 1
EST	Expressed Sequence Tag
FPS	Farnesyl pyrophosphate syntase

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
NTC	No Template Control
OUB	Polyubiquitin
PG	Polygalacturonase
PP2A	Protein Phosphatase 2A
qPCR	Quantitative real-time polymerase chain reaction
RT	Reverse Transcription
SV	Stability Value (NormFinder)
UBC	Ubiquitin conjugating enzyme
UBQ	Ubiquitin

Introduction

Olive (*Olea europaea* L.) is a crop tree widely cultivated in the Mediterranean area for its economical importance as a source of olive fruits for table consumption and olive oil extraction. For its many economical and social implications, research on *O. europaea* represents an important field of study for agricultural and food sciences, plant physiology, biology, nutrition, medical sciences and pharmacology.

An increasing amount of data is becoming available dealing with the characterisation of molecular aspects of olive fruit development and ripening and olive tree responses to environmental stresses. Untargeted systematic approaches, such as EST sequencing, have been recently undertaken pinpointing a significantly high number of differentially expressed genes in a range of developmental and physiological situations in olive fruits, roots and leaves (Alagna et al. 2009; Galla et al. 2009). While providing a global overview of transcriptional regulatory networks, systematic approaches suffer from their intrinsic wide experimental variability of data, thus posing the need for further validation of differential transcription of selected genes of interest by more precise quantitative approaches. The method of choice to assess the transcriptional regulation of a gene, either in terms of its relative or absolute transcriptional changes , is qPCR. For qPCR to be performed reliably and provide reproducible and sound results, extraction of RNA of optimal quality is an absolute requirement along with the careful choice of reference genes displaying stable expression values over the widest range of experimental situations. The identification and choice of the best reference genes are essential for and substantially influence the outcome of qPCR and its reliability (Dheda et al. 2005), by providing internal controls to ensure minimisation of non-biological variation. Ideally, to be useful as a reference a transcript should have a stable level of expression throughout all tissues and experimental conditions under investigation. In the past years, genes involved in basic cellular processes were considered to fulfil these requirements by being stably expressed at a steady-state level in all conditions and were thus defined as “housekeeping genes”. In the last few years, with the rapid spreading of qPCR technology through laboratories, this assumption has been questioned and it has become clear that no gene is transcriptionally stable in all situations leading to the conclusion that strictly speaking housekeeping genes

do not exist (Vandesompele et al. 2002). Thus, the concept of "housekeeping genes" has been substituted with that of "reference genes", underlying the fact that the latter ones must be validated in each experimental set prior to be used as such. These concepts have been highlighted in the last years, with the drafting of the comprehensive rules for minimum information for publication of qPCR data (MIQE) (Bustin et al. 2009; Bustin et al. 2010), described in order to help standardisation of experiments between different laboratories. In the MIQE paper, Bustin et al. (2010) strongly encouraged to submit the MIQE workflow checklist as a supplemental file along with submitted manuscripts. In this list, several details have to be disclosed including those regarding RNA quality and data normalisation.

Since extraction of high quality RNA and identification of reference genes are among the most important factors for reliable qPCR results, in this paper we have compared three different RNA extraction methods and set up an improved extraction protocol yielding high quality RNA from olive fruit mesocarp and stressed leaves.

Furthermore, the stability of 13 putative reference genes was evaluated on 21 samples collected from different developmental and ripening stages of olive fruits and leaf tissues exposed to wounding stress, aiming for the identification of a panel of reference genes useful for future qPCR studies. A number of similar studies already exists for many crop species, but no such study is currently available for *Olea europaea*. Selected candidate genes were evaluated for their performance as reference genes through independent algorithms (genorm^{PLUS} and NormFinder) to assess and quantify transcripts stability. Since the output ranking can be slightly different from one software package to another (e.g. Cruz et al. 2009; Lin and Lai 2010; Tong et al. 2009), most authors use more than one application to analyse data. The two most used algorithms are geNorm (Vandesompele et al. 2002) and NormFinder (Andersen et al. 2004). The first one was improved recently and renamed genorm^{PLUS} and it was embedded in qbase^{PLUS} (Hellemans et al. 2007), a software dedicated to qPCR data analysis. genorm^{PLUS} has many advantages, since result reports are obtained in a fully automatic way, no data handling is needed, the identification of the single best reference gene is possible (instead of the "best two" combination), analysis is faster (http://medgen.ugent.be/~jvdesomp/genorm/#WhatIs). Similarly to the previous version, genorm^{PLUS} provides also the optimal number of reference genes to be used in the considered experimental set.

Since stability of reference genes should be assessed carefully for each experimental condition (Guénin et al. 2009), each tissue (fruit and leaf) was considered independently, giving particular emphasis to the fruit development series, an aspect of outstanding importance for its economical implications. Normalisation of expression data of two target transcripts was also reported to provide further evidence on the importance of having validated reference genes to avoid pitfalls in mRNA quantification and to show the validity of the identified reference genes for gene expression studies on olive fruits.

Materials and methods

Plant material

Olive fruits (*Olea europaea* L., cv. Frantoio) were harvested during the 2009 season in an orchard in Verona province (Italy, 45° 28' 52.85" N, 11° 8' 32.97" E) at ten consecutive sampling dates (ca one week intervals) at 90, 102, 109, 116, 123, 130, 137, 144, 151, 158 DAF. The *véraison* stage was at 123 DAF and olives picked in the last sampling date were overripe. For wounding experiments, lateral branches were collected from olive (*Olea europaea* L., cv. Frantoio) trees in an orchard in Padova province (Italy, 45° 17' 8.02" N, 11° 43' 42.52" E) during the 2010 season and wounding was exerted by pressing the leaves with a blunt metal point. The branches bearing wounded leaves were separated into the following three groups according to the different treatments: 1) branches treated with 500 ppm propylene (ethylene analogue); 2) branches treated with 65 mg of 1-methylcyclopropene (1-MCP) (ethylene inhibitor); 3) untreated branches kept in air. For all treatments branches were kept in large volume sealed vessels though which air was flushed. A negative control was included in the experiments non-wounded leaves were kept in a box flushed with air. Early and late responses were evaluated performing these treatments for both 4 and 20 hours. Olive fruits were picked 109 DAF in Padova province (Italy, 45° 17' 8.02" N, 11° 43' 42.52" E) during the 2009 season and sorted in three groups in three sealed vessels: the first one was kept in laboratory air, the second one was treated with 65 mg 1-MCP, and the third one was treated with 500 ppm propylene. Treatments were performed for 24 hours.

Olives were stoned, and pitted olives and leaves were frozen in liquid nitrogen and stored at –80°C until RNA extraction.

RNA extraction, evaluation of RNA quality and cDNA synthesis

Total RNA was extracted from frozen olive fruit mesocarp and olive leaves using the following three different protocols: 1) column-based RNA extraction by means of illustra RNAspin Mini Isolation Kit (GE Healthcare, Little Chalfont, UK) was performed following the manufacturer's instructions; 2) a protocol suitable for grapevine RNA extraction (Iandolino et al. 2004) was used according to the modifications described in Nonis et al. (2008); 3) a hot borate method, suitable for RNA extraction from recalcitrant species as cotton (Wan and Wilkins 1994) and previously used in olive (Conde et al. 2007), was used and finally improved with the following modifications. Three hundred milligrams of frozen tissue were ground in a mortar with liquid nitrogen. The finely ground powder was incubated in preheated buffer (0.2 M sodium tetraborate decahydrate, 30 mM EGTA, 1% SDS, 1% deoxycholic acid sodium salt, 1% Nonidet P-40 and 2% PVP-40) added with 1 mg/g proteinase K and 0.02 volumes of beta-mercaptoethanol at 42°C for 1 hour, vortexing vigorously every 10 minutes. After incubation, samples were added with potassium chloride (final concentration 160 mM) and kept on ice for 1 hour. Samples were then centrifuged at 18000 g for 10 min at 4°C. The aqueous phase was recovered, kept on ice for 45 min and then centrifuged again at 18000 g for 10 min at 4°C. The aqueous phase was recovered and added with 0.3 volumes of lithium chloride 8 M and kept overnight at 0°C. The following day, samples were centrifuged at 18000 g for 20 min at 4°C. The supernatant was carefully decanted and the pellet was washed twice with cold 80% ethanol and resuspended in TE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA pH 8.0). Samples were added with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), vigorously shaken and centrifuged at 11000 g for 5 min at 4°C. The supernatant was transferred in a new tube and was added with 0.1 volumes of sodium acetate 3 M, pH 4.8 and 1 volume of cold isopropyl alcohol. Samples were kept at –80°C for 1 hour for RNA precipitation and were centrifuged at 18000 g for 15 min at 4°C. The supernatant was carefully decanted and the resulting RNA pellet was washed once with cold 80% ethanol and resuspended in

RNAse-free water. Subsequent DNAse treatment (10U of RQ1 RNAse-free DNAse; Promega, Milano, Italy) and column purification (RNeasy MinElute cleanup kit; Qiagen, Milano, Italy) were performed as described by Falchi et al. (2010). An aliquot of RNA samples extracted with the used protocols was quantified spectrophotometrically, and 500 ng of RNA were separated on a 1% agarose gel to check integrity and accurate quantification.

500 ng of total RNA and 1 µl of Oligo dT 10 µM in a total volume of 12,7 µl were incubated 5 min at 70°C and 5 min at 37°C. Thereafter 100 U of M-MLV reverse transcriptase (Promega, Milano, Italy), 25 U RNAse Inhibitor (Affymetrix, High Wycombe, UK) and dNTPs 10 µM each were added and mixture was kept at 37°C for 85 min, then for 5 min at 94 °C. Two independent reactions were pooled to minimise cDNA synthesis variation.

Primer design

Primers were designed with Primer3 (http://frodo.wi.mit.edu/primer3/; Rozen and Skaletsky 2000) and checked with OligoCalc (http://www.basic.northwestern.edu/biotools/oligocalc.html ; Kibbe 2007). Best resulting pairs were ranked by means of PRaTo (http://prato.daapv.unipd.it; Nonis et al. 2011) and the best hit was selected. OLEAESTdb BLAST (http://140.164.45.140/oleaestdb/blast.php; Alagna et al. 2009) against TCs database was performed to avoid multiple amplifications. Oligonucleotides were ordered deprotected and desalted (Sigma-Aldrich, Milano, Italy). Gene name, accession number, description, primer sequences and product size of reference genes used in this study are given in Table 1. For the two target genes PG (Polygalacturonase) and FPS (Farnesyl Pyrophosphate Synthase) the following primers were used: PG forward 5'-CATGGGAGTTCAGCATCAGA-3'; PG reverse 5'-GACAAGCAGCTATTTGGCTCA-3'; FPS forward 5'-GGGATCCTGAGGTGATTGGT-3'; FPS reverse 5'-TTTTCGCTACACAAGCAGGA-3'.

Gene name (in this study)	OLEAEST TC identifier or GenBank accession no.	Gene description	Primer sequences (forward/reverse)	Product size	PRaTo ALL score
14-3-3	OLEEUCl002244|Contig1	*14-3-3 protein*	TCCTGGGCTGATTTGTAAGC TCCAGTGGTGATTCCAAGGT	134	-4
ACT7a	OLEEUCl025648|Contig2	*Actin-7*	AACGGAATCTCTCAGCTCCA TTGCTTACGTGGCACTTGAC	123	-5
ACT7b	OLEEUCl004175|Contig3	*Actin-7*	GTGCTGAGGGATGCAAGAAT CCATGTTCCCAGGTATTGCT	142	-10
EF1a	OLEEUCl014934|Contig1	*Elongation factor 1-alpha*	CCTCTTGGACGATTTGCTGT CCTGTTGGCTCCTTCTTGTC	86	-1
EF1b	OLEEUCl018061|Contig5	*Elongation factor 1-alpha*	CCAAAGGTGACGACCATACC CTCTCCGTCTCCCACTTCAG	109	-3
GAPDH1	OLEEUCl022518|Contig2	*Glyceraldehyde-3-phosphate dehydrogenase*	CAGCTCTTCCACCTCTCCAG TCCATTGGCAAAGGTTCTTC	131	-4
GAPDH2	OLEEUCl004899|Contig2	*Glyceraldehyde-3-phosphate dehydrogenase*	CCTTCCGTGTGCCTACTGTT GATGGCTGCCTTGATTTCAT	92	-2
PP2A1	OLEEUCl021848|Contig2	*Serine/threonine-protein phosphatase 2A*	TGCAGTGGCTACAGGACAAG TGGACCAAATTCTTCAGCAA	83	-5
PP2A2	OLEEUCl021775|Contig2	*Serine/threonine-protein phosphatase 2A*	GGATGCCATATTCCCACAAC TGGTCCCATGAACAAAAGGT	75	-5
UBC1	OLEEUCl010470|Contig3	*Ubiquitin-conjugating enzyme*	GCCCTTATGCTGGAGGTGTA GGATGGAAAACCTTGGTCCT	100	-4
UBC2	OLEEUCl004061|Contig1	*Ubiquitin-conjugating enzyme*	TTGCAGAAAGACCCTCCTGT CTGTCCGTAGGTCCCATGAT	92	-8
UBQ	OLEEUCl002233|Contig1	*Ubiquitin*	GGTGGAATGCCCTCCTTATC GGGAAAACCATTACCCTTGAG	86	-2
OUB2	AF429430.1	*Polyubiquitin*	GCTGGAGGATGGAAGGACTC CCACGACTCAACAGAGACGA	191	-1

Table 1 Description and accession number of candidate reference genes, primer sequences, product size and PRaTo score (Nonis et al. 2011).

qPCR amplifications

Reactions were made manually mixing 5 µl of Fast SYBR® Green PCR Master Mix (Applied Biosystems, Monza, Italy), 0.2 µl of each primer (200 nM final) and 2.5 µl of cDNA (corresponding to 2.5 ng of total RNA) in a final volume of 10 µl. Each sample was run in triplicate. Reactions were set in 96-well fast plates sealed with optical foils and

loaded into a StepOnePlus (Applied Biosystems, Monza, Italy) platform. A first denaturation step at 95°C for 20 s was followed by 40 cycles made up of a denaturation at 95°C for 1 s and a combined annealing and extension step at 60°C for 15 s. After cycling, a melting curve analysis protocol from 60 to 95 °C was executed. For each sample a –RT sample was run once, and NTC was included in each plate and for each primer pair. Calibration curve was built for each target using 5 cDNA quantities (equivalent to 62.5, 12.5, 2.5, 0.5, 0.1 ng of total RNA). StepOne software (ver. 2.1; Applied Biosystems, Monza, Italy) was used to manage data including outliers identification, threshold settings and C_q exporting. MIQE précis guidelines (Bustin et al. 2010) were followed and details are provided in Supplementary File S1.

Putative reference genes stability and target genes expression determination

Two algorithms were used to assess expression stability: geNorm^{PLUS} (included in qBASE^{PLUS} package; trial version; Biogazelle, Zwijnaarde, Belgium; Vandesompele et al. 2002; Hellemans et al. 2007) and NormFinder (ver. 0.953; Andersen et al. 2004). Amplification cycles (C_q values) were exported with StepOne software, converted into correct input files and uploaded into software applications.

As a result, genorm^{PLUS} returned M and V parameters. M was defined as the average pairwise variation of a particular gene with all other control genes (Vandesompele et al. 2002). Under the assumption that putative reference genes are not co-regulated, a stepwise exclusion of the less stable gene is performed and M values of remaining genes are recalculated accordingly. Finally, the most stable genes were chosen among those displaying lowest M values, considering 0.5 as a good threshold (Hellemans et al. 2007). The parameter V represents the pairwise variation (V_n/V_{n+1}) between two consecutively ranked control genes. V cut-off threshold of 0.15 was recommended (Vandesompele et al. 2002) to determine the optimal number of reference genes.

NormFinder algorithm ranks putative reference genes according to SV parameter defined as the combination of inter- and intra-group variation (Andersen et al. 2004). Similarly to genorm^{PLUS}, genes with lowest SV are most stable ones.

qbase^{PLUS} was also used to calculate expression levels of PG and FPS in order to validate selected reference genes.

Results

Selection of a suitable RNA extraction method

For qPCR to be performed reliably, extracting total RNA of good quality and quantifying it correctly are mandatory requirements. To this end, we have preliminarily evaluated the methods currently used for RNA extraction from olive fruits and leaves for their performance in providing RNA of good quality. A few reports are available showing the successful use of column-based commercial kits to extract total RNA from *Olea europaea* fruit issues(e.g. Alagna et al. 2009; Muzzalupo et al. 2011; Santos Macedo et al. 2009). This method was evaluated for its ability to provide high quality RNA and was confirmed to be a fast and convenient approach for RNA extraction from fruit tissues stored at -80°C for a short period of time (around 1 month; Fig. 1a). However, when fruit samples were stored at -80°C for longer periods (3 months or longer; Fig. 1b) or when olive leaves were used as starting material, this method resulted to be not satisfactory (Fig. 1c). In these cases, even though the extracted RNA displayed distinguishable rRNA bands, a strong smear was visible clearly suggesting the presence of contaminants that may significantly hamper the following steps of cDNA synthesis and amplification. To overcome this technical constrain, two additional alternative RNA extraction methods were evaluated. The first one, based on the use of CTAB buffer and previously employed for grapes (Nonis et al. 2008), significantly improved the quality of the RNA obtained from fruits (Fig. 1d) but not of that obtained from leaves (Fig. 1e). The last method tested was based on the hot borate procedure described by Wan and Wilkins (1994), developed for RNA extraction from recalcitrant plant tissues and already used, with no modifications, on olive fruits (Conde et al. 2007). We have further improved this method to obtain RNA of high quality also from olive leaves by applying modifications, including a final column affinity purification step (as described in the Materials and Methods section). High quality RNA was reproducibly obtained from both long-term stored fruits (Fig. 1f) and leaves (Fig. 1g)

tissues by means of this method, which was used for all RNA extractions for subsequent experiments.

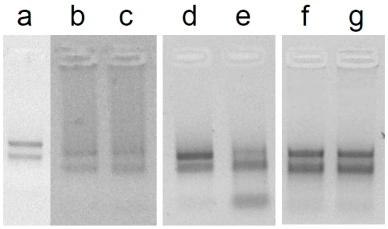

Figure 1. Total RNA integrity displayed by 1% agarose gel electrophoresis and SYBR SAFE (Invitrogen, Milano, Italy) staining. Lanes: a) fruit stored for less than 1 month and extracted by spin-column method; b) fruit stored for more than 1 month and extracted by spin-column method; c) leaf extracted by spin-column method; d) fruit stored for more than 1 month and extracted by Nonis et al. 2008 method; e) leaf extracted by Nonis et al. 2008 method; f) fruit stored for more than 1 month and extracted by this paper improved method; g) leaf extracted by this paper improved method.

Selection of candidate reference genes and evaluation of primer pairs performance

For the selection of candidate reference genes, we could not adopt a data mining approach involving searching publicly available microarray data repositories for genes that may display stable expression through different tissues, conditions and developmental stages, since this kind of data are still unavailable for *Olea europaea*. Therefore, previously validated reference genes from other species were recovered and orthologous genes were searched in the available olive OLEA EST db (http://140.164.45.140/oleaestdb/; Alagna et al. 2009) for their potential use as references for gene expression studies. To this end, an in-depth bibliographical search was performed to gather all studies regarding reference gene validations, with particular regard to those concerning horticultural species. Genes that were frequently ranked as best reference genes for a relatively high number of species were selected as potential candidates (Table 2). Among these genes elongation factor-1-α was reported as transcriptionally stable in at least 13 different species. Additional genes that were selected for their good transcriptional stability on the basis of literature data were Actin (3 species), Glyceraldehyde 3-phosphate dehydrogenase (5 species), Serine/threonine protein phosphatase 2A (4 species), and Ubiquitin (3 species) (Table 2). In this work 14-3-3 protein was considered as well, although it was ranked as one of the best only in coffee.

Best reference genes	Plant species
14-3-3	Coffea (Barsalobres-Cavallari et al. 2009)
Actin	Chicory (Maroufi et al. 2010) Grapevine (Reid et al., 2006) Pea (Die et al. 2009)
Elongation factor 1-α	Chicory (Maroufi et al. 2010) Chinese cabbage (Qi et al. 2011) Chinese cabbage (Qi et al. 2011) Chrysantemum (Gu et al. 2011) Fava bean (Gutierrez et al., 2011) Flax (Huis et al. 2010) Grapevine (Reid et al., 2006) Litchi (Zhong et al. 2011) Poplar (Brunner et al. 2004) Potato (Nicot et al. 2005) Rice (Jain et al. 2006) Tobacco (Schmidt and Delaney 2010) Zucchini (Obrero et al. 2011)
Glyceraldehyde 3-phosphate dehydrogenase	Coffea (Barsalobres-Cavallari et al. 2009) Flax (Huis et al. 2010) Grapevine (Reid et al., 2006) Litchi (Zhong et al. 2011)
Polyubiquitin	Blueberry (Vashisth et al. 2011) Cotton (Artico et al. 2010) Poplar (Brunner et al. 2004)
Serine/threonine protein phosphatase 2A	Arabidopsis (Czechowsky et al. 2005) Chrysantemum (Gu et al. 2011) Pea (Die et al. 2009) Tobacco (Schmidt and Delaney 2010)
Ubiquitin	Banana (Chen et al. 2011) Peach (Tong et al. 2009) Rice (Jain et al. 2006)
Ubiquitin-conjugating enzyme	Arabidopsis (Czechowsky et al. 2005) Blueberry (Vashisth et al. 2011)

Table 2. List of the candidate reference genes selected for this study and corresponding species for which they had been validated as best qPCR reference genes.

Polyubiquitin was tested not only for its good transcriptional stability in blueberry, cotton and poplar, but also for being already adopted in a previous work regarding olive fruit development and ripening (Vezzaro et al. 2012). Similarly, a ubiquitin-conjugating enzyme encoding gene was found to be a useful reference in peach (Nonis et al. 2007), validated with good results in *Arabidopsis* and blueberry and defined as one of the most stable genes in a maize atlas obtained with large scale microarray analyses (Sekhon et al. 2011). Tubulin was not studied since controversial data were reported on its reliability as a reference gene, considered one of the best in certain species, while reported among the worst ones, according to the ranking order, in others (Obrero et al. 2011; Schmidt and Delaney 2010; Zhong et al. 2011).

Since the selected candidate transcripts belonged to multigene families, two genes were chosen and tested for each gene family (Tab. 1). For polyubiquitin, only the already published mRNA was kept for the analysis (Vezzaro et al. 2012).

To retrieve olive expressed sequences, we referred to the Olea EST db (http://140.164.45.140/oleaestdb/; Alagna et al. 2009), selecting contigs made up of a high number of ESTs. Primer pairs were designed on these sequences (Tab. 1), each one producing an amplicon ranging between 75 and 142 bp in length (excluding the already designed pair for polyubiquitin producing an amplicon of 191 bp, as described by Vezzaro et al. 2012).

Primers were first tested on a pool of all olive cDNA samples used in this study. All primer pairs gave a melting curve with a unique peak (supplementary file S2), except for the second transcript chosen as 14-3-3 and UBQ. These two transcripts were discarded from subsequent analyses. To further test the primer pairs, dilution series of pooled cDNAs were amplified to calculate primers efficiency. Values ranged between 1.891 and 2.155, with a calculated standard error always lower than 0.007, and are reported in Table 3.

Gene	**Amplicon T_m (°C)**	**NTC**	**Standard curve slope (± SE)**	**r^2**	**PCR efficiency calculated from slope (± SE)**
14-3-3	78.32	Undetermined	-3.386 ± 0.019	0.996	1.974 ± 0.007
ACT7a	77.27	Undetermined	-3.301 ± 0.008	0.999	2.009 ± 0.003
ACT7b	78.31	Undetermined	-3.225 ± 0.010	0.998	2.042 ± 0.004
EF1a	76.97	37.21	-3.302 ± 0.008	0.999	2.008 ± 0.003
EF1b	78.32	37.15	-3.409 ± 0.005	0.999	1.965 ± 0.002
GAPDH1	77.12	Undetermined	-2.999 ± 0.009	0.998	2.155 ± 0.005
GAPDH2	75.64	Undetermined	-3.253 ± 0.012	0.997	2.029 ± 0.005
PP2A1	73.70	Undetermined	-3.535 ± 0.011	0.998	1.918 ± 0.004
PP2A2	72.34	Undetermined	-3.484 ± 0.016	0.997	1.937 ± 0.006
UBC1	72.80	37.12	-3.236 ± 0.009	0.998	2.037 ± 0.004
UBC2	77.26	Undetermined	-3.422 ± 0.015	0.996	1.960 ± 0.006
UBQ	80.85	Undetermined	-3.613 ± 0.014	0.998	1.981 ± 0.005
OUB2	77.27	37.06	-3.472 ± 0.009	0.999	1.941 ± 0.004

Table 3 Amplicon Tm, C_q of NTC and PCR efficiency calculated from standard curve slope of selected candidate reference genes

Expression stability of candidate reference genes

The selected primer pairs were used to amplify 21 cDNA samples, obtained from leaves and fruits from different developmental stages and treatments, using a sample maximisation strategy (Hellemans et al. 2007). In order to cover most of the olive fruit developmental stages, fruit mesocarp samples were collected starting from pit gardening until complete ripening (overripe fruits) and used for analyses. All data arising from these amplifications were used to calculate expression stability of the candidate reference genes.

A C_q box plot was obtained for each transcript (Fig. 2). The 13 considered genes covered a range between 17 and 31 C_qs. The coverage was quite complete except for a small gap around 22 C_q. This analysis enabled a first evaluation of the most stable genes, since a lower span in C_qs corresponds to a higher gene stability: for example PP2A2 had a limited span, thus a higher transcriptional stability across samples, compared with GAPDH1.

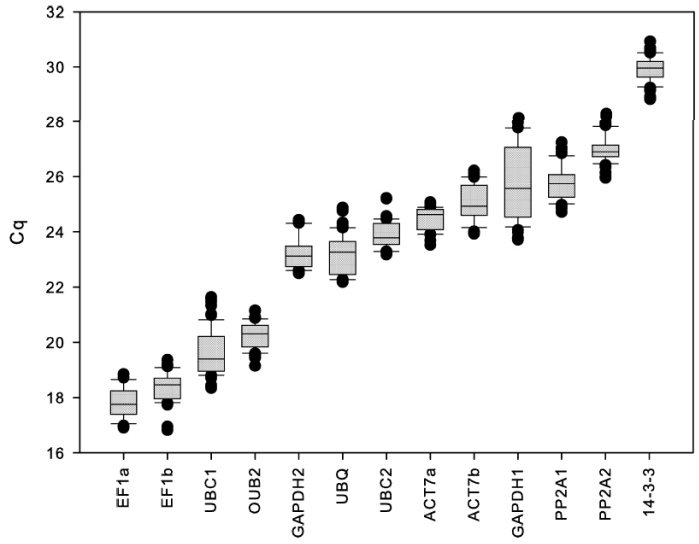

Figure 2. Box plot analysis of C_qs obtained in the whole sample set for each gene. Each box indicates 25/75 percentiles, with the median line dividing the box in two parts. Whisker caps represent 10/90 percentiles. Dots indicate outliers.

To validate quantitatively the putative reference genes in order to identify the most stable ones, two different independent algorithms were used: genorm^{PLUS} (Vandesompele et al. 2002, Hellemans et al. 2007) and NormFinder (Andersen et al. 2004). Analyses were performed first on all samples, then, in a second round, fruit and leaf data were split and considered separately. For what concerns genorm^{PLUS} analysis (Fig. 3a), 12 out of the 13

genes displayed an M value under the acceptable stability threshold of 0.5 indicated by Vandesompele et al. (2002).

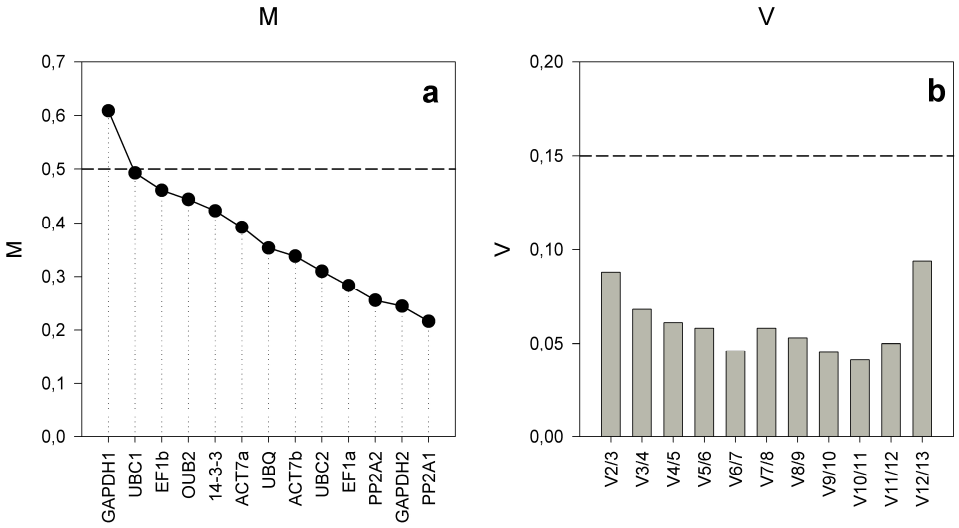

Figure 3. genorm^{PLUS} analysis of candidates reference genes in the whole sample set. Genes are ordered by descending M values (b), and minimum number of genes required for normalisation are indicated by means of V parameter (b). Threshold levels are indicated by dashed lines.

GAPDH1 was the least stable gene with an M greater than 0.5 (M=0.609). According to the V parameter (Fig. 3b), in our samples the optimal minimum number of reference genes for data normalisation resulted to be two ($V_{2/3}$= 0.088), because this was the minimum number of genes required to have a pairwise variation under the threshold of 0.15. Thus the two genes PP2A1 and GAPDH2, that resulted to be the most stable ones by displaying M values of 0.216 and of 0.244, respectively, could be sufficient to reach a good normalisation of data for this experimental set.

The same data processed by NormFinder algorithm showed consistently similar results (Fig. 4a) but with slight differences in ranking position, if compared with genorm^{PLUS}. Stability values (SV) of GAPDH1 (SV=0.694) and PP2A1 (SV= 0.065) confirmed them as the worst and the best genes in terms of transcriptional steadiness, respectively. PP2A2 (SV=0.066) was ranked as second in place of GAPDH2 (SV=0.106) that was fourth. NormFinder also indicated that SV decreased to 0.053 if the best two reference genes were used in combination in place of the best one alone (PP2A1, SV=0.065). Since samples could be divided into two subsets, corresponding to leaf and fruit tissues, NormFinder allowed to calculate both intergroup and intragroup variation (Fig. 4b).

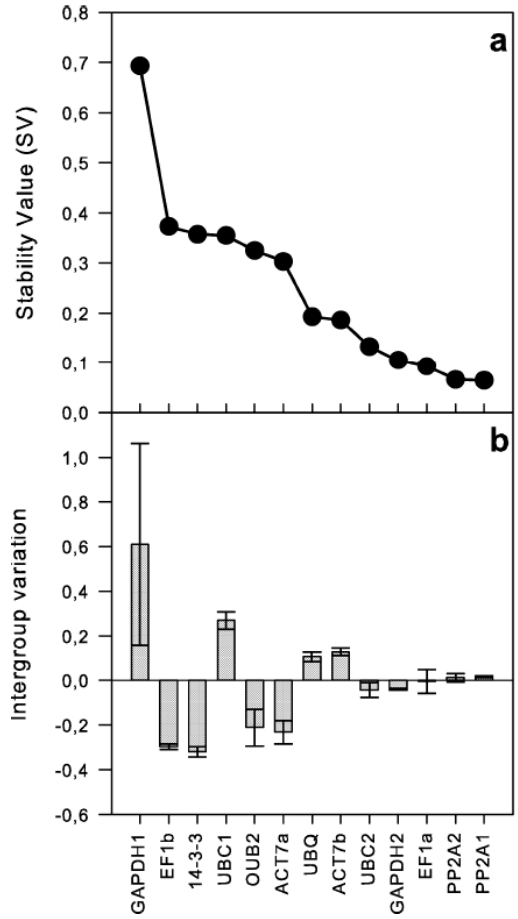

Figure 4. NormFinder analysis of candidates reference genes for the whole sample set. SV (a) and intergroup variation (b) are reported. Error bars (b) represent intragroup variation.

A positive intergroup variation was given when the gene showed systematically higher expression in fruits than in leaves, while a negative intergroup variation was given in the opposite case. Intragroup variation is represented by error bars in Figure 4b. From this analysis it was evident that the most stable genes PP2A1, PP2A2, EF1a, GAPDH2 and UBC2 in Fig. 4a displayed intergroup variation close to zero (Fig. 4b) having comparable stability in fruits and leaves.

genorm^{PLUS} and NormFinder analyses were then conducted separately in the two subsets of fruits and leaves. As far as fruits are concerned, the two algorithms gave the same results: GAPDH1 was the less stable gene (M=0.438; Fig. 5a) (SV= 0.672; Tab. 4) and PP2A1 (M=0.173; Fig. 5a) (SV=0.060; Tab. 4) the most stable one. According to the genorm^{PLUS} V parameter, the optimal number of reference genes was two (Fig. 5c): EF1b (M=0.190;

Fig. 5a) and GAPDH2 (SV=0.062; Tab. 4) were ranked at the second place by genorm^{PLUS} and NormFinder, respectively.

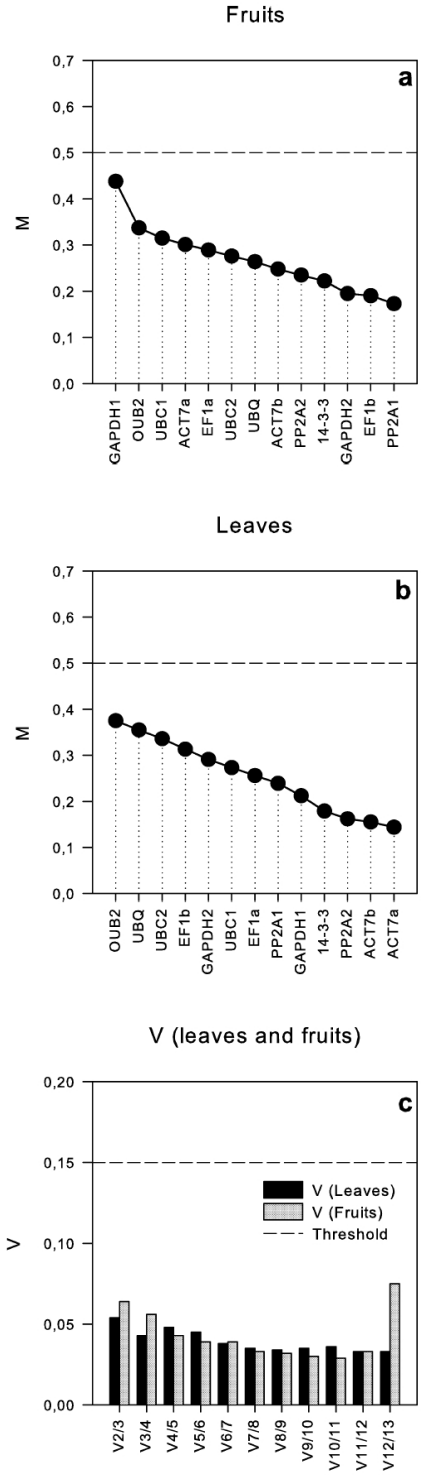

Figure 5. genorm^{PLUS} analysis of candidates reference genes in fruits and leaves samples. For each tested gene parameter M is reported in fruits (a) and leaves (b). Minimum number of reference genes to be used is indicated by V parameter for both fruits and leaves (c). Threshold levels are indicated by dashed lines.

In leaves, genorm^{PLUS} indicated that two genes were needed as well (V=0.054; Fig. 5c). ACT7a (M=0.144) and ACT7b (M=0.155) were the most stable ones (Fig. 5b). OUB2 was the least stable (M=0.375), even if it was still below the stability threshold of 0.5 (Fig. 5b). Comparably, OUB2 was the last in the ranking order in NormFinder too (SV=0.295; Tab. 4). Differently, the two most stable genes for leaves in NormFinder calculations (Tab. 4) resulted to be 14-3-3 (SV=0.045) and PP2A2 (SV=0.106).

Fruits				**Leaves**		
Gene name	**Stability value**	**Standard error**		**Gene name**	**Stability value**	**Standard error**
PP2A1	0.060	0.030		14-3-3	0.045	0.033
GAPDH2	0.062	0.030		PP2A2	0.106	0.037
EF1b	0.116	0.033		GAPDH1	0.120	0.039
ACT7b	0.129	0.035		ACT7a	0.135	0.043
PP2A2	0.138	0.036		ACT7b	0.138	0.043
UBQ	0.147	0.038		PP2A1	0.149	0.046
14-3-3	0.153	0.038		EF1a	0.174	0.051
UBC2	0.183	0.043		GAPDH2	0.222	0.063
UBC1	0.198	0.046		UBC2	0.227	0.064
ACT7a	0.227	0.051		UBC1	0.232	0.066
EF1a	0.232	0.052		UBQ	0.246	0.069
OUB2	0.287	0.062		EF1b	0.263	0.073
GAPDH1	0.672	0.138		OUB2	0.295	0.081

Table 4. NormFinder ranking of putative reference genes for fruit and leaves tissues considered separately.

Reference genes validation in fruit

In order to test the quality of reference genes validated in our sample set, two transcripts were quantified throughout olive fruit development and ripening.

For this aim a putative polygalacturonase(PG; EC 3.2.1.67) and farnesyl pyrophosphate synthase (FPS; EC 2.5.1.1) were selected. These genes have not been previously studied in olive and were chosen on the basis of their specific pattern of accumulation of mRNA levels during development of tomato berry, the model fruit for climacteric fruit ripening. PG is considered a classical marker of fruit ripening and loss of firmness in fleshy fruits, since its expression increases significantly in the last stages of fruit ripening along with firmness decay not only in tomato (Dellapenna et al. 1986; Tacken et al. 2010). FPS was

selected for its peculiar bifasic accumulation pattern in tomato berries: high mRNA levels on young fruits and then a peak at the "late breaker" stage (Gaffe et al. 2000).

The accumulation of PG and FPS transcripts during fruit development was referred to PP2A1 (the best one according to genorm^{PLUS} and NormFinder), GAPDH1 (the worst one) and to the combination of the best two ones (PP2A1 and GAPDH2) to highlight the effect of the choice of reference genes on their expression patterns. For what concerns PG, transcript accumulation was steady until 116 DAFB, then rapidly and progressively increased reaching a maximum at 160 DAFB with an induction that resulted to be 200 or 1600 times the level of the first sampling date, when the worst or the best ranked gene was used as reference, respectively (Fig. 6a).

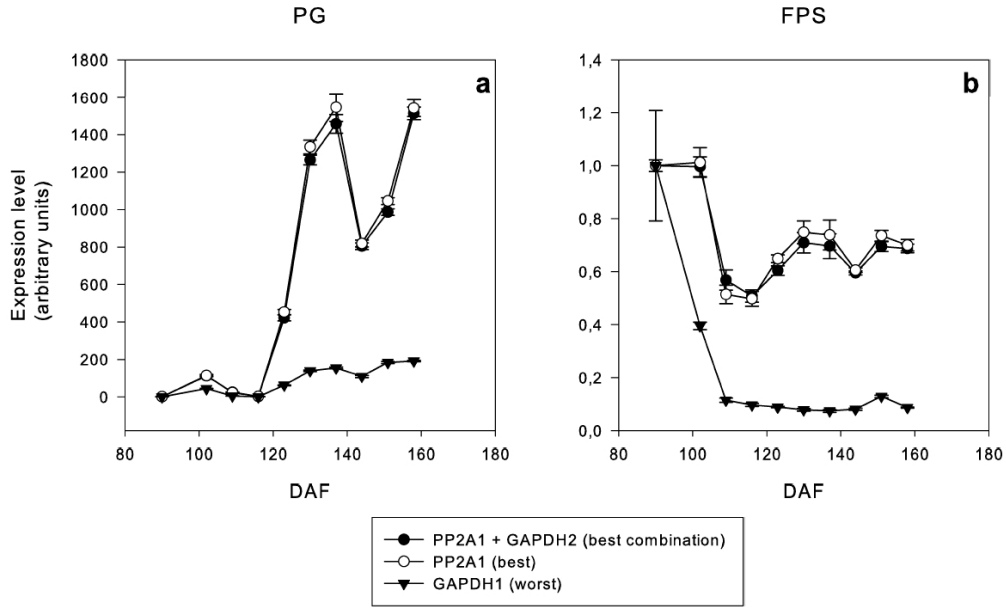

Figure 6. Expression profiles of PG and FPS during fruit development by means of PP2A1 (the best reference gene), GAPDH1 (the worst one) and PP2A1 + GAPDH2 (the best two indicated by genorm^{PLUS}). Expression levels are indicated in arbitrary units and the sample at 90 DAFB is used as a reference setting the expression value to 1. Errors bars represent SE.

FPS showed even more evidently different profiles if GAPDH1 or PP2A1 were used (Fig. 6b). In the first case, FPS expression pattern decreased till 109 DAFB and then remained overall stable throughout ripening. On the contrary, when PP2A1 was used as reference, the olive FPS transcriptional profile reflected the typical bifasic profile of tomato FPS, showing high levels of transcript accumulation during the first phase of fruit growth and then a decline with a transient peak in the late stages of *véraison*.

For both genes no significant differences could be observed when the combination of the top two reference genes was adopted as reference instead of the best one alone.

Discussion

Commercial interest on olives and olive oil is pushing forward molecular studies on this plant species. Recently, significant steps ahead have been made in olive transcripts identification (Alagna et al. 2009; Galla et al. 2009) and well-established strategies for mRNA quantification are therefore needed. In the last years, qPCR has become the preferred method in gene expression studies, even if this technique is far from being defined as a "gold standard". Recent papers have set the minimum information needed for publication of qPCR experiments (MIQE; Bustin et al. 2009, Bustin et al. 2010), but few released studies were performed complying with those rules to date (Huggett and Bustin 2011). Many aspects can affect qPCR experiments importantly, one of these being the quality of the RNA template. Therefore, as a preliminary step, we have evaluated the most commonly used current protocols for RNA extraction from olive fruit and leaf tissues on the basis of the quality of the RNA they provided. A comparison among three different methods pointed out that they could not yield good quality RNA from leaves nor from fruit tissues stored at –80°C for long periods of time. Therefore an optimised protocol was set by applying modifications to the hot borate protocol described by Wan and Wilkins (1994) and adding a final purification step on silica columns that permitted to obtain RNA of satisfactory quality consistently from both leaves and fruit tissues stored for long periods.

An additional major factor that affects dramatically the accuracy of qPCR results is the selection of reference genes for data normalisation to eliminate non-biological variation. It is widely known that the choice of internal controls may be one of the most critical points, because a wrong choice may lead to misleading results (Dheda et al 2005). It has been established that the so called "housekeeping genes" do not exist (Vandesompele et al. 2002), especially when samples coming from very different experimental conditions are compared. Thus, during the last years the number of preliminary screenings on candidate reference genes have grown rapidly. These works have been important as a starting point in qPCR studies, as they have provided guideline for the careful selection of reference

transcripts, allowing a more efficient use of resources and time. To this end, in this paper we have selected 13 potential reference genes and we have tested them in 21 olive samples belonging to 2 different tissues, namely mesocarp of fruits at 10 successive developmental stages ranging from 90 DAFB until full ripening (160 DAFB), fruits treated with propylene or with the inhibitor of ethylene action 1-MCP and leaves subjected to abiotic stress (wounding) in combination with treatments with propylene or 1-MCP. Since extensive microarray expression data are not available for olive, the selection of the candidate reference transcripts for this work was made by choosing olive orthologs of the best ranked reference genes from other crops. All the most common reference genes were included in our analysis, except tubulin and 18S rRNA. These two genes were discarded because their transcriptional stability appeared to be quite controversial (Obrero et al. 2011), especially in the case of 18S (Tricarico et al. 2002; Vandesompele et al. 2002). Overall, this process has lead to the identification of 13 *Olea europaea* potential reference genes, belonging to 8 gene families.

To evaluate these genes in terms of transcriptional stability, qPCR experiments were designed to follow to the best MIQE précis guidelines (Bustin et al. 2010). Due to the lack of genomic data, primers pairs were designed on the available Olea EST db sequences (Alagna et al. 2009). The selected genes were first subjected to melting curve analysis to exclude the presence of multiple amplicons and the amplified products showed a single peak after the melting curve protocol was applied. By plotting the C_q distribution for each selected gene, it has been possible to gain hints on which genes had the lowest variation in all samples. It appeared clear that GAPDH1, which showed the widest variation (roughly a span of 4 C_q) was the least stable gene and this finding was in fact confirmed by the two algorithms used at a later stage. This analysis was also useful to demonstrate that gene expression stability was independent from the average C_q, as more stable genes were not specifically associated to high or low C_qs. Furthermore, the selected genes covered a wide C_q range, thus representing a set of reference genes covering a wide range of transcript abundance that may be chosen on the basis of a similar C_q with differently abundant targets, since an ideal reference gene should have an expression level comparable to that of the target genes under investigation (Bustin 2000).

In order to determine the best reference genes in this experimental plot, expression values were analysed by two algorithms, each based on different calculation principles, namely genorm^{PLUS} (Vandesompele et al. 2002; Hellemans et al. 2007) and NormFinder (Andersen et al. 2004). Considering all samples, genorm^{PLUS} classified 12 genes, out of the 13 selected in total, as stable. This percentage of success was quite high if compared to previous works. Probably this could be explained also by the fact that the whole procedure was optimised, starting from an high-quality RNA to the subsequent steps, thus minimising non-biological errors. The most stable gene was PP2A1, that was firstly identified in whole transcriptomic studies carried on *Arabidopsis* (Czechowski et al. 2005) and shown to have high stability also in other species. Also NormFinder analysis, even though not providing a cutoff value, ranked PP2A1as the most transcriptionally stable and the best one considering the fruit development sample series. The genes that had the best and the worst values in the ranking were consistently identified by both softwares, thus highlighting the robustness of their transcriptional stability and their value as reference genes. Nonetheless, some minor discrepancies could be observed in the resulting lists: for example GADPH2, which ranked second in genorm^{PLUS} order, was fourth in NormFinder. However, this inconsistency is a normal effect due to the different algorithms used, as already observed previously (Cruz et al. 2009; Tong et al. 2009), that results on minor differences in the ranking order between genes characterised by similar stability but that does not reflect significant differences in terms of transcriptional steadiness.

For what concerns leaves, different ranking orders were observed between the two algorithms. It has to be pointed out that all the 13 transcripts were found under the acceptable threshold of 0.5 in genorm^{PLUS}, so the differences in terms of stability were extremely low and this could explain why the two algorithms ranking orders did not overlap in leaves. On the other hand, similar divergences were also experienced previously (Lin and Lai 2010).

As a remark, splitting the two tissue subsets demonstrated once again that it is mandatory to validate internal controls for each experimental plot/subset. This was also supported by NormFinder analysis where it was possible to determine the intergroup variation. For genes having a SV > ~0.2, intergroup variation was quite far from zero, indicating that those

genes showed systematically higher expression levels in one subset compared with the other (Andersen et al. 2004).

Regarding the number of reference genes to be used, in this study two genes were shown to be enough for normalisation according to genorm^{PLUS} analyses. This result was valid for the overall dataset and also for both fruits and leaves considered separately. Notwithstanding multiple reference genes have been considered a gold standard for qPCR data normalisation, it was frequently observed that two was the optimal number in several experimental plots (e.g. Chen et al. 2011; Lin and Lai 2010; Obrero et al. 2011).

To further demonstrate the importance and reliability of the reference genes identified in this work, especially for fruit development studies, the expression profiles of two fruit development- and ripening-related genes (PG and FPS) were studied. The identified profiles were similar to the ones already described for their orthologs in tomato fruit, when either the best reference gene or the best combination (two best reference genes combined) was considered. On the contrary, a strong difference in the resulting pattern was observed when the worst internal control was used instead of the best one, further highlighting the fact that using validated reference genes is mandatory for qPCR studies to be reliable and to avoid pitfalls.

Conclusions

In this work two essential steps for performing optimal qPCR experiments in olive were studied. An optimised protocol for RNA extraction from olive leaf and fruit tissues was implemented. Furthermore a list of validated primers pairs for amplification of putative reference genes for use in future qPCR studies in olive was evaluated and their effectiveness was tested with success on a fruit developmental series. To the best of our knowledge, this is the first study providing validated reference genes in olive. This work will be of general use for future qPCR studies, especially for those dealing with molecular aspects of olive fruit development and ripening.

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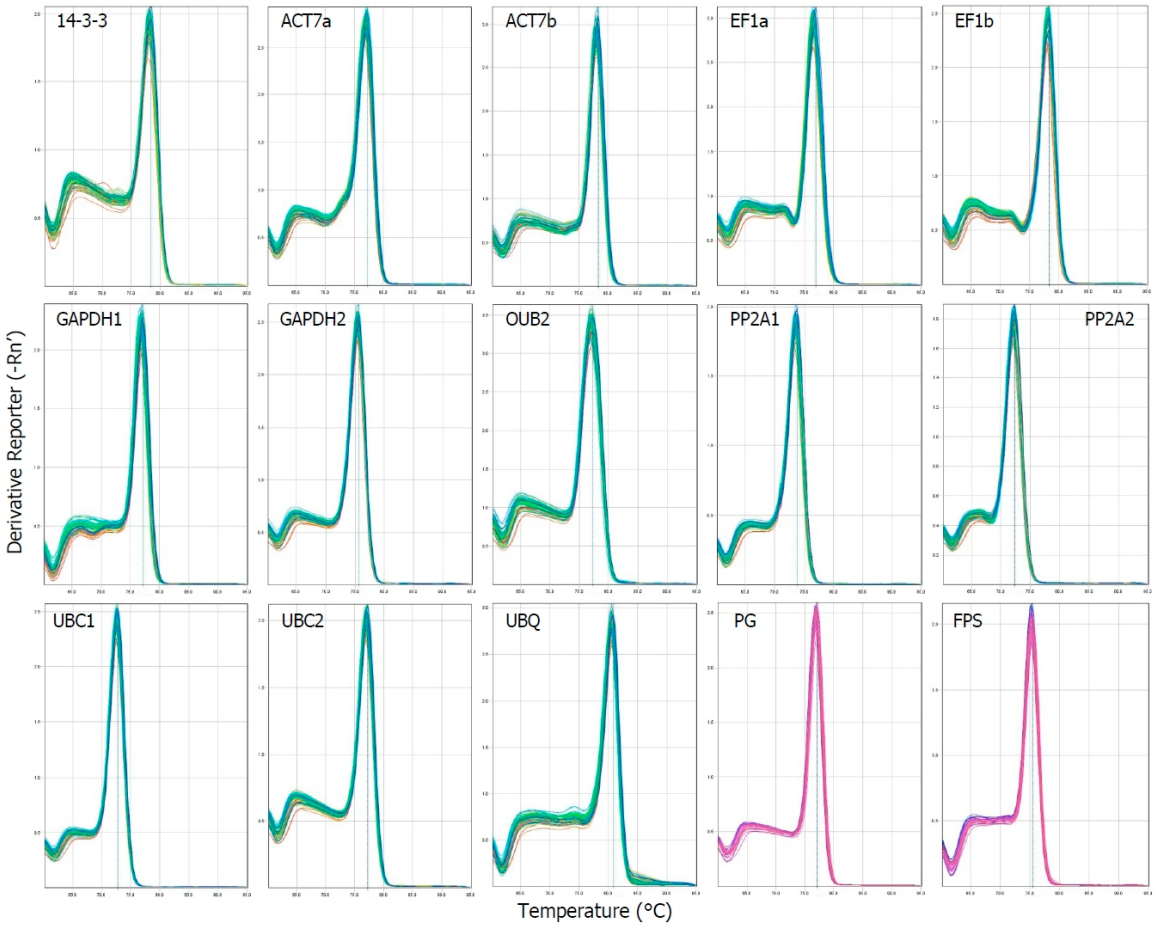
Supplementary material

Supplementary file 1. MIQE précis checklist (Bustin et al., 2010)

Sample/template	
Source	Olive (*Olea europaea* L.) leaves and fruits
Method of preservation	Frozen in N_2 and stored at -80°C
Storage time	Less than 6 months
Handling	Frozen
Extraction method	See Material and Methods section
RNA: DNA-free	-RT control
Concentration	Spectrophotometer
RNA: integrity	Agarose gel electrophoresis
Inhibition-free	Dilution series
Assay optimization/validation	
Accession number	See Tab. 2
Amplicon details	See Tab. 2
Primer sequence	See Tab. 2
Probe sequence	Not applicable
In silico	PRaTo analysis, BLAST against Olea EST db
Empirical	See Material and Methods section and Tab. 3
Priming conditions	Oligo-dT
PCR efficiency	See Tab. 3
Linear dynamic range	3 $\log_{10}$
Limits of detection	Below 0.04 µl of cDNA for each assay
Intra-assay variation	< 0.5 Cq
RT/PCR	
Protocols	See Material and Methods section
Reagents	See Material and Methods section
Duplicate RT	Pooling of three independent RT
NTC	See Tab. 3
NAC	Not applicable
Positive control	Performed as dilution curves
Data analysis	
Specialist software	genorm^{PLUS}, Norm finder
Statistical justification	Sample maximization strategy
Transparent, validated normalisation	genorm^{PLUS}, Norm finder

Supplementary file 2. Melting curves of all the analysed transcripts.



Chapter VI

General conclusions and future perspectives

General conclusions and future perspectives

The comprehension of factors underlying olive oil quality is appealing and intriguing, besides being useful for olive growers and oil producers. Above all, the complex phenomenon of ripening must be fully understood and read properly to close the circle on olive oil quality, since this factor is the most important one and must therefore be taken carefully into consideration.

Different approaches have been adopted for studying olive ripening and some aspects regarding the development of olive oil flavour profile. Starting with technological operations, anoxic extraction has been carried out in each step of olive milling, with the purpose of unravelling the influence of the absence of oxygen on the overall VOC fingerprint of Grignano oils extracted from olives harvested at successive ripening stages, from *véraison* until late ripening. Multivariate analyses allowed to distinguish some clusters of peaks in the PTR-MS profiles which had a clear ripening-dependent regulation. This finding is a preliminary demonstration that the ripening stage has a prevalent effect on the definition of the volatile profile of Grignano, and therefore that technological aspects must integrate a deep knowledge on ripening-related characteristics of fruit to be milled. These findings also suggest that the determinants of olive flavour profiles are significantly dynamic, and show a specific pattern of increase/decrease in mass counts throughout drupe ripening. Moreover, a kind of saturation effect in spectra was observed in ripe and overripe samples, being variability among samples extracted in nitrogen atmosphere higher for olives picked at the onset of ripening.

These results taken together clearly point out that in Grignano ripening related events are extremely rapid and dynamic, since significant changes in volatile profile take place in short time frames.

Ripening parameters analyses further reinforced this concept, showing that some of the classical ripening markers such as chlorophyll content and loss of firmness change rapidly and too early with regard to fruit ripening and thus are not suitable to describe properly the progression of ripening. Oil accumulation in drupes showed different trends in two successive harvest seasons, confirming that seasonal effects may affect significantly the definition of ripening-related variables. Frantoio olives showed more variability in oil content data in both seasons compared to cv. Grignano, and for Grignano samples across

and after *véraison* important changes in oil content occurred at a more rapid rate, being sampling at weekly intervals not enough to capture the variations in this parameter. This phenomenon anyway did not show a comparable trend in colorimetric data. These results globally underline that Grignano cultivar has a peculiar ripening profile, which must be monitored carefully to obtain high quality oils. This cultivar seems in fact to ripen in a shorter time if compared to cv. Frantoio, confirming to undergo an earlier onset of ripening.
The findings discussed above suggest that a model of the ripening physiology of olive cultivars in Veneto can be designed in the future by taking into consideration oil accumulation and colorimetric parameters. An exhaustive sampling of drupes for more successive seasons and subsequent analyses will allow to exclude seasonal/climatic effects on ripening events, helping to build an objective model of olive ripening. Correlation analyses between oil content data and the different colour indices considered in this study will be helpful to define the best colour index, easily available to olive growers, which may assist them in the choice of the best period for harvesting.
Finally, part of this dissertation has been devoted to the first study of terpene metabolism in olive. Terpenes are a fascinating class of molecules, synthesised by the a wide group of enzymes (terpene synthases – TPS) and characterised by a huge diversity and chemical complexity, and are important components of the aromatic profile of many fruits. Three olive terpene synthases were isolated which showed a clear induction in gene expression with the progression of ripening, and also in response to wounding stress. Geraniol, actively produced *in vitro* by one of the isolated enzymes, has not been to date reported among olive oil volatiles, and therefore it will be of primary importance to define whether this compound may be present in olive oil headspace in another chemical structure possibly by undergoing chemical modifications. The isolation and characterisation of three novel *Olea europaea* terpene synthases reported in this work lay the foundations for future studies on olive terpene biochemistry. The isolation and characterisation of further olive TPSs will be useful for a deeper understanding of olive terpenic metabolism, which is at present largely unknown, with relation to genetic differences and ripening stages.

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