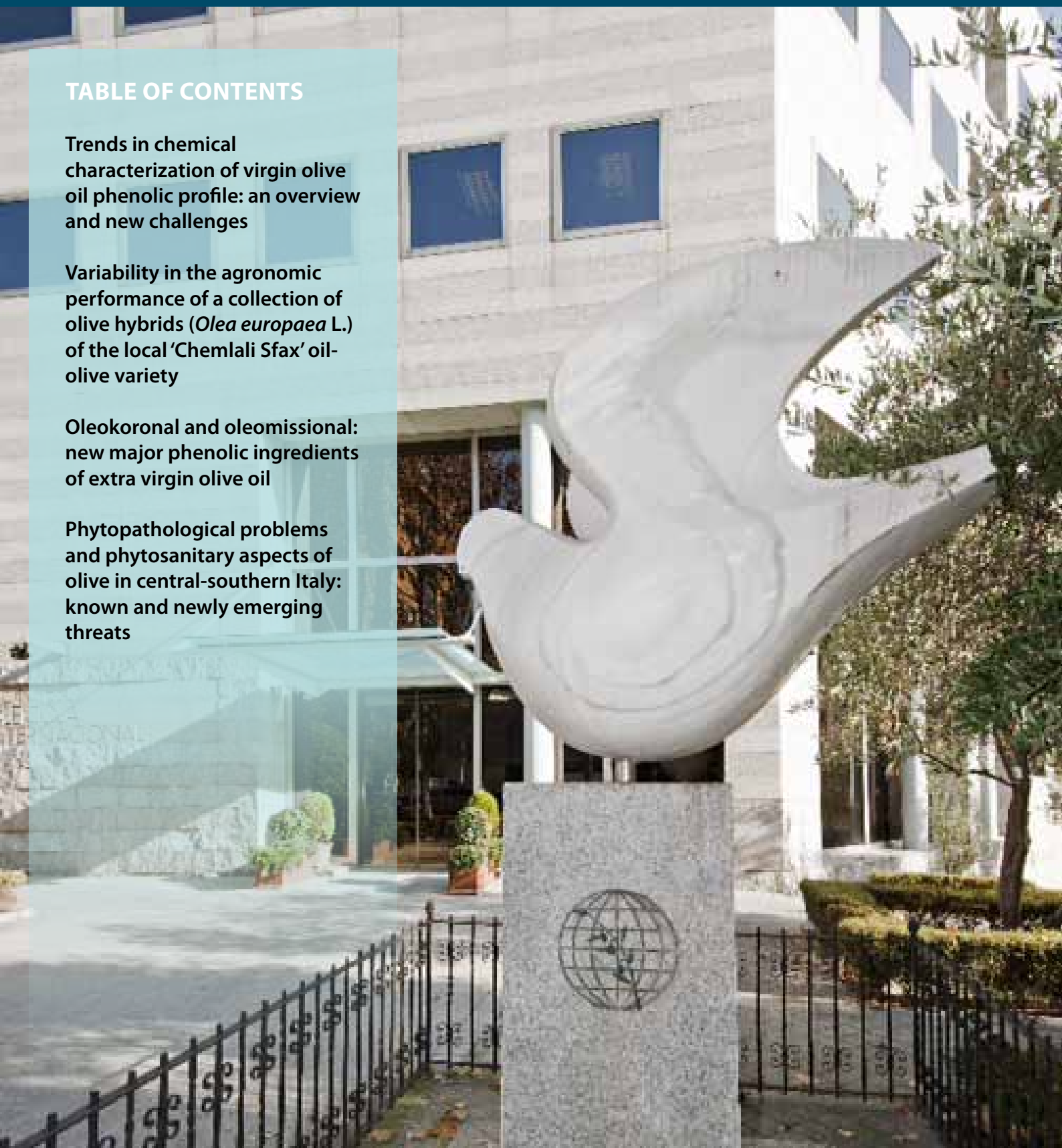
LIVAE

No 122
ENGLISH ED.
NOVEMBER 2015

OFFICIAL JOURNAL OF THE INTERNATIONAL OLIVE COUNCIL



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OLIVAE I No122

OLIVAE
Official Journal of the International Olive Council

Published in Arabic, English, French, Italian, Spanish.
Peer reviewed journal

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ISSN: 0255-996X
Registration: M-18626-1984

Trends in chemical characterization of virgin olive oil phenolic profile: an overview and new challenges

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Abstract

Due to the important role played by phenolic compounds in the nutritional value of virgin olive oil (VOO), it is necessary to develop efficient and accurate analytical methods for their qualitative and quantitative analysis. This review presents an overview of different analytical approaches to the determination of phenolic compounds in VOO. In principle, the analytical procedure for the determination of individual phenolic compounds in VOO involves three basic steps: extraction from the oil sample, chromatographic separation, and characterization. The extraction systems in widest use are liquid-liquid extraction (LLE) and solid-phase extraction (SPE). Among the separation techniques reported, high performance liquid chromatography (HPLC) was the most widespread technique applied for the analysis of phenolic compounds. However, it was demonstrated that gas chromatography (GC) and capillary electrophoresis (CE) are able to achieve the same aims as HPLC while providing alternative methodologies for the characterization of phenolic compounds in VOO. The optimized parameters, advantages and disadvantages of each technique are reported in this review. In addition, the different detectors coupled to the separation techniques are reviewed. Finally, the current analytical problems in the determination of phenolic compounds in VOO are also presented. In order to overcome these problems, researchers have to take into consideration the drawbacks of the previous methods. The future challenge will be to establish one single method for application to all VOO studies relating to phenolic compounds.

Keywords

VOO, phenolic compounds, extraction, separation techniques, analytical problems.

Received: June 2015 / **Accepted:** July 2015 / **Published:** November 2015

Introduction

Virgin olive oil (VOO) is increasingly popular worldwide, not only because of its unique sensory characteristics but also because of the beneficial health effects associated with its consumption, particularly as part of the Mediterranean diet. The health-promoting effects of olive oil have been attributed to its fatty acid profile, as well as to the presence of many bioactive components such as tocopherols, phospholipids and phenolic compounds. In fact, several biological functions and properties have been ascribed to phenolic compounds. In human studies, olive oil rich in phenolic compounds has been shown to improve antioxidant and anti-inflammatory effects and to reduce the proliferation of cell adhesion molecules compared with low-phenolic compound olive oils (Covas 2007; Fitó and de la Torre *et al*., 2007). In 2011, the European Food Safety Authority (EFSA) endorsed a claim regarding the effectiveness of olive oil phenolic compounds (5 mg/day) in protecting blood lipids from oxidative damage (Franco and Galeano-Díaz *et al*., 2014).

At least thirty-six structurally distinct phenolic compounds have been identified in VOO. Hydrophilic phenols, such as phenolic alcohols, phenolic acids, lignans, flavonoids and secoiridoids, are the most important class of natural antioxidants found in VOOs. Unfortunately, their concentration is not constant in VOO but varies depending on many factors including olive cultivar, fruit ripening stage, irrigation management, and pedoclimatic conditions in the growing area (Bajoub and Carrasco-Pancorbo *et al*., 2015; Bakhouche and Lozano-Sánchez *et al*., 2015; Dabbou and Chehab *et al*., 2010). Additionally, several studies have reported the effect of different stages of VOO processing, such as crushing, malaxation, centrifugation, storage and filtration, on VOO phenol composition (Bakhouche and Lozano-Sánchez, *et al*., 2014a; Frankel and Bakhouche *et al*., 2013).

Owing to the continuous variation in phenolic compounds in VOO under the effects of the factors mentioned above and to the need for correct discrimination of the richest VOOs from the poorest ones in terms of phenolic compound content, several analytical methods have been proposed to determine phenolic compounds using different extraction, separation and qualitative and quantitative characterization techniques. Two main methods have been employed for phenolic recovery: liquid-liquid extraction (LLE) and solid-phase extraction (SPE). Different solvent mixtures have been tested in the former method and different types of sorbent in the latter to maximize the recovery of phenolic compounds from VOO (Bendini and Bonoli *et al*., 2003). In the case of phenolic characterization, high performance liquid chromatography (HPLC) is the main technique used for the separation of phenolic compounds (Bayram and Esatbeyoglu *et al*., 2012). Other techniques such as gas chromatography (GC) and capillary electrophoresis (CE) have also been reported (Ballus and Meinhart *et al*., 2011; García-Villalba and Pacchiarotta *et al*., 2011). The separation techniques cited are coupled to different detectors. UV–visible diode array detection (DAD) is the standard method used for phenolic compounds and, together with mass spectrometry, is the predominant system nowadays (Bakhouche and Lozano-Sánchez *et al*., 2013). Nuclear magnetic resonance spectroscopy (NMR) has also been used as a detector in the characterization of phenolic compounds in VOO; however, due to its high cost, it is only available at a limited number of institutions (Etrakis and Giomyrgianaki *et al*., 2008).

Although many methods have been optimized to determine the concentration of phenolic compounds in VOO, direct comparison of the data available in the literature is still difficult because the reported concentrations often differ greatly. The explanations put forward, for instance that various agronomical and technological factors might affect VOO phenolic concentration, may account for these discrepancies, but only in part. However, some authors have suggested that the discrepancies observed in VOO concentrations might be caused by the analytical methods used (Bakhouche and Lozano-Sánchez *et al*., 2014b; Karkoula and Skantzari *et al*., 2012). Consequently, the objectives of this review were: first, to provide an overview of the main extraction and separation methods used in the analysis of phenolic compounds in VOO; second, to highlight the drawbacks of the most cited methods in order for them to be taken into account in future studies of phenolic compounds in VOO.

Methods for the extraction of phenolic compounds from virgin olive oil

Isolation from the sample matrix is generally a prerequisite for any comprehensive analysis scheme. Its main objective is to prepare a sample extract that is uniformly enriched in all the compounds concerned and free from the interfering matrix component. LLE and SPE are the two systems reported the most for the extraction of phenolic compounds from VOO. These systems vary not only in the solvents and/or solid-

phase sorbents used but also in the quantities of sample needed for analysis, and the volumes of the solvents.

Liquid-liquid extraction

The phenolic compounds in olive oil have mostly been isolated by extracting an oil solution with methanol or methanol/water mixtures. Before extraction, the liquid-liquid procedure involves a pre-step in which the VOO is dissolved with an apolar solvent in order to remove the lipid fraction and make it easier to extract the phenolic compounds with the polar solvent. Hexane, petroleum ether, and chloroform are used for this purpose; however, hexane is the most widely reported (Lerma-García and Lantano *et al*., 2009; Montedoro and Servili *et al*., 1992). In the case of extraction solvents, controversial data have been reported in the literature as regards the best solvent for the complete recovery of the phenolic compounds from VOO. Initially, extraction with methanol/water 80:20 seemed to give better results than absolute methanol or methanol/water 60:40 (Montedoro and Servili *et al*., 1992). However, five years later, absolute methanol was chosen to extract phenolic compounds from VOO instead of methanol/water owing to incomplete extraction of some phenolic compounds when the latter mixture was used as extraction solvent. This hypothesis could be attributed to the considerable formation of emulsion between the water and oil (Angerosa and D'Alessandro *et al*., 1995). Other studies demonstrated that reducing the percentage of methanol to 60% increased the recovery efficiency of phenolic compounds (Ballus and Meinhart *et al*., 2014; Pirisi and Cabras *et al*., 2000). The use of organic solvents other than methanol in LLE has also been reported. Examples are ethanol, acetonitrile, and *N*, *N* dimethylformamide (DMF), the last of which seemed to show interesting results in terms of recovery efficiency (Brenes and Garcı *et al*., 2000).

The LLE system could be divided into different categories depending on the amount of sample and solvent used in the extraction of phenolic compounds from VOO. The conventional system was characterized by the use of large amounts of sample and organic solvents, which made it laborious, expensive and time-consuming (Gómez-Caravaca and Carrasco Pancorbo *et al*., 2005). Due to these disadvantages, a new LLE system named liquid–liquid microextraction (LLME) was developed in place of the conventional one. It could be considered a miniature version of conventional LLE because it required a smaller amount of sample and generated a smaller volume of residues; it was also faster. The application of this method to extract phenolic compounds from VOO was reported in the literature. In a comparative study, LLE and LLME were evaluated in terms of repeatability, reproducibility, and phenolic compound recovery. The results showed that both methods had a good repeatability and reproducibility. However, LLE gave lower values than LLME for the total phenols extracted from VOO (Pizarro and Becerra *et al*., 2013). In a more recent study, the same LLME was improved by reducing the volume of extractant from 1 ml to 0.5 ml in order to characterize the phenolic compounds in VOO using ultra high performance liquid chromatography-triple-quadrupole mass spectrometry (Becerra-Herrera and Sánchez-Astudillo *et al*., 2014). Other liquid-liquid extraction techniques have also been developed and applied for the extraction of phenolic compounds from VOO, such as dispersive liquid–liquid microextraction (DLLME), and reversed phase dispersive liquid–liquid microextraction (RP-DLLME) (Godoy-Caballero and Acedo-Valenzuela *et al*., 2013).

Solid-phase extraction

The SPE technique has become more popular in the last decade as a step for the isolation of phenolic compounds from VOO. It has been applied using several types of sorbents. For instance, C8 cartridges (500 mg, 3.5 mL, Alltech) were used for the isolation of phenolic compounds from VOO and were found to be fast and simple (Pirisi and Cabras *et al*., 2000). A year later, diol-bonded phase cartridges and amino-phase cartridges were compared to determine which type was the best for the extraction of these analytes. The authors ruled out the amino-phase cartridge due to the appearance of some compounds in the extract as a result of an artifact originated during extraction through interactions between the solvent and the amino phase (Mateos and Espartero *et al*., 2001). Two other commercial cartridges, octadecyl C_{18} (2g, 6ml) and octadecyl $C_{18\ EC}$ (end capped; 2g, 6 ml) have been applied for the extraction of phenolic compounds from VOO. A comparative study of both cartridges was carried out. The results reported unsatisfactory recoveries with $C_{18\ EC}$ whereas the C_{18} cartridges resulted in practically full quantitative recovery of all the compounds examined. The authors attributed the differing behavior of the C_{18} and $C_{18\ EC}$ cartridges to the interaction between the sorbent material and the analyte. The mechanism behind the eluent-induced release of the analyte probably depends on the interaction between the residual Si-OH groups located on the surface of the silica and the absorbed compounds. In the case of $C_{18\ EC,}$ the residual polar groups are suppressed and this feature apparently worsens the analyte release mechanism (Liberatore and Procida *et al*., 2001). In another interesting study, all the cartridges cited above (C_8, C_{18} and diol) were compared in terms of their recovery efficiency of phenolic compounds from VOO. The results showed that extraction using

diol cartridges gave higher recoveries of total phenols, *o*-diphenols, tyrosol, hydroxytyrosol and secoiridoids than the other extraction procedures (Bendini and Bonoli *et al*., 2003). In a more recent study, diol-SPE was also chosen as the most efficient cartridge in comparison with C18-SPE and Sax-SPE (Gómez-Caravaca and Carrasco Pancorbo *et al*., 2005). Another important aspect to take into account during SPE extraction is the equilibrium between the amount of VOO and the volume of sorbent used. In this respect, initially 30 g of VOO were spiked with the analytes, and SPE was performed using different capacity (500 mg and 1 g) diol cartridges. The results obtained showed that when 500 mg cartridges were employed, extraction recovery values were low. However, they recorded a substantial increase with the 1 g cartridges. Subsequently, in the same study, a 1 g diol cartridge was used to extract phenolic compounds from 10 g, 20 g and 30 g of VOO, the objective being to detect the saturation point of the diol cartridge used. The results showed that most of the compounds recorded a linear increase in peak area as the amount of olive oil increased; however, as of 20 g of VOO, a curvature of the signal, probably related to cartridge saturation, was observed for tyrosol, vanillic acid and syringic acid (Godoy-Caballero and Acedo-Valenzuela *et al*., 2012). It was reported that 500 mg are generally employed for small scale SPE, and 1 g for large scale (Laura Capriotti and Cavaliere *et al*., 2014).

Comparisons of both extraction techniques, SPE and LLE, have also been carried out and have led to the publication of controversial data. While many comparative studies have shown that LLE achieves a higher recovery than SPE (Bendini and Bonoli *et al*., 2003; Hrncirik and Fritsche, 2004), other authors have considered SPE to be the reference method for the extraction of phenolic compounds from VOO instead of LLE because of its ease of use and short extraction time (Gómez-Caravaca and Carrasco Pancorbo *et al*., 2005; Pirisi and Cabras *et al*., 2000). However, other research has reported the SPE method to be problematic because of its selectivity towards the individual phenolics, particularly the aglycone ones (Hrncirik and Fritsche, 2004). Furthermore, when studying the retention effects of oxidized phenolic compounds during analytical extraction of the phenolic compounds in VOO, the authors came to the conclusion that SPE only seems to be effective for fresh VOO because in the case of oxidized VOO, the stationary phase of the SPE columns interacts with the oxidized phenols. This interaction would lead to further nonselective retention of non-oxidized phenolic compounds, thus reducing the total recovery of these analytes (Armaforte and Mancebo-Campos *et al*., 2007).

Separation techniques for the analysis of phenolic compounds in VOO

Liquid chromatography

Liquid chromatography (LC) is considered the most popular and reliable technique for the separation of phenolic compounds. Normal phase LC and reverse phase LC are reported in the literature. The first uses silica gel as the stationary phase in the column and a non-polar solvent as eluent while the second uses a non-polar octadecylsilane (C18) bonded stationary phase and a polar solvent as the mobile phase. Reverse phase LC was reported to be the technique in greatest use due to its better reproducibility and separation of polar compounds (Carrasco-Pancorbo and Cerretani *et al*., 2005; Laura Capriotti and Cavaliere *et al*., 2014). A wide variety of columns are employed. The preferred columns are 100 to 250 mm in length, with 2-4.6 mm inner diameter and 1.8-5 µm particle size (Selvaggini and Servili *et al*., 2006; Taamalli and Abaza *et al*., 2013).

An isocratic elution was developed for the separation of phenolic compounds and an adequate resolution was achieved using a suitable composition of mobile phase (Akasbi and Shoeman *et al*., 1993). However, recent studies exclusively use gradient elution mode. This fact confirms the complexity of the phenolic profile which cannot be properly separated in isocratic elution mode. Numerous mobile phases have been employed, but binary systems comprising water and a less polar organic solvent such as acetonitrile or methanol remain common (Bakhouche and Lozano-Sánchez *et al*., 2015; De la Torre-Carbot and Jauregui *et al*., 2005). Acids such as acetic, formic, and perchloric acid are usually added to water to maintain a constant acid concentration during gradient runs (Bayram and Esatbeyoglu *et al*., 2012; De la Torre-Carbot and Jauregui *et al*., 2005; Taamalli and Abaza *et al*., 2013). Decreasing the pH partly helped to improve the resolution. Nevertheless, the lack of separation between peaks and the long analysis time are still the main drawbacks of the LC technique. In fact, the separation of complex VOO phenolic compounds has required longer run times using conventional HPLC methods. However, the development of columns with small particle sizes in the stationary phase has enhanced resolution and reduced analysis time. The high pressures produced by the use of small particle sizes made it necessary to develop new equipment such as rapid resolution liquid chromatography (RRLC) and ultra performance liquid chromatography (UPLC) to support the higher pressures. The application of the new column for the separation of VOO phenolic compounds, together

with the optimization of gradient, temperature, and flow rate were reported in the literature. In a recent study, a new RRLC method has been optimized (Column Zorbax C18: 4.6 mm×150 mm and 1.8 µm particle size) on the basis of the chromatographic conditions of a previous high performance liquid chromatography (HPLC) method (Gemini C18 column: 3 mm×250 mm, 5 µm particle size). Firstly, a gradient elution was optimized using water + 0.5% of acetic acid as mobile phase A, and acetonitrile as mobile phase B. In the next step, flow rate and temperature values ranging from 0.5 to 2 ml/min and 25 ºC to 40 ºC, respectively, were tested. The results showed that increasing the flow and temperature shortened the run time without excessively compromising resolution, but temperatures above 40 ºC led to overlapping of some peaks and loss of compounds. Finally, an optimum flow of 1.5 mL/min and temperature of 30 ºC were chosen for the analysis. With the optimized method, the run time could be reduced from 60 min (HPLC) to 20 min (RRLC), and a good resolution was obtained by using a column with a small particle size (García-Villalba and Carrasco-Pancorbo *et al.*, 2010). In an even more recent study, optimum chromatographic separation was obtained with the same equipment (RRLC) using water + 0.25% acetic acid as mobile phase A and methanol as mobile phase B. The testing temperature and the flow rate were 25 ºC and 0.8 ml/min, respectively. Applying all these conditions, the phenolic compounds were correctly separated in only 27 min (Lozano-Sánchez and Segura-Carretero *et al.*, 2010).

Gas chromatography

Since it was invented, and especially when the fused-silica capillary column was introduced in gas chromatography (GC), this technique has become one of the most effective techniques in analytical chemistry on account of the significant improvement in separation quality (Carrasco-Pancorbo and Nevedomskaya *et al.*, 2009). GC has been used to perform qualitative and quantitative determinations of the phenolic compounds in VOO (García-Villalba and Pacchiarotta *et al.*, 2011; Saitta and Curto *et al.*, 2002). However, its use has been restricted by the limited volatility of many phenolic compounds. In order to overcome this problem, the analysis requires a pre-step named derivatization in which the phenolic compounds are converted into more volatile compounds using different reagents. There are many derivatization methods, although trimethylsilylation is the most reported one (Angerosa and D'Alessandro *et al.*, 1996; Zafra-Gómez and Luzón-Toro *et al.*, 2010).

In a recent study, the derivatization step was carefully optimized by comparing different reagents and testing their efficiency in both standard solutions and real samples. Firstly, the derivatization step was optimized in a mixture of tyrosol and hydoxytyrosol standard solution, using N, O-Bis (trimethylsilyl)trifluoroacetamide (BSTFA), N,O-Bis(trimethylsilyl)trifluoroacetamide + trimethylchlorosilane (BSTFA+TMCS), N,O-Bis(trimethylsilyl)acetamide + trimethylchlorosilane (BSA+TMCS), and tert-Butyldimethylchlorosilane (TBDMSCl) as derivatization reagents. Pyridine and ACN were compared as reaction solvents. Despite the good results obtained using BSTFA and BSTFA+TMCS, the mixture of BSA+TMCS showed the best efficiency. However, BSTFA was chosen for the analysis of phenolic compounds in VOO samples to avoid injection of TMCS, which can reduce column life (Purcaro and Codony *et al.*, 2014).

The literature reports many attempts to achieve good separation using GC in the analysis of phenolic compounds. The GC methods use different columns, oven temperature programs, injection temperatures, injection modes, and injection volumes of the extract (Angerosa and D'Alessandro *et al.*, 1996; García-Villalba and Pacchiarotta *et al.*, 2011; Ríos and Gil *et al.*, 2005). In almost all the methods reported, helium was employed as the carrier gas with a linear velocity. Besides separation quality, shorter or longer analysis times were achieved in the published methods depending on the analysis parameters mentioned above. However, the need to perform derivatization makes this technique more laborious. Furthermore, incomplete derivatization may be accompanied by the formation of several chemical species from the same compound, giving confusing results. Another drawback of this technique is the use of high temperatures, which can damage the analytes (Carrasco-Pancorbo and Cerretani *et al.*, 2005).

Capillary electrophoresis

In recent years, capillary electrophoresis (CE) has proved to be a fast technique combining short analysis times and high separation efficiency for the analysis of food components. It has been used in particular for the analysis of phenolic compounds in VOO (Bendini and Bonoli *et al.*, 2003). Different electrophoretic methods have been optimized in order to find the best separation conditions to perform the analysis in the shortest time with sufficient resolution. Typical parameters for optimization were the buffer (type, concentration and pH), capillary temperature, voltage, effective capillary diameter, and type of sample injection. The first optimized methods were laborious because of the need for individual optimization of all the parameters cited above (Bonoli and Montanucci *et al.*, 2003; Carrasco-Pancor-

bo and Gómez-Caravaca *et al*., 2006a; Gómez-Caravaca and Carrasco Pancorbo *et al*., 2005). However, recently the use of multi-criteria methods, which simultaneously take into account all the critical separations, makes the development of new electrophoretic methods easy and fast (Ballus and Meinhart *et al*., 2011; Ballus and Meinhart *et al*., 2014). Furthermore, in order to reduce the number of steps involved in the analysis of phenolic compounds using capillary electrophoresis, the use of a new technique named non-aqueous capillary electrophoresis (NACE) has been reported. Its main advantage in comparison with the conventional technique is its ability to work with a large variety of organic solvents (methanol, ethanol, 1-propanol, 2-propanol, or acetonitrile, among others). As it is known, LLE or SPE is often needed to achieve pre-concentration with organic solvents before the analysis of complex matrices. After pre-concentration, the analytes concerned are commonly diluted in an organic solvent. In aqueous CE, the extract is usually evaporated and the analytes are dissolved in aqueous media. However, this step can be bypassed when NACE is used (Godoy-Caballero and Acedo-Valenzuela *et al*., 2012b). In spite of the efforts of several research groups to develop reliable CE methods, only a few papers have been published on the application of this technique in the field of *Olea europaea*, probably because of some disadvantages of CE such as poor reproducibility, low UV detection sensitivity, and difficulties with the MS coupling (Carrasco-Pancorbo and Gómez-Caravaca *et al*., 2006a; Godoy-Caballero and Acedo-Valenzuela *et al*., 2012b).

Detectors

Phenolic compounds are commonly determined using UV/VIS and DAD detectors, particularly coupled to LC and CE. In LC phenolic studies, 280 nm is useful for routine analysis since most VOO phenolics absorb at this wavelength, whereas 240 nm is used for some secoiridoids and their derivatives, 310-320 nm for hydroxycinnamic acids, and 350 nm for flavones (Bakhouche and Lozano-Sánchez *et al*., 2013; Garcia and Coelho *et al*., 2013; Godoy-Caballero and Acedo-Valenzuela *et al*., 2012a). In the official phenolic analysis method published by the International Olive Council (IOC), UV-Vis is used for detection. The method establishes the maximum absorbance values of 27 different phenolic compounds and gives a procedure for the quantification of these analytes on the basis of the data provided by the UV detector (IOC 2009). UV detectors have also been used to study the variation of phenolic content in VOO under the influence of different agronomical and technological factors (Gómez-Rico and Salvador *et al*., 2006; Parenti and Spugnoli *et al*., 2008). With regard to CE analysis, the literature also reports the performance of UV detection at 200, 240, 280 and 330 nm for the characterization of different phenolic groups in VOO, although diode-array detection was used over the range of 190–600 nm to achieve spectral data (Carrasco-Pancorbo and Gómez-Caravaca *et al*., 2006a). In more recent studies, CE coupled to a UV detector was used to develop a new electrophoretic method for the separation of the phenolic compounds of VOO extracts, to compare different extraction systems in terms of phenolic recovery and to study the phenolic composition of VOO obtained from different olive varieties (Ballus and Meinhart *et al*., 2014; Godoy-Caballero and Galeano-Díaz *et al*., 2012c; Gómez-Caravaca and Carrasco Pancorbo *et al*., 2005).

In GC, the flame ionization detector (FID) is the most common detector coupled to this separation technique. In fact, GC-FID has been used to analyze the phenolic profiles of different oils, including VOO. The method proposed for this purpose has made it possible to estimate the phenolic content of sunflower oil, colza oil, and VOO (Farajzadeh and Yadeghari *et al*., 2014). Other authors have used GC-FID to analyze the total hydroxytyrosol and tyrosol content of extra virgin olive oils, after hydrolysis of the linked forms (Purcaro and Codony *et al*., 2014).

Recently, mass spectrometry detectors (MS) have been coupled to different separation techniques for further characterization of the phenolic compounds in VOO. Time-of-flight (TOF-MS), quadrupole time-of-flight (QTOF-MS), and ion trap (IT-MS) MS detectors are the types reported most widely in the literature (Bakhouche and Lozano-Sánchez *et al*., 2014b; Fu and Segura-Carretero *et al*., 2009; Laura Capriotti and Cavaliere *et al*., 2014). Apart from fast data acquisition and a wide mass detection range, another important characteristic of the MS detector is its great accuracy in mass measurements. It provides high selectivity in the determination of phenolic compounds using the extracted ion chromatogram (EIC) mode when there are overlapping peaks, and it permits rapid and efficient confirmation of the elemental composition of ions when fragmentation is carried out. Coupling LC, GC, or CE to a mass spectrometry detector has permitted detailed characterization of the phenolic fraction in different studies on VOO quality (Bengana and Bakhouche *et al*., 2013; Carrasco-Pancorbo and Arráez-román *et al*., 2006b; Saitta and Curto *et al*., 2002). Furthermore, the use of a mass spectrometry detector enables investigation of the relationship be-

tween the chemical nature or concentration of individual phenolic compounds in VOO extracts and their ability to reduce some chronic diseases (García-Villalba and Carrasco-Pancorbo *et al.*, 2010). Other detectors such as fluorescence and NMR detectors are also reported (Etrakis and Giomyrgianaki *et al.*, 2008; Tena and García-González *et al.*, 2009); however, they are not as common as UV and MS detectors.

Analytical problems of phenolic compound characterization in VOO

In all the studies aimed at quantification of the phenolic compounds in VOO, the concern of researchers has been to achieve the total recovery of these analytes from the matrix. Initially, a mixture of commercial standards spiked in refined peanut oil was used to compare the efficiency recovery of various isolation techniques. The spiked oil was then subjected to different extraction systems (LLE and SPE). The resultant extracts were analyzed by HPLC, and the amount of each standard was compared with that of the standard mixture that had not undergone any extraction procedure (Bendini and Bonoli *et al.*, 2003). However, when considering the results obtained, it should be borne in mind that most of the naturally occurring phenolic compounds in olive oil are not commercially available. Consequently, research continued on the basis of other compounds having similar structures. The response of the standards can be different from that of the analytes present in the oil samples; hence, the recovery results could only be estimates. To overcome this problem, other authors proposed an extraction method based on spiking refined sunflower oil (phenolic-free) with an exact dose of an LLE-prepared phenolic extract of VOO. The spiked oil was extracted using SPE, and its recovery efficiency was calculated (Gómez-Caravaca and Carrasco Pancorbo *et al.*, 2005). This approach provided a partial solution for estimating the recovery of phenolic compounds from VOO. The previous attempts where different extraction systems were compared in terms of recovery efficiency did not consider the interactions between both matrix and target compounds. In fact, the effect of VOO water content on phenolic extraction should be taken into account in both systems (LLE and SPE). Several studies have demonstrated the relationship between water content and phenolic compounds. It is common knowledge that olive oil contains a small quantity of water; for this reason it can be considered a water-in-oil-emulsion. Phenolic compounds are located in the water/oil interface (Ambrosone and Cinelli *et al.*, 2006; Frankel and Huang *et al.*, 1994).

The effect of water content on the extraction of phenolic compounds from VOO was observed for the first time on a laboratory scale by studying the effect of the filtration system on VOO phenolic content (Gómez-Caravaca and Cerretani *et al.*, 2007). In this research, LLE was used to extract the phenolic compounds. The results obtained by the authors showed that compounds belonging to the secoiridoid group, such as ligstroside aglycone and oleuropein aglycone, increased significantly after reducing water content by filtration with cotton in comparison with unfiltered VOO. In effect, in a water-in-oil emulsion, phenolic compounds are stabilized around water droplets, and the affinity of the phenolic compounds for solvent extraction is low in a more polar matrix (olive oils with high water content), making their recovery more difficult. However, the partial elimination of water during the filtration process makes more phenolic compounds available for extraction with the apolar solvent mixture, which results in the apparent increase in their concentration in filtered VOO. Five years later, the effect of water content on phenolic compound extraction from VOO samples was confirmed using SPE (Lozano-Sánchez and Cerretani *et al.*, 2012). Working on a pilot-plant scale using filter bags, the authors found that secoiridoids in filtered VOO were responsible for the apparent increase in the total phenolic content. Lastly, the apparent increase in different compounds from the secoiridoid group due to the variation in water content in VOO was confirmed in a more recent study (Bakhouche and Lozano-Sánchez *et al.*, 2014a). The chemical structures of the main compounds whose recovery was affected by the variation in the water content of VOO are shown in **Figure 1**.

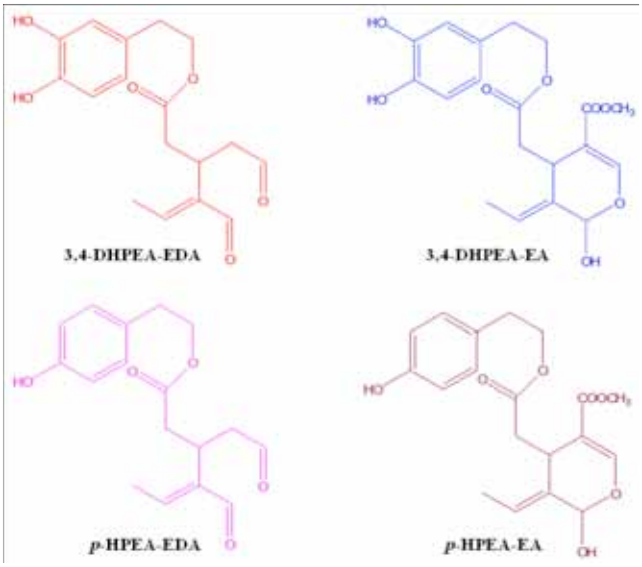

Figure 1: Structure of some phenolic compounds affected by the variation in VOO water content during extraction: 3,4-DHPEA-EDA, decarboxymethyl oleuropein aglycone; 3,4-DHPEA-EA, oleuropein aglycone; p-HPEA-EDA, decarboxymethyl ligstroside aglycone; p-HPEA-EA , ligstroside aglycone

These studies confirmed the effect of the filtration-induced variation in VOO water content on the extraction of phenolic compounds. However, it is common knowledge that VOO water content also varies according to fruit ripening stage, olive variety, and geographical area (Motilva and Tovar *et al*., 2000; Taamalli and Gómez-Caravaca *et al*., 2010). Many studies have used phenolic profiles as a fingerprint to distinguish between olive varieties as well as to classify VOOs according to their geographical origin (designation of origin) and to determine the best harvest period for obtaining high-phenolic VOO while other studies have been conducted on the bioactivity of VOO phenolic compounds (García-Villalba and Carrasco-Pancorbo *et al*., 2010; Karkoula and Skantzari *et al*., 2012; Ouni and Taamalli *et al*., 2011; Rotondi and Bendini *et al*., 2004; Taamalli and Gómez-Caravaca *et al*., 2010). Unfortunately, in all of these studies, the variation in VOO water content during phenolic extraction was not considered. This could affect the accuracy of data reported in different publications for the concentration of these analytes in VOO. Recently, in an attempt to resolve this problem, a new approach was developed to correct the effect exerted by moisture reduction after VOO filtration on the recovery of phenolic compounds by using an internal standard during extraction. As a result, the apparent increase of secoiridoids was corrected and the phenolic compounds in filtered VOO were correctly quantified (Bakhouche and Lozano-Sánchez *et al*., 2014b). However, the optimization of the proposed method was based solely on the change in VOO moisture content after filtration. Consequently, future investigations are warranted to develop a new extraction method which can be applied to all kinds of VOO studies relating to the phenolic fraction and water content. Until then, the extraction of phenolic compounds from VOO will continue to be problematic.

As it is well known, after the isolation of the phenolic compounds from VOO, the next challenge is to draw up a reliable method for the analysis of the resultant extracts. Although powerful analytical equipment and methods have been developed, the total structural characterization of the phenolic fraction is still sometimes impossible because of the complexity of the wide group of secoiridoids. The main compounds identified in this group are oleuropein aglycone, ligstroside aglycone and their derivatives such as the hydroxylated, decarboxymethylated, dehydrated, and methylated forms. Today, the isomers of these compounds are the subject matter of scientific research. The first research characterized eleven isomers of oleuropein aglycone in Spanish VOO (Fu and Segura-Carretero *et al*., 2009) using rapid-resolution liquid chromatography coupled to electrospray time-of-flight and ion trap tandem mass spectrometry. In a more recent study, eighteen, seventeen and nine isomers were detected for oleuropein aglycone, ligstroside aglycone and elenolic acid, respectively, using fused-core reverse phase chromatography coupled to high resolution mass spectrometry (HRMS) and high resolution tandem mass spectrometry (HRMS/MS), in positive and negative electrospray ionization (ESI) modes (Vichi and Cortés-Francisco *et al*., 2013). These isomers were considered to be the result of oleuropein and ligstroside isomerization after hydrolysis during olive ripening and olive oil processing. However, a study using HPLC-UV with reversed phase columns showed that isomers of decarboxymethyl ligstroside aglycone and decarboxymethyl oleuropein aglycone were formed by the reaction of these two compounds with water or methanol used as a mobile phase (Karkoula and Skantzari *et al*., 2012). In addition, in a more recent study, the artificial formation of oleuropein and ligstroside aglycone isomers was proved by the same authors (Karkoula and Skantzari *et al*., 2014). Therefore, isomers could also be formed during chromatographic analysis depending on the mobile phase used. This finding confirmed that classic chromatographic measurement of these compounds is problematic, especially in aqueous media, and that many of the previous measurements reported in the literature are more or less questionable. It should be taken into account that the validated and official methods proposed by several authors and international committees use water as the eluent for the mobile phase. This can affect

the results owing to the interaction between water and some phenolic compounds, which casts doubts over the estimation of this fraction as shown for example in **Figure 2** for the main isomers detected in VOO.

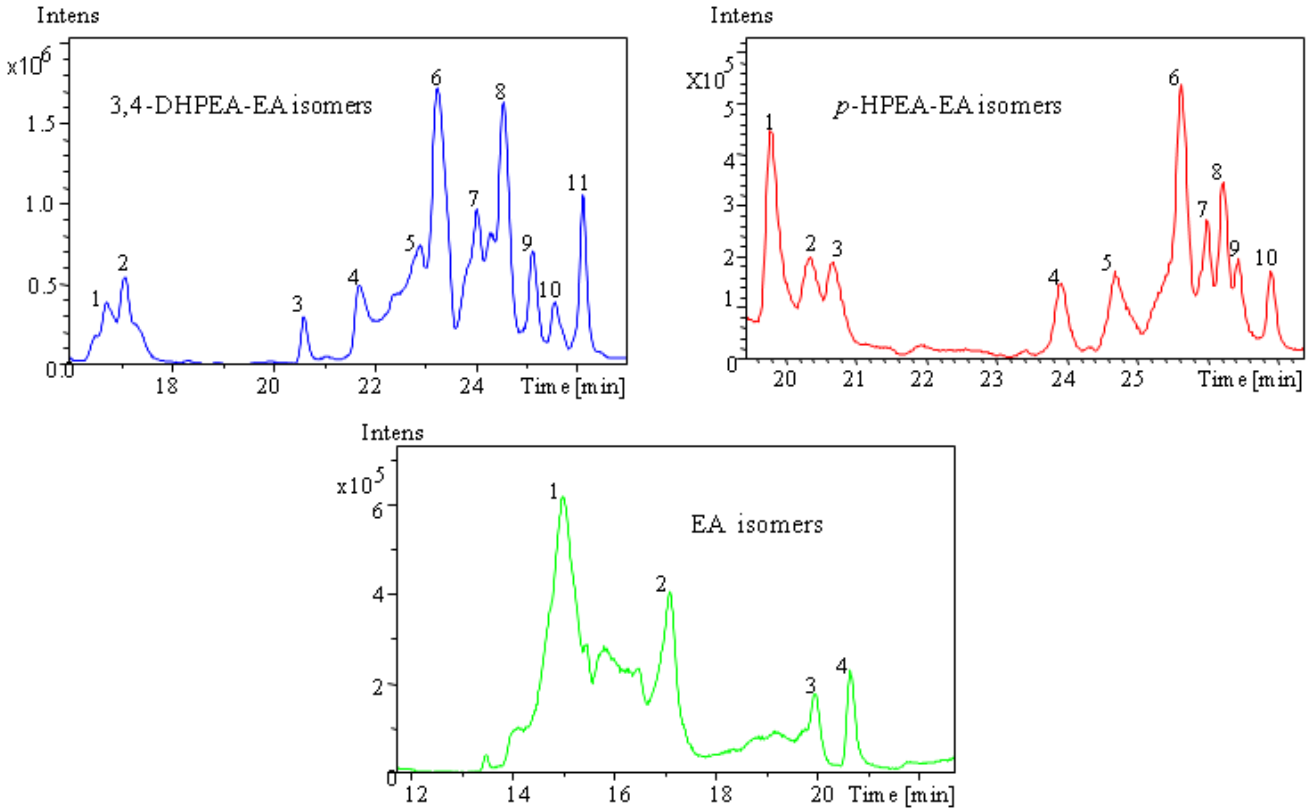

Figure 2: Isomers of oleuropein aglycone (3,4-DHPEA-EA), ligstroside aglycone (p-HPEA-EA), and elenolic acid (EA) obtained using HPLC-ESI-TOF/MS

Conclusions

The different methods for the isolation and separation of phenolic compounds resolved many problems related to the estimation of this fraction in VOO. Having done so, the nutritional value and healthy properties of VOO were easily proven. However, some aspects of the cited methods need to be improved. For instance, the controversial data reported in the literature make it difficult to compare VOOs produced in different parts of the world in terms of their phenolic content. New investigations are therefore warranted in order to devise accurate, harmonized methods aimed at avoiding confusion when different published data are compared. The improvements required need to take into account the current problems, especially the effect of VOO water content on the isolation of phenolic compounds, and the artificial formation of some isomers during chromatographic separation due to the mobile phase used.

Acknowledgments

We are grateful to the Spanish Ministry of Economy and Competitiveness (project BFU2014-52433-C3-2-R) the Andalusian Regional Government Council of Innovation and Science (P11-CTS-7625), the University of Granada, the Functional Food Research and Development Center, and to the International Olive Council (IOC) for granting a scholarship to the PhD student.

References

Akasbi, M., Shoeman, D. M. and Saari Csallany, A. 1993. High-Performance Liquid Chromatography of Selected Phenolic Compounds in Olive Oils. J. Am. Oil Chem. Soc. 70: 367–370.

Ambrosone, L., Cinelli, G., Mosca, M. and Ceglie, A. 2006. Susceptibility of water-emulsified extra virgin olive oils to oxidation. J. Am. Oil Chem. Soc. 83: 165–170.
Angerosa, F., D'Alessandro, N., Corana, F. and Mellerio, G. 1996. Characterization of phenolic and secoiridoid aglycons present in virgin olive oil by gas chromatography-chemical ionization mass spectrometry. J. Chromatogr. A. 736: 195–203.
Angerosa, F., D'Alessandro, N., Konstantinou, P. and Di Giacinto, L. 1995. GC-MS Evaluation of Phenolic Compounds in Virgin Olive Oil. J. Agric. Food Chem. 43: 1802–1807.
Armaforte, E., Mancebo-Campos, V., Bendini, A., Desamparados Salvador, M., Fregapane, G. and Cerretani, L. 2007. Retention effects of oxidized polyphenols during analytical extraction of phenolic compounds of virgin olive oil. J. Sep. Sci. 30: 2401–2406.
Bajoub, A., Carrasco-Pancorbo, A., Ajal, E. A., Ouazzani, N. and Fernández-Gutiérrez, A. 2015. Potential of LC-MS phenolic profiling combined with multivariate analysis as an approach for the determination of the geographical origin of north Moroccan virgin olive oils. Food Chem. 166: 292–300.
Bakhouche, A., Lozano-Sánchez, J., Ballus, C. A., Bendini, A., Gallina-Toschi, T., Fernández-Gutiérrez, A. and Segura-Carretero, A. 2014b. A new extraction approach to correct the effect of apparent increase in the secoiridoid content after filtration of virgin olive oil. Talanta. 127: 18–25.
Bakhouche, A., Lozano-Sánchez, J., Ballus, C. A., Martínez-García, M., Velasco, G. M., Govantes, Á. O., Gallina-Toschi, T., Fernández-Gutiérrez, A. and Segura-Carretero, A. 2014a. Monitoring the moisture reduction and status of bioactive compounds in extra-virgin olive oil over the industrial filtration process. Food Control. 40: 292–299.
Bakhouche, A., Lozano-Sánchez, J., Beltrán-Debón, R., Joven, J., Segura-Carretero, A. and Fernández-Gutiérrez, A. 2013. Phenolic characterization and geographical classification of commercial Arbequina extra-virgin olive oils produced in southern Catalonia. Food Res. Int. 50: 401–408.
Bakhouche, A., Lozano-Sánchez, J., Bengana, M., Fernández-Gutiérrez, A. and Segura-Carretero, A. 2015. Time course of Algerian Azeradj extra-virgin olive oil quality during olive ripening. Eur. J. Lipid Sci. Technol. 117: 0000–0000.
Ballus, C. A., Meinhart, A. D., Bruns, R. E. and Godoy, H. T. 2011. Use of multivariate statistical techniques to optimize the simultaneous separation of 13 phenolic compounds from extra-virgin olive oil by capillary electrophoresis. Talanta, 83: 1181–1187.
Ballus, C. A., Meinhart, A. D., de Souza Campos, F. A., Bruns, R. E. and Godoy, H. T. 2014. Doehlert design-desirability function multi-criteria optimal separation of 17 phenolic compounds from extra-virgin olive oil by capillary zone electrophoresis. Food Chem. 146: 558–68.
Bayram, B., Esatbeyoglu, T., Schulze, N., Ozcelik, B., Frank, J. and Rimbach, G. 2012. Comprehensive analysis of polyphenols in 55 extra virgin olive oils by HPLC-ECD and their correlation with antioxidant activities. Plant Foods Hum. Nutr. 67: 326–36.
Becerra-Herrera, M., Sánchez-Astudillo, M., Beltrán, R. and Sayago, A. 2014. Determination of phenolic compounds in olive oil: New method based on liquid–liquid micro extraction and ultra high performance liquid chromatography-triple–quadrupole mass spectrometry. LWT - Food Sci. Technol. 57: 49–57.
Bendini, A., Bonoli, M., Cerretani, L., Biguzzi, B., Lercker, G. and Toschi, T. G. 2003. Liquid–liquid and solid-phase extractions of phenols from virgin olive oil and their separation by chromatographic and electrophoretic methods. J. Chromatogr. A. 985:425–433
Bengana, M., Bakhouche, A., Lozano-Sánchez, J., Amir, Y., Youyou, A., Segura-Carretero, A. and Fernández-Gutiérrez, A. 2013. Influence of olive ripeness on chemical properties and phenolic composition of Chemlal extra-virgin olive oil. Food Res. Int. 54: 1868–1875.
Bonoli, M., Montanucci, M., Toschi, T. G. and Lercker, G. 2003. Fast separation and determination of tyrosol, hydroxytyrosol and other phenolic compounds in extra-virgin olive oil by capillary zone electrophoresis with ultraviolet-diode array detection. J. Chromatogr. A. 1011: 163–172.
Brenes, M., Garcı, A., Garcı, P. and Garrido, A. 2000. Rapid and Complete Extraction of Phenols from Olive Oil and Determination by Means of a Coulometric Electrode Array System. J. Agric. Food Chem. 48: 5178–5183.
Carrasco-Pancorbo, A., Arráez-Román, D., Segura-Carretero, A. and Fernández-Gutiérrez, A. 2006b. Capillary electrophoresis-electrospray ionization-mass spectrometry method to determine the phenolic fraction of extra-virgin olive oil. Electrophoresis. 27: 2182–2196.
Carrasco-Pancorbo, A., Cerretani, L., Bendini, A., Segura-Carretero, A., Gallina-Toschi, T. and Fernández-Gutiérrez, A. 2005. Analytical determination of polyphenols in olive oils. J. Sep. Sci. *28*: 837–858.
Carrasco-Pancorbo, A., Gómez-Caravaca, A. M., Cerretani, L., Bendini, A., Segura-Carretero, A. and Fernández-Gutiérrez, A. 2006a. A simple and rapid electrophoretic method to characterize simple phenols, lignans, complex phenols, phenolic acids, and flavonoids in extra-virgin olive oil. J. Sep. Sci. 29: 2221–2233.
Carrasco-Pancorbo, A., Nevedomskaya, E., Arthen-Engeland, T., Zurek, G., Baessmann, C., Deelder, A.

M. and Mayboroda, O. A. 2009. Gas Chromatography/ Atmospheric Pressure Chemical Ionization-Time of Flight Mass Spectrometry: Analytical Validation and. Anal. Chem. 81: 10071–10079.

Covas, M. I. 2007. Olive oil and the cardiovascular system. Pharmacol. Res. 55: 175–86.

Dabbou, S., Chehab, H., Faten, B., Dabbou, S., Esposto, S., Selvaggini, R., Taticchi, A., Servili, M., Montedoro, G. F. and Hammamia, M. 2010. Effect of three irrigation regimes on Arbequina olive oil produced under Tunisian growing conditions. Agric. Water Manage. 97: 763-768

De la Torre-Carbot, K., Jauregui, O., Gimeno, E., Castellote, A. I., Lamuela-Ravento, R. M. and López-Sabater, M. C. 2005. Characterization and Quantification of Phenolic Compounds in Olive Oils by Solid-Phase Extraction , HPLC-DAD , and HPLC-MS / MS. J. Agric. Food Chem. 53: 4331–4340.

Etrakis, P. A. V. P., Giomyrgianaki, A. L. A., Hristophoridou, S. T. C., Pyros, A. P. S. and Ais, P. H. D. 2008. Geographical Characterization of Greek Virgin Olive Oils (Cv. Koroneiki) Using 1 H and 31 P NMR Fingerprinting with Canonical Discriminant Analysis and Classification Binary Trees. J. Agric. Food Chem. 56: 3200–3207.

Farajzadeh, M. A., Yadeghari, A., Khoshmaram, L. and Ghorbanpour, H. 2014. Analytical Methods Development and validation of a rapid and sensitive gas chromatographic method for the analysis of some phenolic compounds in vegetable oils. Anal. Methods. 6: 5314–5321.

Fitó, M., de la Torre, R. and Covas, M. I. 2007. Olive oil and oxidative stress. Mol. Nutr. Food Res. 51: 1215–24.

Franco, M. N., Galeano-Díaz, T., López, O., Fernández-Bolaños, J. G., Sánchez, J., De Miguel, C., Gil, M. V. and Martín-Vertedor, D. 2014. Phenolic compounds and antioxidant capacity of virgin olive oil. Food Chem. 163: 289–98.

Frankel, E., Bakhouche, A., Lozano-Sánchez, J., Segura-Carretero, A. and Fernández Gutiérrez, A. 2013. Literature Review on Production Process To Obtain Extra Virgin Olive Oil Enriched in Bioactive Compounds. Potential Use of Byproducts as Alternative Sources of Polyphenols. J. Agric. Food Chem. 61: 5179–5188.

Frankel, E., Huang, S., Kanner, J. and German, J. B. 1994. Interfacial Phenomena in the Evaluation of Antioxidants: Bulk Oils vs Emulsions. J. Agric. Food Chem. 42: 1054–1059.

Fu, S., Segura-Carretero, A., Arráez-Román, D., Menéndez, J. A., De La Torre, A. and Fernández-Gutiérrez, A. 2009. Tentative characterization of novel phenolic compounds in extra virgin olive oils by rapid-resolution liquid chromatography coupled with mass spectrometry. J. Agric. Food Chem. 57: 11140–11147.

Garcia, B., Coelho, J., Costa, M., Pinto, J. and Paiva-Martins, F. 2013. A simple method for the determination of bioactive antioxidants in virgin olive oils. J. Sci. Food Agric. 93: 1727–32.

García-Villalba, R., Carrasco-Pancorbo, A., Oliveras-Ferraros, C., Vázquez-Martín, A., Menéndez, J. A., Segura-Carretero, A. and Fernández-Gutiérrez, A. 2010. Characterization and quantification of phenolic compounds of extra-virgin olive oils with anticancer properties by a rapid and resolutive LC-ESI-TOF MS method. J. Pharm. Biomed. Anal. 51: 416–29.

García-Villalba, R., Pacchiarotta, T., Carrasco-Pancorbo, A., Segura-Carretero, A., Fernández-Gutiérrez, A., Deelder, A. M. and Mayboroda, O. A. 2011. Gas chromatography-atmospheric pressure chemical ionization-time of flight mass spectrometry for profiling of phenolic compounds in extra virgin olive oil. J. Chromatogr. A. 1218: 959–971.

Godoy-Caballero, M. D. P., Acedo-Valenzuela, M. I. and Galeano-Díaz, T. 2013. New reversed phase dispersive liquid-liquid microextraction method for the determination of phenolic compounds in virgin olive oil by rapid resolution liquid chromathography with ultraviolet-visible and mass spectrometry detection. J. Chromatogr. A. 1313: 291–301.

Godoy-Caballero, M. D. P., Acedo-Valenzuela, M. I. and Galeano-Díaz, T. 2012a. Simple quantification of phenolic compounds present in the minor fraction of virgin olive oil by LC-DAD-FLD. Talanta. 101: 479–87.

Godoy-Caballero, M. D. P., Acedo-Valenzuela, M. I., Durán-Merás, I. and Galeano-Díaz, T. 2012b. Development of a non-aqueous capillary electrophoresis method with UV-visible and fluorescence detection for phenolics compounds in olive oil. Anal. Bioanal. Chem. 403: 279–90.

Godoy-Caballero, M. D. P., Galeano-Díaz, T. and Acedo-Valenzuela, M. I. 2012c. Simple and fast determination of phenolic compounds from different varieties of olive oil by nonaqueous capillary electrophoresis with UV-visible and fluorescence detection. J. Sep. Sci. 35: 3529–39.

Gómez-Caravaca, A. M., Carrasco Pancorbo, A., Cañabate Díaz, B., Segura Carretero, A. and Fernández Gutiérrez, A. 2005. Electrophoretic identification and quantitation of compounds in the polyphenolic fraction of extra-virgin olive oil. Electrophoresis. 26: 3538–51.

Gómez-Caravaca, A. M., Cerretani, L., Bendini, A., Segura-Carretero, A., Fernández-Gutiérrez, A. and Lercker, G. 2007. Effect of filtration systems on the phenolic content in virgin olive oil by HPLC-DAD-MSD. Am. J. Food Technol. 2: 671–678.

Gómez-Rico, A., Salvador, M. D., La Greca, M. and Fregapane, G. 2006. Phenolic and Volatile Compounds

of Extra Virgin Olive Oil (*Olea europaea* L. Cv. Cornicabra) with Regard to Fruit. J. Agric. Food Chem. 54: 7130–7136.
Hrncirik, K. and Fritsche, S. 2004. Comparability and reliability of different techniques for the determination of phenolic compounds in virgin olive oil. Eur. J. Lipid Sci. Technol. 106: 540–549.
International Olive Council. 2009. Determination of biophenols in olive oils by HPLC. COI/T.20/Doc No 29.
Karkoula, E., Skantzari, A., Melliou, E. and Magiatis, P. 2012. Direct measurement of oleocanthal and oleacein levels in olive oil by quantitative (1)H NMR. Establishment of a new index for the characterization of extra virgin olive oils. J. Agric. Food Chem. 60: 11696–703.
Karkoula, E., Skantzari, A., Melliou, E. and Magiatis, P. 2014. Quantitative Measurement of Major Secoiridoid Derivatives in Olive Oil Using qNMR. Proof of the Artificial Formation of Aldehydic Oleuropein and Ligstroside Aglycon Isomers. J. Agric. Food Chem. 62: 600–607.
Laura Capriotti, A., Cavaliere, C., Crescenzi, C., Foglia, P., Nescatelli, R., Samperi, R. and Laganà, A. 2014. Comparison of extraction methods for the identification and quantification of polyphenols in virgin olive oil by ultra-HPLC-QToF mass spectrometry. Food Chem. 158: 392–400.
Lerma-García, M. J., Lantano, C., Chiavaro, E., Cerretani, L., Herrero-Martínez, J. M. and Simó-Alfonso, E. F. 2009. Classification of extra virgin olive oils according to their geographical origin using phenolic compound profiles obtained by capillary electrochromatography. Food Res. Int. 42: 1446–1452.
Liberatore, L., Procida, G., D'Alessandro, N. and Cichelli, A. 2001. Solid-phase extraction and gas chromatographic analysis of phenolic compounds in virgin olive oil. Food Chem. 73: 119–124.
Lozano-Sánchez, J., Cerretani, L., Bendini, A., Gallina-Toschi, T., Segura-Carretero, A. and Fernández-Gutiérrez, A. 2012. New filtration systems for extra-virgin olive oil: effect on antioxidant compounds, oxidative stability, and physicochemical and sensory properties. J. Agric. Food Chem. 60: 3754–62.
Lozano-Sánchez, J., Segura-Carretero, A., Menendez, A. J., Oliveras-Ferraros, C., Cerretani, L. and Fernández-Gutiérrez, A. 2010. Prediction of Extra Virgin Olive Oil Varieties through Their Phenolic Profile. Potential Cytotoxic Activity against Human Breast Cancer Cells. J. Agric. Food Chem. 58: 9942–9955.
Mateos, R., Espartero, L., Trujillo, M., Rı, J. J. and Leo, M. 2001. Determination of Phenols, Flavones, and Lignans in Virgin Olive Oils by Solid-Phase Extraction and High-Performance Liquid Chromatography with Diode Array Ultraviolet Detection. J. Agric. Food Chem. 49: 2185–2192.
Montedoro, G., Servili, M., Baldioli, M., Miniati, E., Agrarie, I. and Costanzo, V. S. 1992. Simple and Hydrolyzable Phenolic Compounds in Virgin Olive Oil. 1. Their Extraction, Separation, and Quantitative and Semiquantitative Evaluation by HPLC. J. Agric. Food Chem. 40: 1571–1576.
Motilva, M. J., Tovar, M. J., Romero, M. P., Alegre, S. and Girona, J. 2000. Influence of regulated deficit irrigation strategies applied to olive trees (Arbequina cultivar) on oil yield and oil composition during the fruit ripening period. J. Sci. Food Agric. 80: 2037–2043.
Ouni, Y., Taamalli, A., Gómez-Caravaca, A. M., Segura-Carretero, A., Fernández-Gutiérrez, A. and Zarrouk, M. 2011. Characterisation and quantification of phenolic compounds of extra-virgin olive oils according to their geographical origin by a rapid and resolutive LC-ESI-TOF MS method. Food Chem. 127: 1263–7.
Parenti, A., Spugnoli, P., Masella, P. and Calamai, L. 2008. The effect of malaxation temperature on the virgin olive oil phenolic profile under laboratory-scale conditions. Eur. J. Lipid Sci. Technol. 110: 735–741.
Pirisi, F. M., Cabras, P., Cao, C. F., Migliorini, M. and Muggelli, M. 2000. Phenolic compounds in virgin olive oil. 2. Reappraisal of the extraction, HPLC separation, and quantification procedures. J. Agric. Food Chem. 48: 1191–1196.
Pizarro, M. L., Becerra, M., Sayago, A., Beltrán, M. and Beltrán, R. 2013. Comparison of Different Extraction Methods to Determine Phenolic Compounds in Virgin Olive Oil. Food Anal. Methods. 6: 123–132.
Purcaro, G., Codony, R., Pizzale, L., Mariani, C. and Conte, L. 2014. Evaluation of total hydroxytyrosol and tyrosol in extra virgin olive oils. Eur. J. Lipid Sci. Technol. 116: 805–811.
Ríos, J. J., Gil, M. J. and Gutíerrez-Rosales, F. 2005. Solid-phase extraction gas chromatography-ion trap-mass spectrometry qualitative method for evaluation of phenolic compounds in virgin olive oil and structural confirmation of oleuropein and ligstroside aglycons and their oxidation products. J. Chromatogr. A. 1093: 167–176.
Rotondi, A., Bendini, A., Cerretani, L., Mari, M., Lercker, G. and Toschi, T. G. 2004. Effect of olive ripening degree on the oxidative stability and organoleptic properties of cv. Nostrana di Brisighella extra virgin olive oil. J. Agric. Food Chem. 52: 3649–54.
Saitta, M., Curto, S. Lo, Salvo, F., Bella, G. D. and Dugo, G. 2002. Gas chromatographic–tandem mass spectrometric identification of phenolic compounds in Sicilian olive oils. Anal. Chim. Acta. 466: 335–344.
Selvaggini, R., Servili, M., Urbani, S., Esposto, S., Taticchi, A. and Monterdero, G. F. 2006. Evaluation of phenolic compounds in virgin olive oil by direct injection in high-performance liquid chromatography

with fluorometric detection. J. Agric. Food Chem. 54: 2832–2838.
Servili, M., Montedoro, G. F. and Hammami, M. 2010. Effect of three irrigation regimes on Arbequina olive oil produced under Tunisian growing conditions. Agric. Water Manag. 97: 763–768.
Taamalli, A., Abaza, L., Arráez Román, D., Segura Carretero, A., Fernández Gutiérrez, A., Zarrouk, M. and Nabil, B. Y. 2013. Characterisation of phenolic compounds by HPLC-TOF/IT/MS in buds and open flowers of "Chemlali" olive cultivar. Phytochem. Anal. 24: 504–512.
Taamalli, A., Gómez-Caravaca, A. M., Zarrouk, M., Segura-Carretero, A. and Fernández-Gutiérrez, A. 2010. Determination of apolar and minor polar compounds and other chemical parameters for the discrimination of six different varieties of Tunisian extra-virgin olive oil cultivated in their traditional growing area. Eur. Food Res. Technol. 231: 965–975.
Tena, N., García-González, D. L. and Aparicio, R. 2009. Evaluation of Virgin Olive Oil Thermal Deterioration by Fluorescence Spectroscopy. J. Agric. Food Chem. 57: 10505–10511.
Vichi, S., Cortés-Francisco, N. and Caixach, J. 2013. Insight into virgin olive oil secoiridoids characterization by high-resolution mass spectrometry and accurate mass measurements. J. Chromatogr. A. 1301: 48–59.
Zafra-Gómez, A., Luzón-Toro, B., Jiménez-Diaz, I., Ballesteros, O. and Navalón, A. 2010. Quantification of phenolic antioxidants in rat cerebrospinal fluid by GC-MS after oral administration of compounds. J. Pharm. Biomed. Anal. 53: 103–108.

Variability in the agronomic performance of a collection of olive hybrids (*Olea europaea* L.) of the local 'Chemlali Sfax' oil-olive variety

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Résumé

L'étude de quelques paramètres agronomiques dans une collection d'hybrides de la variété locale d'olive *Chemlali Sfax* a révélé une variabilité assez importante. La collection renferme 145 hybrides de *Chemlali Sfax* avec plusieurs géniteurs locaux et étrangers et surtout avec la variété italienne *Coratina* (90). La production en olives par année de la collection varie de 1,9 kg en 2010 à 9 kg en 2005, avec une large variabilité intra-annuelle. La production moyenne par croisement varie entre 3,35 kg *(Chemlali/Sigoise)* et 4,86 kg *(Chemlali/Souri)*, avec une variabilité moins importante que celle par année. L'indice d'alternance de la production est élevé et varie de 0,71 *(Chemlali/Souri)* à 0,87 *(Arbéquina/Chemlali)* et il est peu variable au sein de chaque croisement. Pour le port de l'arbre, 90 % des hybrides de la collection ont un port étalé (55 %) ou dressé (35 %), le port retombant étant faiblement représenté (10 %). La caractéristique des hybrides issus des croisements entre *Chemlali Sfax* et *Coratina* est d'avoir un taux de port retombant nettement plus élevé que celui de toute la collection (26 %). L'état de l'arbre (productif ou végétatif) des hybrides varie selon l'année et l'état productif sur deux années consécutives est plus élevé chez les hybrides de *Chemlali Sfax* et *Coratina* (58 %) que dans toute la collection (17,5 %).

Abstract

Study of a number of agronomic parameters in a collection of hybrids of the local 'Chemlali Sfax' olive variety revealed considerable variability. The collection contains 145 hybrids of 'Chemlali Sfax' obtained from several local and foreign genitors, in particular the Italian 'Coratina' variety (90). The annual crop production of the collection varied from 1.9 kg in 2010 to 9 kg in 2005 and showed wide intra-annual variability. Average production per cross varied between 3.35 kg ('Chemlali'/'Sigoise') and 4.86 kg ('Chemlali'/'Souri'), thus recording smaller variability than annual production. The alternate bearing index was high, ranging from 0.71 ('Chemlali'/'Souri') to 0.87 ('Arbequina'/'Chemlali'), and showed little variability within each cross. The growth habit of the hybrids in the collection was spreading (55%) or erect (35%) in 90% of the cases and drooping in only 10%. The hybrids obtained by crossing 'Chemlali Sfax' and 'Coratina' were characterised by a higher percentage of trees with a drooping growth habit (26%) compared with the collection as a whole. Hybrid tree status (on-crop or off-crop) differed according to year. The percentage of 'Chemlali Sfax'/'Coratina' hybrids with on-crop status in two consecutive years (58%) was higher than in the collection as a whole (17.5%).

Received: February 2015 / **Accepted:** August 2015 / **Published:** November 2015

Introduction

Controlled hybridisation is quite recent in olive, dating back to the second half of the 20th century. Controlled crossbreeding was used in Israel to develop varieties capable of responding to "luxury" growing conditions and adapted to intensive, profitable olive growing (Lavee *et al.*, 2003). This research led to the development of several new varieties: 'Kadesh' (Lavee, 1978), 'Barnea' (Lavee *et al.*, 1986), 'Maalot' (Lavee *et al.*, 1999), 'Askal' (Lavee *et al.*, 2003) and 'Kadeshon', 'Sepoka' and 'Masepo' (Lavee *et al.*, 2004).

In Spain, a reciprocal crossbreeding programme undertaken in 1991 aimed at shortening the juvenile period and increasing olive and oil yields led to the recent selection of a new variety known as 'Chiquitita' (Rallo *et al.*, 2008).

The same technique was used in China to try to select varieties adapted to local soil and climatic conditions following the failure of trials conducted on foreign varieties introduced from the Mediterranean (Fontanazza and Baldoni, 1990). As a result, new cold-resistant cultivars were obtained (Ying *et al.*, 1984).

In Italy, a genetic improvement programme started in 1971 resulted in the production of 5,000 hybrids through controlled crossbreeding (Bellini *et al.*, 2000 a). Selection based on the criteria of vigour, productivity and fruit quality led to the identification of three new dual-purpose cultivars called 'Arno', 'Tevere' and 'Basento' (Bellini *et al.*, 2000 b).

In France, a new variety known as 'Moncita' and characterised by a short non-bearing period and good quality oil was registered in 1998 in the Official Catalogue of Fruit Species and Varieties (www.Olivettes.fr) after selection of seedlings obtained from non-controlled hybridisation.

In 1993, the International Olive Council embarked on a genetic improvement project entailing controlled crossbreeding in five southern Mediterranean countries, two of which were Tunisia and Morocco (Trigui and Msallem, 2002). Research conducted in Morocco into the resultant hybrids enabled the selection of five high-performance cultivars (Ministry of Agriculture, 2013).

In the case of Tunisia, between 1993 and 1996 this programme focused on the widest grown varieties, namely 'Chemlali Sfax', 'Chétoui' and 'Meski'. The cv. 'Chemlali Sfax' is grown for oil production and is characterised by its vigour, adaptability to different environments and yield (Trigui, 1996). However, there are problems with the fatty acid composition of its oil which has a low content of oleic acid and a high content of palmitic acid (Grati-Kamoun and Khlif, 2001). Crossbreeding of this variety therefore aims to obtain new genotypes that have a better fatty acid composition but still retain the good attributes of this variety.

Since 1997, the resultant hybrids have been planted in a collection housed at two sites in the Sfax region, one at the Research Station of the *Institut de Taous* and the other at the head offices of the *Institut de l'Olivier* (IO). Approximately one hundred hybrids underwent preliminary selection on the basis of their oleic acid content (> 65%) (IO, 2005) and were later characterised with a view to final selection.

The purpose of this paper is to report the agronomic performance of a number of parameters in the hybrid collection relating to tree growth habit, tree status and crop production.

Materials and methods

The plant material for this study comprised 145 hybrids planted at the head offices of the *Institut de l'Olivier* in Sfax (Tunisia) located at the following geographical coordinates: latitude 34° 44' 02" North; longitude 10° 43' 59" East; altitude 6 m. Several local and foreign varieties were used for crossbreeding with the 'Chemlali Sfax' variety.

The trees were planted at a density of 1,250 trees/ha, i.e. on a 2 m x 4 m layout, under localised irrigation.

The following parameters were monitored in this collection:

- Olive production/hybrid (kg) was recorded in November from 2005 to 2013, thus permitting calculation of average production per year for the entire collection and per cross for the entire monitoring period.
- The alternate bearing index of each cross was calculated for the entire monitoring period according to the formula of Hoblyn *et al.* (1936).
- Tree growth habit was recorded in November according to IOC standards (1997), i.e. erect, spreading or drooping.

- Tree status was monitored in two consecutive years and was recorded as "on-crop" or "off-crop".

The data collected are reported for the collection as a whole and for each cross. Each mean is given along with the standard deviation and the variability of each mean is measured by the coefficient of variation (standard deviation/mean)*100).

Results & discussion

Collection

The collection at the *Institut de l'Olivier* houses a total of 145 hybrids belonging to 13 different crosses (Table 1). Apart from the two hybrids obtained by open pollination of the 'Chemlali Sfax' variety, the other crosses were bred from different parent plants from Mediterranean countries. Specifically, the genitors were from France ('Picholine' and 'Lucques'), Lebanon ('Souri'), Algeria ('Sigoise'), Tunisia ('Chemchali ' and 'Sig 4'), Spain ('Arbequina' and 'Manzanilla') and Italy ('Coratina').

It should be noted that 62% of the hybrids were obtained by reciprocal crossbreeding of 'Chemlali Sfax' and 'Coratina'. This percentage is one of the strong points of this hybridisation programme owing to the good flowering and oil properties of the 'Coratina' variety. Al-Kasasbeh *et al.* (2005) report that this variety has good pollen and flower characteristics since it records high rates of perfect flowers, pollen per flower and fertile flowers when cross pollinated. In addition, Zarrouk *et al.* (2009) showed that 'Coratina' oil has a balanced fatty acid composition with a high content of oleic acid (70%) and quite a low level of palmitic acid (9%). Hybrid selection from this cross should prove effective.

Table 1. Breakdown, by cross, of the hybrids housed in the *Institut de l'Olivier* collection

Cross	Number of hybrids
'Arbequina'/'Chemlali'	6
'Chemlali'/'Arbequina'	2
'Chemlali'/'Coratina'	84
'Coratina'/'Chemlali'	6
'Chemlali'/'Lucques'	8
'Chemlali'/'Sigoise'	13
'Chemlali'/'Souri'	17
'Koroneiki'/'Chemlali'	3
Open pollinated 'Chemlali'	2
'Chemlali'/'Manzanilla'	1
'Chemlali'/'Picholine'	1
'Chemlal'i/'Sig 4'	1
'Chemlali'/'Chemchali'	1
Total	145

Annual olive production

Olive crop production in the hybrid collection varied widely from year to year, ranging from 1.9 kg in 2010 to 9 kg in 2005 (Figure 1). Pronounced between-hybrid variability was noted for each year since the coefficient of variation was more than 200% in 2013. Intra-annual variability denoted quite diversified yield performance between the hybrids, which enables effective selection in terms of productivity in the conditions in which the orchard was managed. Inter-annual variability may reveal a tendency towards alternate bearing. Special attention should be paid to this criterion in the final selection of the hybrids.

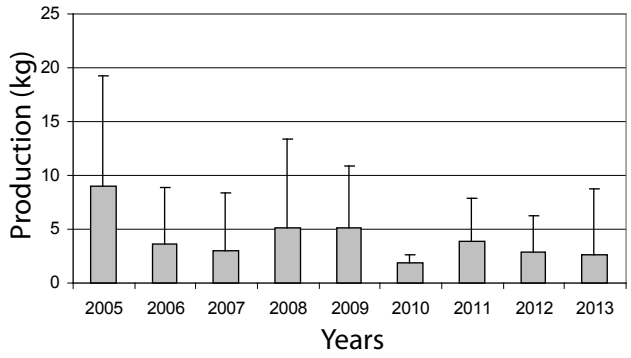

Figure 1: Hybrid olive production by year (horizontal bar = standard deviation)

Olive production by cross

Average production per cross was calculated for six crosses with at least three hybrids in the collection (Figure 2). This criterion was less variable than yearly production since the values oscillated between 3.35 kg ('Chemlali/Sigoise') and 4.86 kg ('Chemlali'/'Souri'). The variability between the hybrids of each cross was also smaller than annual variability, recording a maximum coefficient of variation of 83.7%. The variability observed will enable effective selection of the hybrids from most of the crosses.

It is noteworthy that the production performance of the 'Chemlali Sfax' and 'Coratina' reciprocal crosses was practically the same. This observation may indicate that the genetic determinism of the production trait is independent of the genitor in this cross. Hence, selection for olive crop production may be effective in both reciprocal crosses.

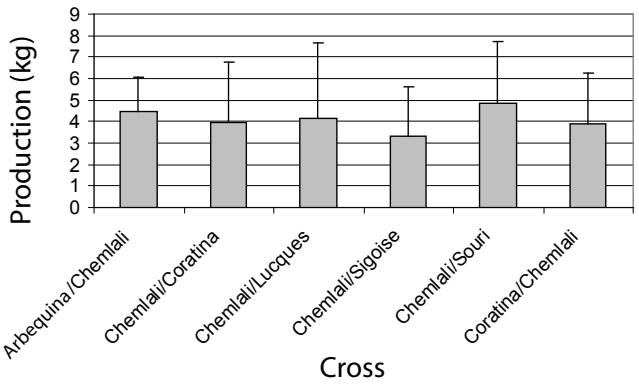

Figure 2: Hybrid olive production by cross

Alternate bearing

The alternate bearing index was calculated for the same six hybrids (Figure 3). Although it varied very little between the crosses it was quite high since the values were between 0.71 ('Chemlali'/'Souri') and 0.87 ('Arbequina'/'Chemlali'). The variability between the hybrids of each cross was also quite small, with a maximum coefficient of variation of 16.2%. These results indicate that the hybrid collection studied was characterised by strict alternate bearing in the conditions of the study. This implies that the selection of productive hybrids will be accompanied by a high degree of alternate bearing. This on/off aspect of the hybrid collection may have its roots in the variety undergoing genetic improvement, i.e. the 'Chemlali Sfax' variety, which is known for its alternate bearing pattern according to Trigui and Msallem (2002).

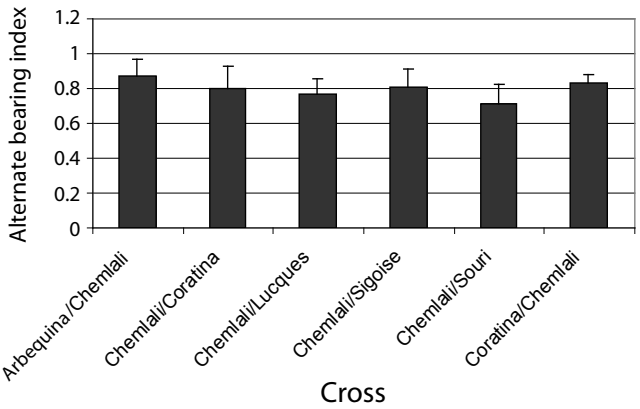

Figure 3: Hybrid alternate bearing index by cross

Tree growth habit

As can be seen from Figure 4, 90% of the hybrids in the collection have a spreading (55%) or erect (35 %) growth habit. Only a small percentage (10%) has a drooping stance. The percentage of trees with a spreading growth habit was highest (45%) among the hybrids crossbred from the 'Chemlali Sfax' and 'Coratina' varieties (Figure 5), although it was lower than the percentage for the collection as a whole. The percentage of the same hybrids with an erect growth habit was similar to the rate for the collection (29%) while it was distinctly higher in the case of those with a drooping growth habit (26%).

The dominance of the spreading growth habit in both cases concurs with the growth stance of the 'Chemlali Sfax' variety reported by IOC (2000) and Trigui and Msallem (2002). The fact that some of the hybrids in the collection have an erect growth habit may be due to the genitors used, such as 'Souri' and 'Lucques' which have such a stance as reported by IOC (2000). In addition, the significantly higher percentage of hybrids with a drooping growth habit among the 'Chemlali Sfax' and 'Coratina' crosses may be indicative of a very specific instance of genetic determinism in these two varieties. A genetic and molecular study of these two genitors and their hybrids must be undertaken to shed light on this determinism. The results of this study will be very useful for the selection of varieties with a drooping growth habit in genetic olive improvement programmes. Such varieties are in great demand nowadays for hyper-intensive olive growing, which Tombesi (2013) reports is gaining more and more ground around the world. Selection for the different types of growth habit is possible in our collection.

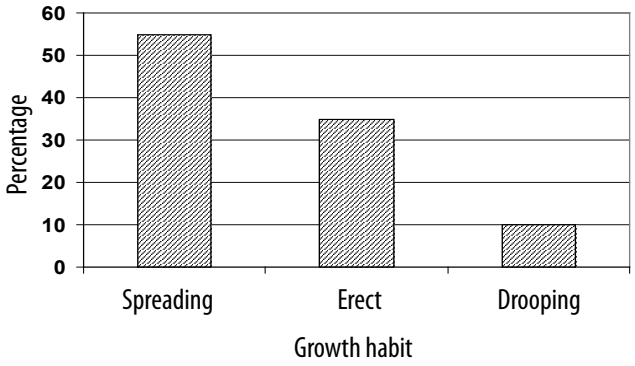

Figure 4: Variability in the growth habit of the hybrids in the collection

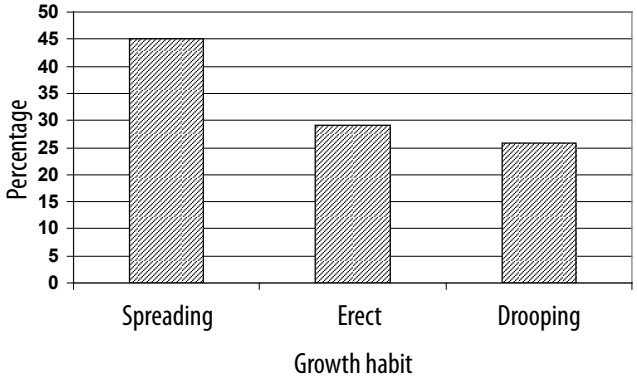

Figure 5: Variability in the growth habit of the hybrids in the collection

Tree status

As can be seen from Table 2, most of the hybrids (72%) in the collection as a whole were off-crop in the first year whereas the hybrids bred from the 'Chemlali Sfax' and 'Coratina' crosses were quite on-crop (58%). In the second year, the tree status of 50% of the hybrids in the collection was on-crop whereas this percentage rose to 100% in the hybrids bred from the 'Chemlali Sfax' and 'Coratina' crosses. Consequently, in the two years, only 17.5% of the hybrids in the collection as a whole were on-crop versus 58% of the group crossbred from the 'Chemlali Sfax' and 'Coratina' varieties.

These values confirm the alternate bearing observed in this hybrid collection. However, this phenomenon is less pronounced in the hybrids crossbred from 'Chemlali Sfax' and 'Coratina'. Selection on the grounds of alternate bearing will therefore be more effective using hybrids of the 'Chemlali Sfax' and 'Coratina' varieties than the other crosses.

Table 2. Tree status of hybrids in two consecutive years (%)

Plant material	Status	Year 1	Year 2	Both years
Hybrid collection	Off-crop	72	48	
	On-crop	28	52	17.5
'Chemlali Sfax' and 'Coratina' crosses	Off-crop	42	0	
	On-crop	58	100	58

Conclusion

This study of the agronomic parameters of the collection of hybrids of the 'Chemlali Sfax' variety reveals that selection on the basis of these parameters shows promise. Moreover, work undertaken since 2005 has led to the preliminary selection of hybrids which are undergoing final evaluation. The plan for the near future is to register certain hybrids in the National Catalogue of Varieties.

References

Al-Kasasbeh M.F., Atteyyeh A.F., Qrunfleh M.M., 2005. A study on self and cross-pollination of three olive varieties in Jordan. Dirasat Agricultural Sciences 32 (2): 222-227.

Bellini E., Giordani E., Parlati M.V., Pandolfi S., 2000 a. Olive genetic improvement: Thirty years of research. Acta Horticulturae 586: 105-108.
Bellini E., Giordani E., Parlati M.V., 2000 b. Three new olive cultivars obtained by cross breeding. Acta Horticulturae 586: 221-223.
Fontanazza G., Baldoni L., 1990. Proposition pour un programme d'amélioration génétique de l'olivier. Olivae 34 : 32-40.
Grati-Kamoun N., Khlif M., 2001. Caractérisation technologique des variétés d'olivier cultivées en Tunisie. Revue Ezzitouna (numéro spécial). 69 p.
IO, 2005. Rapport d'activité de l'Institut de l'Olivier pour l'année 2005.
IOC, 1997. Methodology for the primary and secondary characterisation of olive varieties. RESGEN-CT project (96/97). European Union–IOC.
IOC, 2000. World catalogue of olive varieties. Barranco D., Cimato A., Fiorino P., Rallo L., Touzani A., Castañeda C., Serafini F., Trujillo I., 360 pp, illustrated.
Lavee S. 1978. Kadesh table olive. HortScience 131 (1): 62-63.
Lavee S., Haskal A., Wodner M. 1986. 'Barnea': a new olive cultivar from first breeding generation. Olea 17: 95-99.
Lavee S., Harshemesh H., Haskal A., Meni Y., Wodner M., Ogrodovich A., Avidan B., Wiesman Z., Avidan N., Trapero-Casus A. 1999. « Maalot », un nouveau cultivar résistant à l'œil de paon (*Spilocaea oleagina,* Cast). Olivae 78 : 51-59.
Lavee S., Avidan B., Meni Y. 2003. « Askal » : une nouvelle variété performante d'olivier à huile pour les oliveraies intensives et super-intensives. Olivae 97 : 53-59.
Lavee S., Avidan B., Meni Y., Haskal A., Wodner M. 2004. Trois nouvelles variétés d'olivier demi-naines pour la table. Olivae 102 : 33-41.
Ministry of Agriculture and Sea Fisheries. 2013. Veille économique du secteur oléicole. Note stratégique n° 95. Septembre 2013. 14 p.
Rallo L., Barranco D., De La Rosa L., Leon L. 2008. Chiquitita olive. HortScience 43: 529-531.
Trigui A. 1996. L'amélioration quantitative et qualitative de la production oléicole en Tunisie : l'incontournable nécessité et les perspectives de l'identification et de l'amélioration génétique de l'olivier. Olivae 61 : 34-40.
Trigui A., et Msallem M. 2002. Catalogue des variétés autochtones et types locaux, 159 p.
Tombesi A. 2013. Advances in harvesting and pruning of olive trees. La Rivista di Scienza dell'Alimentazione. 42 (1): 97-103.
Ying G., Shu-zhi H., Ziu-jun S., Hui-chan B, Hou-jun W., Shan-an H., 1984. Studies on variation and selection of olive seedlings for cold resistance. In: Shan-an H, Ying G. (eds): Olive acclimatisation and breeding.
Zarrouk W., Baccouri B., Taamalli W., Trigui A., Daoud D., Zarrouk M. 2009. Oil fatty acid composition of eighteen Mediterranean olive varieties cultivated under the arid conditions of Boughrara (southern Tunisia). Grasas y aceites. 60 (5): 498-506.
www.olivettes.fr/fr/biodiversité/olives.pdf
(site consulted on 03/02/2015).

Oleokoronal and oleomissional: new major phenolic ingredients of extra virgin olive oil

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Abstract

Extra virgin olive oil contains significant quantities of polar phenolic ingredients. The large majority is made up of esters of tyrosol or hydroxytyrosol with secoiridoid derivatives from oleuropein or ligstroside. In the current study we describe a number of new or incompletely characterized forms of ligstroside and oleuropein aglycons. Two of them which are stable enolic forms are described for the first time as real olive oil ingredients although their presence in olive oil had been postulated. To minimize the confusion with the complicated names of the aglycon isomers we propose the names oleokoronal and oleomissional for the two ingredients. After screening 2000 samples of olive oil from most major varieties we were able to identify samples of olive oil in which oleokoronal and oleomissional were the major phenolic ingredients and could be used as starting material for their isolation. Interestingly, during normal or reversed phase chromatography both compounds were transformed to the known forms of monoaldehydic closed ring aglycons, which offers an explanation as to why those compounds had not been identified so far. Their real presence in olive oil was confirmed by direct NMR observation without the use of any solvent.

Key words

Phenolics, secoiridoids, ligstroside aglycon, oleuropein aglycon, olive oil, NMR.

Received: August 2015 / **Accepted:** October 2015 / **Published:** November 2015

Introduction

The traditional Mediterranean diet, which is attracting continuous interest from the scientific community because of its health protecting properties, is based on the daily consumption of olive oil as the major source of lipids. Secoiridoid phenolic derivatives are one of the most important classes of constituents in olive oil which present an increasing potential for health protection. European Union legislation (EU 432/2012) based on the scientific opinion of EFSA has recently permitted specific health claims related to the levels of specific phenolic compounds found in olive oil.

The key compounds that are responsible for the recognized health claim "protection of blood lipids from oxidative stress" are hydroxytyrosol (**1**), tyrosol (**2**) and their derivatives. For this reason, it is very important to obtain accurate knowledge of the chemical identity of all these ingredients and to perform their quantitative measurement in olive oil. As yet there is no officially adopted method for the measurement of the ingredients related with the health claim because of well known technical difficulties. Hydroxytyrosol (3,4-DHPEA) and tyrosol (p-HPEA) are found in olive oil mainly in the esterified forms of oleacein (3,4-DHPEA–EDA) (**3**) and oleocanthal (p-HPEA-EDA) (**4**) as well as oleuropein aglycon (3,4-DHPEA-EA) (**5a**) and ligstroside aglycon (p-HPEA-EA) (**6a**), which all have significant biological activities.

However, the terms oleuropein aglycon and ligstroside aglycon are not accurately defined and are often used in a misleading way. In fact, there are many possible isomers of the aglycons and many of them are not well characterized. They are often reported with complicated descriptive names like: hydroxylated form, monoaldehydic form, dialdehydic form, hydrated form, open ring, closed ring, carboxylated, decarboxylated etc. The lack of accurate and official definitions of names and of robust NMR data is the source of several problems in the related literature. In the current paper we discuss the isolation and structure elucidation of a series of previously undescribed forms of oleuropein aglycon and ligstroside aglycon and we clarify the terms and the NMR characterization of the previously described members of this family of compounds.

Materials and methods

General

Almond β-glucosidase was purchased from Sigma-Aldrich. Oleuropein was isolated from leaves of wild olive trees with a high oleuropein content (15% per dry weight) as previously described (Andreadou *et al* 2006). NMR spectra were recorded on an Avance 700 spectrometer; chemical shifts were expressed in ppm and the axes were calibrated on the residual signal of $CDCl_3$. Column chromatography was performed on columns containing RP-18 Si gel 60 (40–63 μm) (Merck, Darmstadt, Germany). Thin layer chromatography (TLC) was performed on plates coated with RP-18 Si gel 60 F254 Merck, 0.25 mm.

Olive oil

The isolation of the studied compounds was performed in two types of oil: the first was provided by the Cooperative of Paleopanagia, Lakonia, Greece and was produced in November 2013 from the Koroneiki variety in a three phase mill operating at 25 °C with a 30 min malaxation period. The second was provided by Berkeley olive grove, Oroville, California and was produced from the Mission variety in November 2013.

Other olive oils used for screening came from the sample database as previously described (Karkoula *et al* 2014).

Extraction and isolation

Olive oil (100 g) was mixed with cyclohexane (400 mL) and acetonitrile (500 mL) and the mixture was homogenized and centrifuged at 4,000 rpm for 5 min. The acetonitrile phase was collected using a separation funnel, and evaporated under reduced pressure using a rotary evaporator. The residue was subjected to reversed phase silica gel column chromatography with acetonitrile 100% to remove the residual lipids. All the collected fractions that were free of lipids were pooled, evaporated and re-chromatographed on a reversed phase preparative TLC, (H_2O/acetonitrile 60:40), resulting in the isolation of two zones: A (2.3 mg/ rf = 0.5) and B (3.3 mg/ rf = 0.7). The structure of the isolated compounds (**Figure 1**) was studied using a combination of 1D and 2D NMR spectra and the assigned peaks are presented in **Tables 1-4**.

Table 1. ^{1}H-NMR data of oleokoronal (14) and ligstrodials (12a,b) at concentration 3 mg/0.6 mL. Axis calibration based on $CDCl_3$ = 7.26 ppm

	12a (5S,4R)	12b (5S,4S)	14
1	9.212, d, 2.0	9.218, d, 2.0	9.225, d, 1.7
3	9.68, d, 2.7	9.46, d, 2.7	7.386, dd, 12.6, 0.8
4	4.06, dd, 10.5, 2.7	4.11, dd, 10.5, 2.7	-
5	3.83, m, (overlap)	3.83, m (overlap)	4.16, ddd (overlap)
6a	2.81, (overlap)	2.82, (overlap)	2.97, dd, 16.1, 9.6
6b	2.62, (overlap)	2.58, (overlap)	2.78, dd, 16.1, 6.3
8	6.70, q. 7.1	6.72, q, 7.1	6.56, q, 7.1
10	2.055, d, 7.0	2.062, d, 7.1	2.062, d, 7.0
3-OH	-	-	11.75, d, 12.6
OCH3	3.65, s	3.77, s	3.75, s
1′	4.20, m	4.18, m	4.18, m
2′	2.81, m	2.81, m	2.81, m
4′,8′	7.05, d (overlap)	7.05, d (overlap)	7.05, d (overlap)
5′, 7′	6.76, d (overlap)	6.76, d (overlap)	6.76, d (overlap)

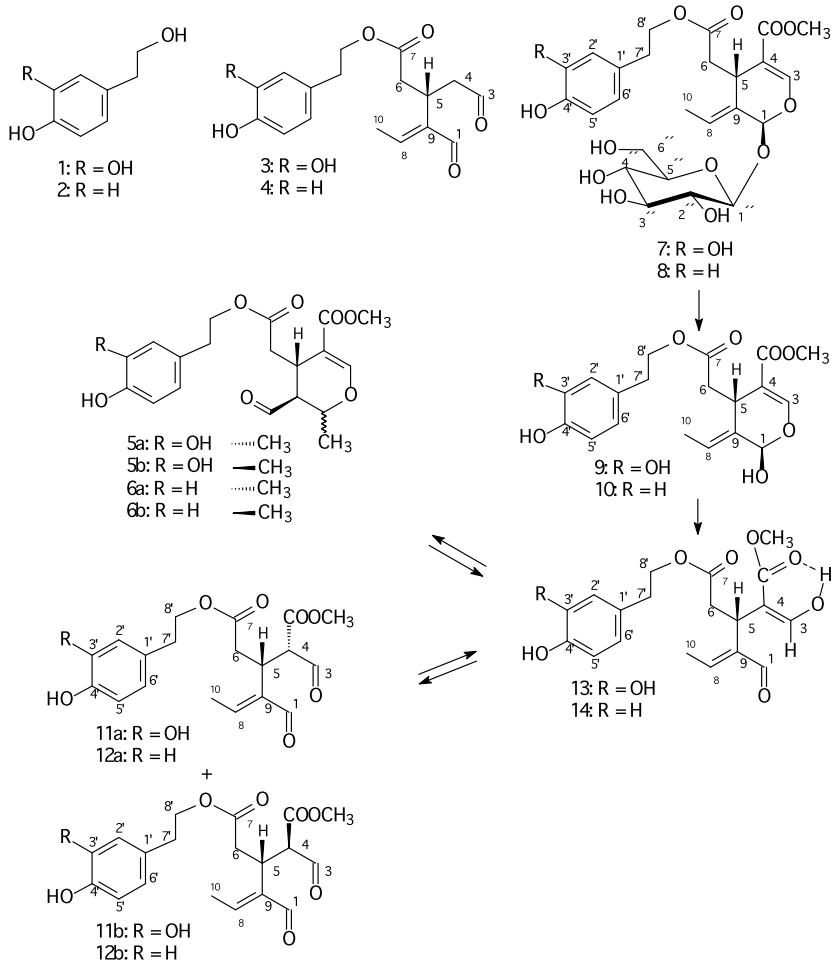

Figure 1: Structures of the studied compounds

NMR analysis of olive oil with extraction

The extraction and analysis of the olive oil was performed as previously described (Karkoula *et al.* 2014). Briefly, olive oil (5.0 g) was mixed with cyclohexane (20 mL) and acetonitrile (25 mL) and the mixture was homogenized using a vortex mixer for 30 sec and centrifuged at 4,000 rpm for 5 min. A part of the acetonitrile phase (25 mL) was collected, mixed with 1.0 mL of a syringaldehyde solution (0.5 mg/mL) in acetonitrile and evaporated under reduced pressure using a rotary evaporator. The residue was dissolved in $CDCl_3$ (750 μL) and an accurately measured volume of the solution (550 μL) was transferred to a 5 mm NMR tube. Typically, 50 scans were collected into 32K data points over a spectral width of 0-16 ppm with a relaxation delay of 1 s and an acquisition time of 1.7 s. Prior to Fourier transformation, an exponential weighting factor corresponding to a line broadening of 0.3 Hz was applied. The spectra were phased, corrected and integrated automatically using TopSpin software (Bruker). Accurate integration was performed manually for the peaks of interest.

Selective excitation pulse NMR analysis of olive oil without extraction

600 μL of olive oil were transferred to a 5mm NMR tube without any deuterated solvent. NMR spectra of the neat samples were obtained without deuterium lock, setting the field offset to the same value as that obtained for the extracted sample locked in $CDCl_3$. The DPFGPE sequence was executed utilizing a 1.26 ms 180 degree reburp selective refocusing pulse, affording a 4900 Hz excitation window from 14 to 7 ppm. Data from 16 scans were collected. The spectra were phase corrected automatically using TopSpin software (Bruker).

Synthesis of oleuropein aglycon

In a 25-mL round bottomed flask containing acetate buffer (pH = 5, 5 mL), oleuropein (100 mg) was dissolved. Chloroform (5 mL) was added and the resulting biphasic system was stirred gently. To this system, *β*-glucosidase (3.4 Units/mg, 70 mg) was added and the resulting reaction mixture was stirred gently at 30 °C for 20 h, or until TLC (12 % MeOH in dichloromethane) showed complete consumption of oleuropein. The organic phase was separated and the aqueous phase was extracted with chloroform (3x5 mL). The combined organic extracts were washed with brine (20 mL), dried over sodium sulfate, then filtered and evaporated under reduced pressure. The crude product (66 mg, 94 %) was analyzed by ^{1}H NMR.

Results and discussion

Oleuropein aglycon and ligstroside aglycon are terms that are not accurately defined and are often used in a misleading way. Both terms correspond to a large number of isomers which in many cases are not well described. In fact most secoiridoid phenolic derivatives in olive oil come from oleuropein (**7**) and ligstroside (**8**), which are the major secoiridoids in the olive fruit. During crushing and malaxation for the production of olive oil, these two ingredients come into contact with *β*-glucosidase (Koudounas *et al* 2015) and are initially transformed to the corresponding real aglycons **9** and **10**. These two forms are unstable and have only been observed under very specific conditions (Christophoridou and Dais 2009). Inside the matrix of olive oil or generally in a non aqueous medium the real aglycons **9** and **10** do not exist and are mainly transformed to the more stable closed ring monoaldehydic forms **5a** and **6a** by rearrangement (Limiroli *et al* 1995) or to open ring dialdehydic forms. The closed ring forms have also been described in different forms of steroisomers e.g. **5b** and **6b** but others also exist (Perez-Trujillo *et al* 2010). As we have recently shown, the closed ring forms of oleuropein and ligstroside aglycons that dominate in olive oil are found in the **5a** and **6a** form, while the other steroisomers are artificially increased during chromatographic purification or analysis (Karkoula *et al.* 2014) and are normally found at a very low ratio or are totally absent.

We have recently published a methodology for the observation and quantitation of the closed ring monoaldehydic forms **5a** and **6a** using qNMR, together with oleacein (**3**) and oleocanthal (**4**) (Karkoula *et al* 2012; Karkoula *et al* 2014). During the application of this methodology to 2000 samples of olive oil obtained from several different varieties in different production conditions we were able to observe in several cases a number of peaks corresponding to aldehydic protons which did not correspond to any described structure but which we could tentatively relate to isomers of oleuropein and ligstroside aglycons. Interestingly, in some samples of olive oils those peaks corresponded to major phenolic ingredients. For this reason, we decided to isolate the unknown ingredients and characterize their structure, using as starting material two specifically selected olive oils from the Koroneiki and Mission varieties.

Surprisingly, all initial efforts at isolation with column chromatography or thin layer chromatography, either normal or reversed phase, led only to the known structures **5a**,**b** and **6a,b** although the NMR analysis showed only small concentrations of these structures in the original oil. Based on this observation, we assumed that the

unknown ingredients were structures which were transformed to **5a**,**b** and **6a,b** during purification**.** Careful re-investigation of the two TLC zones A and B revealed the presence of a small remnant of the initial form, identical to that observed in the olive oil extract before purification. NMR analysis of the aforementioned zones showed that it was an inseparable mixture of three compounds in each case. Since the mixture was inseparable and was transformed during any attempt at purification we studied it as a mixture with extended 2D NMR experiments in order to elucidate the structure of each compound and attribute the observed peaks (**Tables 1**-**4**).

Table 2. ^{13}C-NMR data of oleokoronal (14) and ligstrodials (12a,b) at concentration 3 mg/0.6 mL. Axis calibration based on $CDCl_3$ = 177.16 ppm

	12a (5S,4R)	12b (5S,4S)	14
1	194.75	194.56	194.88
3	195.72	195.08	164.23
4	60.27	59.30	103.33
5	31.42	31.42	29.95
6	34.52	34.98	35.61
7	172.07	171.33	171.98
8	156.11	155.74	153.53
9	142.40	141.87	142.68
10	15.20	15.20	15.20
11	168.18	168.55	172.18
OCH3	52.38	52.61	51.59
1′	65.24	65.24	65.24
2′	34.02	34.02	34.02
3′	129.88	129.88	129.95
4′,8′	130.03	130.03	130.05
5′,7′	115.29	115.29	115.29
6′	154.13	154.13	154.13

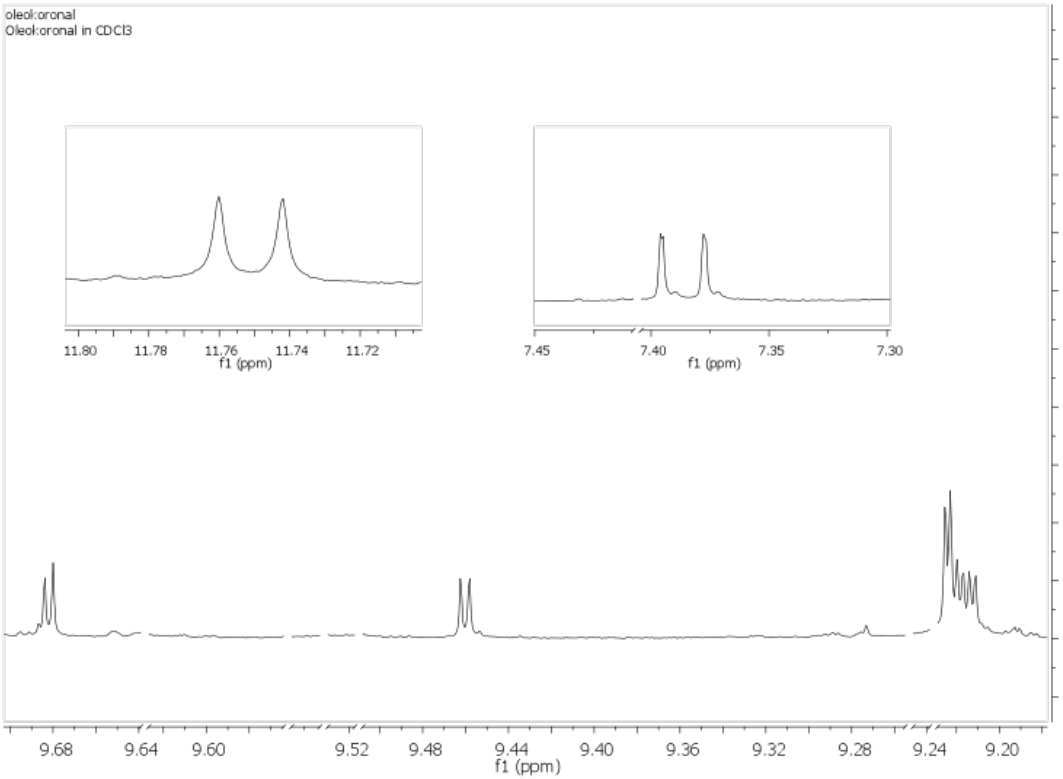

Figure 2: ^{1}H NMR spectrum of the aldehydic and olefinic region of oleokoronal and 4S/4R-ligstrodial mixture

Structure elucidation revealed that the major ingredient in each mixture was an open ring monoaldehydic form while the other two corresponded to open ring dialdehydic forms. The ratio between the three compounds as determined by the integration of the three aldehydic protons e.g. at 9.21-9.22 ppm for zone A was 2:1:1 **(Figure 2**). The first mixture of the three compounds of zone A showed the characteristic signals of the tyrosol moiety while the second one of zone B showed the characteristic signals of the hydroxytyrosol moiety. Using HSQC, HMBC, COSY and TOCSY experiments we were able to assign all the peaks of the three compounds in each mixture. All the compounds showed the characteristic peaks of a methyl attached to a double bond adjacent to an olefinic proton as well as all the other peaks expected for the open ring form of elenolic acid esterified with either tyrosol or hydroxytyrosol. The two minor constituents of each mixture corresponded to the *4R,5S* and *4S,5S* pairs of the open ring dialdehydic forms of oleuropein aglycon and ligstroside aglycon **11a,b** and **12a,b.** The third major ingredient in each mixture corresponded to a very similar structure with a main difference; the absence of a second aldehyde. Interestingly, instead of a second aldehyde each molecule showed a highly deshielded doublet around 11.8 ppm which in the HSQC was not correlated to any carbon. Moreover, an additional olefinic proton was observed around 7.3 ppm as a doublet (J = 16 Hz) correlated with the doublet at 11.8 ppm. The olefinic proton in the HMBC experiment was correlated with a carbonyl bearing a methoxy group. It was obvious from all these data that the major ingredient in each zone was the monoenolic form of dialdehydes **11a,b** and **12a,b.** Contrary to expectations, the enol forms **13** and **14** were stable due to the hydrogen bond between the enol hydroxyl and the adjacent carbonyl of the carbomethoxy group.

The existence of the enol form of the ligstroside aglycon **14** had been postulated in the past (e.g Angerosa *et al* 1996); however, it is undoubtedly reported here for the first time as a natural product and ingredient of olive oil which we propose naming oleokoronal. The dialdehydic forms of the ligstroside aglycon **12a,b** have been reported several times as olive oil ingredients through MS identification (e.g. De Nino *et al* 2000) but their structure elucidation with NMR has never been reported. Similarly, they have been reported through MS identification in olive mill waste water by Sanchez de Medina *et al* (2011) and named as p-HPEA-FA. In some cases they have been reported as ligstroside aglycone dialdehyde but in fact they refer to p-HPEA-EDA or oleocanthal, which is confused with the name decarboxymethyl ligstroside aglycon dialdehyde (e.g. Gomez del Campo and Garcia 2012). To minimize the use of long descriptive and ambiguous names we propose that the most appropriate name for **12a,b** would be *4R/4S*-ligstrodial, by association with ligstral which refers to the closed ring monaldehydic form of the ligstroside aglycon **6a,b** (Calis *et al* 1993).

The enolic form of the oleuropein aglycon **13** has never been reported as an olive oil ingredient but only as an ingredient of unprocessed olive fruits (Bianco & Uccella 2000) or as an enzymatic hydrolysis product of oleuropein (Bianco *et al* 1999a). However, there are several discrepancies in the NMR description of this compound (Bianco & Uccella 2000; Bianco *et al* 1999a) which need to be revised. Most significantly, the authors did not report the critical peak of the enolic hydroxyl at 11.78 ppm. They also reported the chemical shift of the olefinic carbon bearing the carbomethoxy group at 130.37 ppm whereas we observed it at 103.69 ppm. The enolic olefinic proton at 7.36 ppm was given as a doublet with 6 Hz coupling while the real coupling is 16 Hz and obviously there is no OH signal at 7.40 ppm as previously reported. There are several other differences in the ^{1}H and ^{13}C NMR as well as several missing coupling constants, all of which makes a more complete and precise description necessary. It should be noted that this compound and **11a,b** present significant drift of specific ^{1}H-NMR chemical shifts depending on the concentration of the sample (**Table 4** and **Figure 3**). This can explain some differences with regard to previous literature data but not the major ones. Interestingly, the same authors in a parallel paper (Bianco *et al* 1999b) assign the peaks observed at 11.79-11.83 ppm to oxidation derivatives (aldehyde to carboxyl) arising slowly in $CDCl_3$. Based on our results, those peaks are not due to oxidation but to equilibrium between aldehyde and enol.

Table 3. ^{1}H-NMR data of oleomissional (13) and oleuropeindials (11a,b) at concentration 20 mg/0.6 mL.[a] Axis calibration based on $CDCl_3$ = 7.26 ppm

	11a (5S,4R)	11b (5S,4S)	13
1	9.17, d, 2.0	9.17, d, 2.0	9.17, d, 2.0
3	9.67, d, 2.7	9.44, d, 2.7	7.36, dd, 12.6, 0.8
4	4.04, dd, 10.5, 2.7	4.10, dd, 10.5, 2.7	-
5	3.84, tdd, 10.5, 4.7, 2.0	3.80, tdd, 10.5, 4.3, 2.0	4.17, ddd (overlap)
6a	2.82, dd, 16.0, 10.5	2.83, dd, 15.8, 10.5	2.98, dd, 15.8, 9.6
6b	2.63, dd, 16.0, 4.7	2.58, dd, 15.8, 4.3	2.77, dd, (overlap)
8	6.70, q. 7.1	6.73, q, 7.1	6.59, q, 7.1
10	2.02, d, 7.1	1.99, d, 7.1	2.05, d, 7.1
3-OH	-	-	11.78, d, 12.6
OCH_3	3.65, s	3.78, s	3.74, s
1′	4.21, m	4.12, m	4.16, m
2′	2.75, m	2.75, m	2.75, m
4′	6.68, d, 1.8[b]	6.67, d, 1.8[b]	6.71, d, 1.8[b]
7′	6.78, d (overlap)	6.78, d (overlap)	6.78, d (overlap)
8′	6.58	6.58	6.60 dd

[a] Small differences in chemical shifts were observed for **11a**, **11b** and **13** when the spectra were recorded at concentration 1 mg/0.6 mL (e.g H-3: 9.70/9.46/7.39, H-10: 2.04/1.98/2.07, $COOCH_3$: 3.66/3.80/3.76 respectively)

[b] may interchange

Table 4. ^{13}C-NMR data of oleomissional (13) and oleuropeindials (11a,b) at concentration 20 mg/0.6 mL. Axis calibration based on $CDCl_3$ = 177.16 ppm

	11a (5S,4R)	11b (5S,4S)	13
1	195.53	195.65	196.04
3	196.24	195.41	164.07
4	60.45	59.50	103.69
5	31.58	31.97	30.14
6	34.80	35.44	35.92
7	171.71	171.46	172.18
8	156.78	157.29	155.05
9	141.14	140.52	142.40
10	15.40	15.49	15.41
11	168.23	169.02	172.12
OCH3	52.69	53.02	51.86
1′	65.52	65.52	65.52
2′	34.28	34.28	34.28
3′	130.46	130.46	130.53
4′	116.29	116.24	116.38
5′	143.60	143.65	143.68
6′	143.05	143.00	142.92
7′	115.39	115.39	115.39
8′	121.27	121.33	121.27

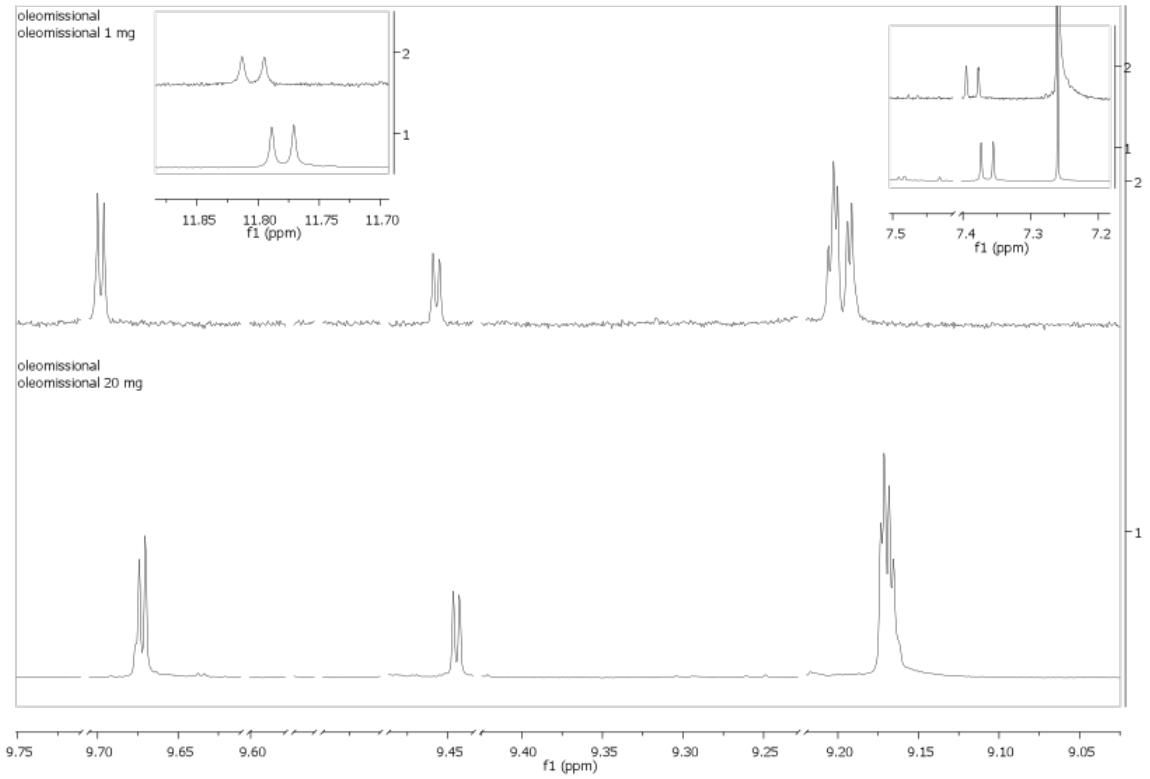

Figure 3: ^{1}H NMR spectrum of the aldehydic and olefinic region of oleomissional and 4S/4R-oleuropeindial mixture (top: 1 mg/0.6 ml, bottom: 20 mg/0.6 ml).

The same confusion seems to appear in the case of the paper by Paiva-Martins & Gordon (2001) where two peaks at 11.76 and 11.82 ppm are assigned to the carboxyl groups of two geometric isomers arising from the hydrolysis of the carbomethoxy groups of **13**. Similarly, the cause is not hydrolysis or oxidation but the equilibrium between the aldehyde and the enol form; the proposed structure should therefore be considered questionable. The same authors in the same work also describe the open ring dialdehydic forms **11a,b** isolated from olive leaves but the reported NMR data are incomplete for many coupling constants. Also, in several cases there is confusion in the ^{13}C data between the peaks assigned to each of the two diastereoisomers.

Compounds **11a,b** have been reported in olive oil by De Nino *et al* (2000) but the NMR description is very misleading and contains several inconsistent assignments that also need revision. The description by Bianco *et al* (1999a) in oleuropein hydrolysis is more accurate but there are still several peaks that are not observed (e.g at 56 ppm) or observed at quite different chemical shifts (>5 ppm difference for the carbon bearing the carbomethoxy group) or for which the description of the splitting pattern and the corresponding coupling constants are incomplete. The most accurate description has been given by Christophoridou & Dais (2009) in a section of a paper related to the study of the enzymatic hydrolysis of oleuropein, but there are still differences and no carbon NMR data are provided.

To avoid confusion and to simplify the nomenclature of the enol form **13**, we propose the name oleomissional instead of the erroneously described enololeuropeindial (Bianco *et al* 1999a). For the open ring dialdehyde forms of the oleuropein aglycon the most appropriate name would be *4S/4R*-oleuropeindial as previously reported (Bianco *et al* 1999).

In order to confirm that oleokoronal is a real ingredient of olive oil and not an artifact produced during the extraction or dilution of olive oil with the NMR solvent ($CDCl_3$) we performed a selective excitation pulse experiment in an olive oil sample without solvent at all as described in the experimental part and compared it with the extract of the same olive oil diluted in $CDCl_3$. The selective excitation pulse permits observation of low concentration peaks in the presence of other ingredients at a very high concentration (e.g. lipids). The methodology applied has been described in detail previously (Melliou *et al* 2015). In this experiment we were able to observe the deshielded hydroxyl at 11.78 ppm of the enol form **14** of ligstroside aglycon in intact olive oil and in extract from the same

olive oil (**Figure 4**) proving that it is a real ingredient of olive oil. The observed drift of the chemical shifts in the neat oil spectrum is caused by the presence of high amounts of lipids.

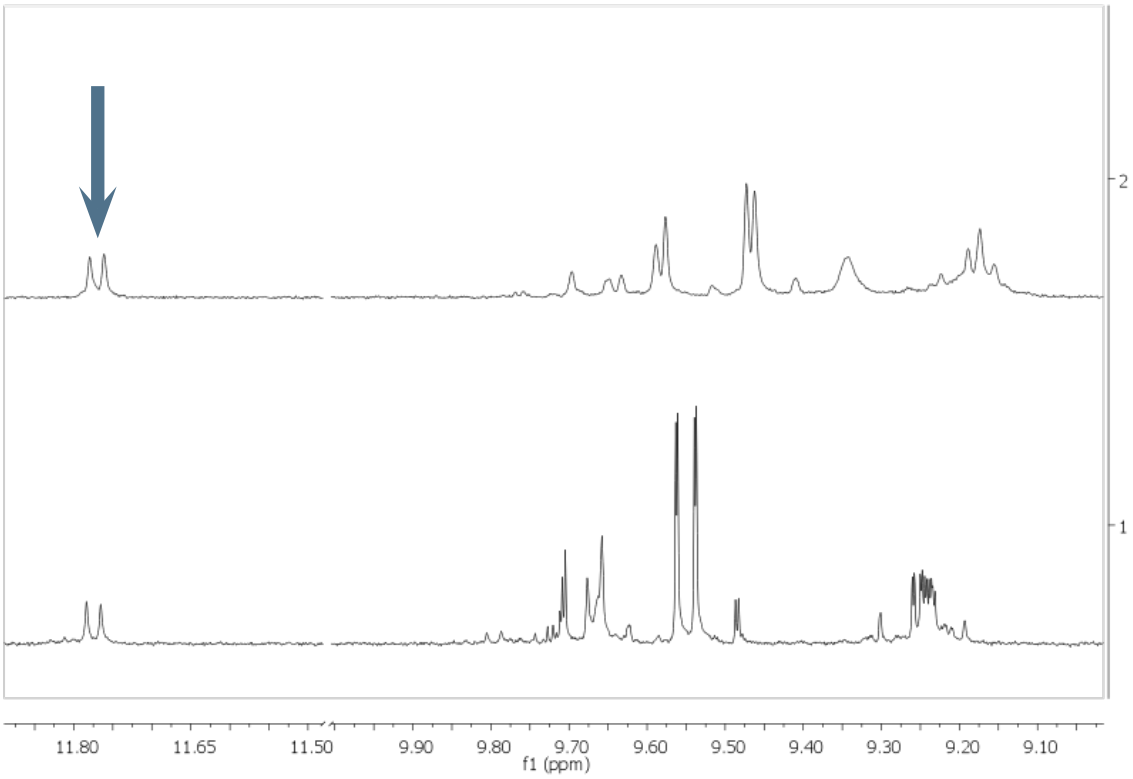

Figure 4: Selective excitation pulse ^{1}H NMR spectrum of neat olive oil (top) or olive oil extract diluted in $CDCl_3$ (bottom). The peak of oleokoronal at 11.78 ppm is observable in both cases showing that oleokoronal is a real ingredient of olive oil.

To further confirm the proposed structures we performed the enzymatic transformation of oleuropein to the open ring forms. Under appropriate pH conditions it was possible to minimize the transformation of oleuropein to the closed ring form **5a** and to direct the transformation only to the open ring forms. The resultant mixture of compounds showed exactly the same peaks as the mixture isolated from olive oil and again the major derivative was the enol form **13** accompanied by the two stereoisomers **11a** and **11b**.

Concerning the quantitative measurement of the oleuropein and ligstroside aglycons, in a previous work we presented a validated method for the measurement of the closed ring monoaldehyde forms **5** and **6** (Karkoula *et al*. 2014). Using the same methodology and calibration curves we were able to obtain quantitative data for each of the dialdehydic and enolic forms. Complete separation of each peak is achieved only with high magnetic field NMRs (>600 MHz); however, even at 400 MHz, NMR integration of the whole region of the aldehydic protons can provide quantitative data for all the forms of open ring aglycons as a total.

As regards the abundance of oleokoronal and oleomissional it should be mentioned that they were first detected in olive oil from the Koroneiki variety (**Figure 5**) and Mission variety (**Figure 6a**). It should be noted that in most of the oils studied the concentration of oleokoronal and oleomissional and of the related dialdehydes was lower than that of oleocanthal and oleacein and in many cases they were totally absent (**Figure 6b**). However, it seems that in specific varieties and also depending on the oil production parameters, oleokoronal or oleomissional can be major phenolics in olive oil. More data on this issue as well as more quantitative data are the subject of a separate paper.

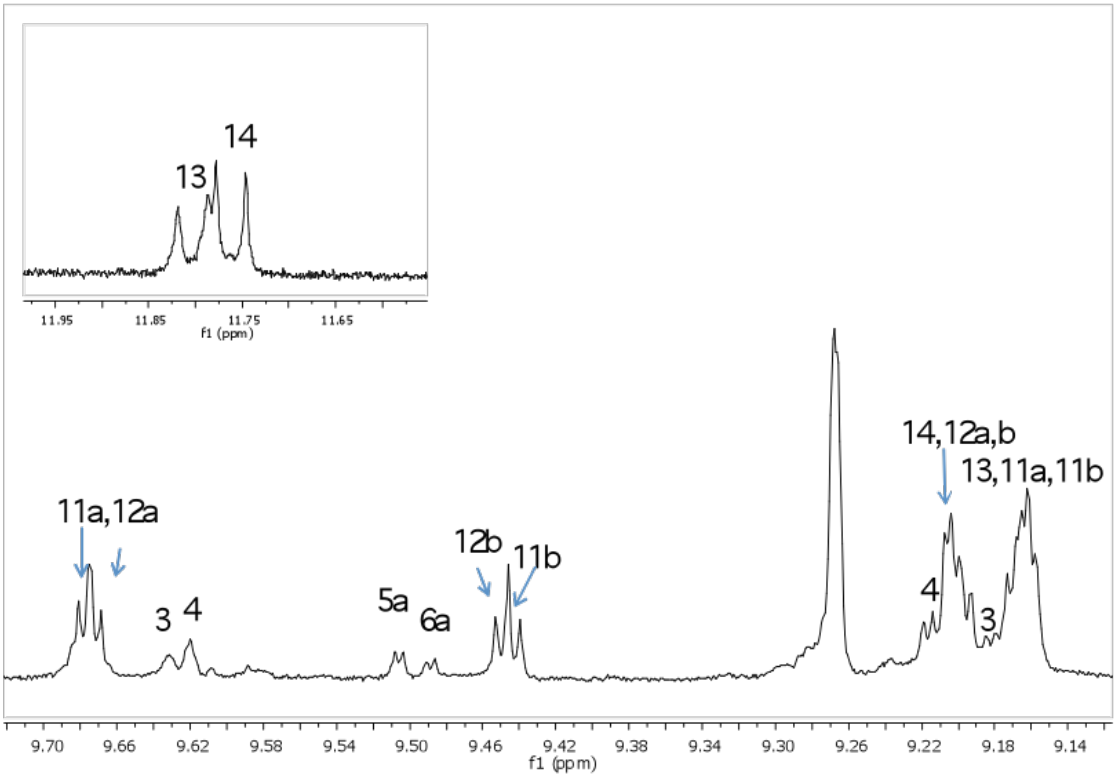

Figure 5: [1]H NMR spectrum of the aldehydic region of Koroneiki olive oil produced at low temperature and with a short malaxation time (recorded at 400 MHz).

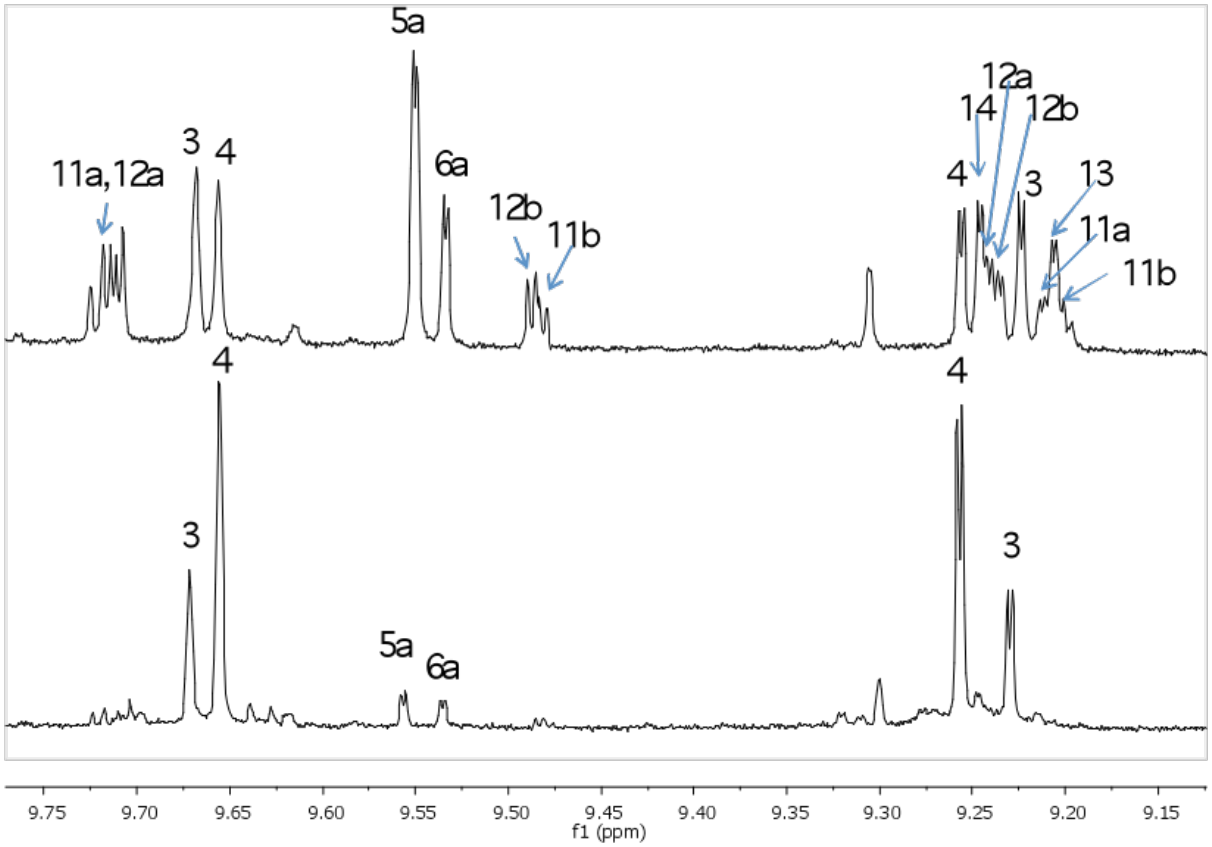

Figure 6: [1]H NMR spectrum of the aldehydic region of Mission olive oil extract sample (a: top) and typical Koroneiki (b: bottom) showing the peaks corresponding to compounds **3, 4, 5a, 6a, 11a,b, 12a,b, 13, 14.** The chemical shift of the observed peaks in the olive oil extract is slightly different from that of the pure compounds due to the presence of lipids in the extract (not shown).

Conclusion

In the current work we isolated and described by NMR a series of compounds belonging to the oleuropein and ligstroside aglycon family, some of which have been found for the first time as olive oil ingredients. New names like oleokoronal, oleomissional and ligstrodial have been proposed in order to minimize the confusion arising from the use of complicated or abbreviated names. Direct NMR study of neat olive oil has proven that the enolic forms of the aglycons are major phenolic ingredients, at least of specific olive oils. The complete characterization of the new compounds and the simplicity of the quantitative NMR analysis offer a new perspective for the quality control of the health protecting properties of olive oil.

Acknowledgements

We would like to thank the Cooperative of Paleopanagia in Lakonia and Berkeley Olive Grove in California for the olive oil samples, Mrs. Marcomichelaki from Volvi Estate for the offer of olive leaves, Venia Smyrli and Stella Konstantopoulou for the isolation of oleuropein and Prof. Diomedes Logothetis for the offer of the NMR facility at VCU. A.V. and T.G. acknowledge funding by the SYNERGASIA 2011 PROGRAM. This Program is co-funded by the European Regional Development Fund and National Resources (Project Code 11SYN_2_1613). P.D., E.M. and P.M. acknowledge the Latsis foundation for financial support.

References

Andreadou, I., Iliodromitis, E., Mikros, E., Constantinou, M., Agalias, A., Magiatis, P., Skaltsounis, A.L., Kamber, E., Tsantili-Kakoulidou, A., and Kremastinos, D. 2006. *The olive constituent oleuropein exhibits anti-ischemic, antioxidative, and hypolipidemic effects in anesthetized rabbits*. J. Nutrition 136: 2213-2219.

Angerosa, F., d'Alessandro N., Corana, F., and Mellerio, G. 1996. *Characterization of phenolic and secoiridoid aglycons present in virgin olive oil by gas chromatography-chemical ionization mass spectrometry.* J. Chromatogr. A, 736: 195-203.

Bianco, A. D. and Uccella, N. 2000. *Biophenolic components of olives*. Food Res. Intl. 33: 475-485

Bianco, A. D., Muzzalupo, I., Piperno, A., Romeo, G. and Uccella, N. 1999. *Bioactive Derivatives of Oleuropein from Olive Fruits.* J. Agric. Food Chem. 47: 3531-3534.

Bianco, A. D., Piperno, A., Romeo, G. and Uccella, N. 1999. *NMR Experiments of Oleuropein Biomimetic Hydrolysis.* J. Agric. Food Chem. 47: 3665-3668.

Caliş, I., Hosny, M., Khalifa, T., and Nishibe, S. 1993. *Secoiridoids from Fraxinus angustifolia*. Phytochem. 33: 1453-1456,

Christophoridou S. and Dais, P. 2009. *Detection and quantification of phenolic compounds in olive oil by high resolution 1H nuclear magnetic resonance spectroscopy*. Anal. Chim. Acta 633: 283–292.

De Nino, A., Mazzotti, F., Perri, E., Procopio, A., Raffaelli, A., and Sindona, G. 2000. *Virtual freezing of the hemiacetal-aldehyde equilibrium of the aglycones of oleuropein and ligstroside present in olive oils from Carolea and Coratina cultivars by ionspray ionization tandem mass spectrometry*. J. Mass Spec. 35: 461-467

Gomez-del-Campo, M. and Garcia, J. M. 2012. *Canopy Fruit Location Can Affect Olive Oil Quality in 'Arbequina' Hedgerow Orchards.* J. Am. Oil Chem. Soc. 89: 123-133.

Karkoula, E., Skantzari, A., Melliou, E. and Magiatis, P. 2012. *Direct measurement of oleocanthal and oleacein levels in olive oil by quantitative 1H NMR. Establishment of a new index for the characterization of extra virgin olive oils.* J. Agric. Food Chem. 60: 11696-11703.

Karkoula, E., Skantzari, A., Melliou, E., and Magiatis, P. 2014. *Quantitative Measurement of Major Secoiridoid Derivatives in Olive Oil Using qNMR. Proof of the Artificial Formation of Aldehydic Oleuropein and Ligstroside Aglycon Isomers.* J. Agric. Food Chem. 62: 600-607.

Koudounas, K., Banilas, G., Michaelidis, C., Demoliou, C., Rigas, S., and Hatzopoulos, P. 2015. *A defence-related Olea europaea β-glucosidase hydrolyses and activates oleuropein into a potent protein cross-linking agent.* J. Exp. Bot. doi:10.1093/jxb/erv002

Limiroli, R., Consonni, R., Ottolina, G., Marsilio, V., Bianchi, G., and Zetta, L. 1995. *1H and 13C NMR characterisation of new oleuropein aglycone.* J. Chem. Soc., Perkin Trans. 1, 1519-1523.

Melliou, E., Killday, K.B. and Magiatis, P. 2015. *A New Ultra Rapid Screening Method for Olive Oil Health Claim Evaluation Using Selective Pulse NMR Spectroscopy* From the book: Magnetic Resonance in Food Science: Defining Food by Magnetic Resonance. Royal Society of Chemistry

Paiva-Martins, F., Gordon, M. H. 2001. *Isolation and Characterization of the Antioxidant Component 3,4-Dihydroxyphenylethyl 4-formyl-3-formylmethyl-4-hexenoate from Olive (Olea europaea) Leaves.* J. Agric. Food Chem. 49: 4214-4219.

Pérez-Trujillo, M., Gómez-Caravaca, A.M., Segura-

Carretero, A., Fernández-Gutiérrez, A., and Parella; T. 2010. *Separation and identification of phenolic compounds of extra virgin olive oil from Olea europaea L. by HPLC-DAD-SPE-NMR/MS. Identification of a new diasteroisomer of the aldehydic form of oleuropein aglycon.* J. Agric. Food Chem. 58: 9129-9136.

Sanchez de Medina, V., Priego-Capote, F., Jimenez-Ot, C. and Luque de Castro, M. D. 2011. *Quality and stability of edible oils enriched with hydrophilic antioxidants from the olive tree: The role of enrichment extracts and lipid composition*. J. Agric. Food Chem. 59: 11432-11441.

Phytopathological problems and phytosanitary aspects of olive in central-southern Italy: known and newly emerging threats

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Riassunto

La rilevanza economica, sociale, culturale ed eco-ambientale che l'olivo riveste in Italia ed in altri Paesi mediterranei, il mantenimento degli oliveti esistenti, la coltivazione in nuove aree o addirittura la sua introduzione in aree extra-europee richiedono una grande attenzione per le problematiche fitopatologiche della coltura ed un continuo aggiornamento sugli aspetti agro-colturali ed epidemiologici di fitopatie e fitofagi. La presente nota illustra, pertanto, l'attuale situazione delle problematiche fitopatologiche dell'olivo anche alla luce dei cambiamenti climatici, evidenziando come questi possano incidere sulla recrudescenza di malattie già note, focalizzando l'attenzione sulle nuove emergenze e fornendo indicazioni sulle più moderne strategie di difesa integrata ecosostenibile.

Abstract

The economic, social, cultural and eco-environmental importance of olive growing in Italy and the other Mediterranean countries, coupled with the maintenance of existing olive orchards and the spread of olive cultivation to new areas or its introduction in areas outside Europe, makes it necessary to pay close attention to crop phytopathological problems and to update the cultural and epidemiological aspects of plant pests and diseases on a continuing basis. This paper describes the current phytopathological problems in olive, including in the light of climate change. It shows how these problems can affect the re-emergence of diseases that are already known and focuses attention on newly emerging threats, besides informing on the latest eco-sustainable integrated protection strategies.

Received: February 2015 / **Accepted:** July 2015 / **Published:** November 2015

Introduction

The very differing scenarios found in olive growing across Italy may be the key to its loss of competitiveness. In Italy, unlike in Spain, olives are still grown on marginal land although in some parts of the South this may be the only option open. Some weak points such as the small average size of olive farms – 70% are just under 2 ha – are offset by strong points such as the extensive range of varieties even although only 10 or so are found nationwide, accounting for 475,000 ha or approximately 42% of total olive area (Madau, 2009). The most notable of these varieties are 'Coratina', 'Ogliarola Salentina' and 'Cellina di Nardò', which are grown widely in Apulia, and others such as 'Carolea', 'Frantoio' and 'Leccino', which are cultivated in a large part of Italy. However, although grown on a very limited scale (between 1,000 and 10,000 m^2), at least another 100 varieties are a valuable resource because of the characteristics of their fruit for table consumption or the quality of their oil. They are particularly valuable in the search for new sources of resistance to the main biotic threats to olive production as well as in the drive to orient olive cultivation towards more suitable areas more readily adaptable to the different soil and climatic conditions found in Italy.

This paper follows on from an earlier paper published a decade ago (Tosi and Zazzerini, 2005). It intends to provide an update on the phytosanitary status of the olive with the focus on two known plant diseases – verticillium wilt, a vascular disease that is still difficult to combat, and anthracnose, considered to be a minor disease but attacks of which have recently become more intense in some olive growing environments – and two emerging pests/diseases. The review then aims to show that the development and adoption of control measures must be accompanied by up-to-date knowledge of agricultural crop practices which, when they change, affect the olive agrosystem and have repercussions on the incidence and severity of pests and diseases like those mentioned above. Modern pest and disease control such as that required under Directive 128/2009/EC, which has been worked into Italian legislation in Decree Law 150/2012, takes a broad and systematic approach to the aspects of the impact of agricultural chemicals on the environment and food healthiness. The National Action Plan (known by the Italian acronym PAN) introduced on 1 January 2014 maps out the general principles of integrated plant protection, which is now compulsory, and makes provision for two levels of application (integrated protection and organic agriculture). In short, in the new regulation, plant protection is prevention-based. It entails synergically harnessing the potential and distinctive features of the various forms of control in order to ensure comprehensive crop protection instead of limiting protection to containing a single plant disease and to take immediate action when new pests emerge (for instance, olive quick decline syndrome). Hence, rational crop protection calls for continuing, constant updating of knowledge, technologies and techniques and ever increasing integration of different fields of expertise.

Epidemiological aspects of verticillium wilt and effect of phytosanitary practices

This cryptogamic disease has been widespread for many years in olive orchards in central and southern Italy and several other Mediterranean countries (Spain, Greece, Tunisia, Morocco, Syria, etc.) where it is causing problems in old groves as well as young, often irrigated orchards. Research conducted in olive orchards in central (Umbria, Tuscany) and southern Italy (Calabria, Sicily, Apulia) from the 1990s onwards (Tosi and Zazzerini, 1998; 2000; Ciccarese 2003; Vizzarri, 2005) has shown that climatic, agricultural crop practices and geno-phenotypical factors linked to increasing disease incidence and intensity and the not infrequent phenomenon of natural recovery of infected plants may have a differing effect on the development of verticillium wilt (Fig. 1A and B) and hence frustrate phytosanitary measures.

Figure 1A: Verticillium wilt of olive. Characteristic symptoms of chronic or slow decline

Figure 1B: Verticillium wilt of olive. Characteristic symptoms of acute decline or apoplexy (B)

Phytosanitary plant certification is very important not only when establishing new orchards but also when restructuring old orchards and increasing orchard density, as has been occurring in the last two decades in Greece and Spain – and Italy too – in rainfed and irrigated orchards (Navarro and Parra, 2008). Relevant research in Apulia (Nigro *et al.*, 2005) revealed the presence of *Verticillium dahliae* in 50% of the nurseries analysed. Known cases of verticillium wilt which can be traced back to infected propagation material have also been observed in Umbria in both young and restructured orchards (Tosi, personal communication).

In the two situations described above (new orchards and orchards where trees killed by the disease have been replaced), the use of healthy propagating material should go hand in hand with the choice of resistant cultivars or the use of varieties that have been grafted on to verticillium-resistant rootstock. However, this control measure is heavily dependent on certain biological characteristics of the pathogen (soil inoculum density, isolate virulence) and environmental conditions, as well as on resistance screening results which do not always coincide because of differences in the *V. dahliae* isolates and inoculation methods used and in the experimental conditions of resistance appraisal. This last aspect is the reason why standardised, internationally recognised experimental protocols must be adopted.

V. dahliae isolates are traditionally classified as defoliating (D) or non-defoliating (ND) on the basis of their virulence in olive and their ability to cause total green leaf loss (Rodríguez-Jurado *et al.*, 1993). In particular, D pathotypes isolated from olive and cotton can be virulent in both hosts and cause symptoms such as plant withering, chlorosis, defoliation and die-back while ND pathotypes produce similar symptoms but of mild or moderate severity.

In general, differences in virulence are also observed among different olive cultivars, and infections caused by the D-pathotype start earlier and develop more quickly and intensely (Rodríguez-Jurado *et al.*, 1993; Lopez-Escudero *et al.*, 2004; Martos-Moreno *et al.*, 2006; Dervis *et al.*, 2010).

Since the reproduction of the pathogen is exclusively agamic, it is also known that the only possibilities ensuring safe exchange of genetic material are hyphal anastomosis (fusion of hyphae between vegetatively compatible isolates) and subsequent heterocaryosis (fusion of nuclei). Although much research has highlighted an association between vegetative compatibility groups (VCG) and genetic (molecular) diversity, the correlation between host specificity, pathogen virulence and geographical distribution appears to be more complex and difficult to interpret. In point of fact, although the defoliating pathotype has been classified in group VCG 1A in all the countries where it has been reported (Pérez-Artés *et al.*, 2000; Collado-Romero *et al.*, 2006; Dervis *et al.*, 2010) and the ND pathotype has been assigned to the other groups (VCG 2, VCG 4), some research has revealed that olive isolate pathogenicity may not be correlated with VCG groups (Tanatoui *et al.*, 2002) and that, when artificially inoculated, some D isolates in the VCG 1A/D group/pathotype may exhibit a virulence (severity of symptoms) similar to that of the ND pathotypes (Dervis *et al.*, 2010).

Characterisation and genetic differentiation of *V. dahliae* isolates from olive using molecular techniques (RAPD, AFLP and PCR with specific markers) appear to show molecular similarity within a given VCG group. These groups of isolates are related to host specificity and geographical origin (Collado-Romero *et al.*, 2006; 2008) although VCG 1A/D pathotypes isolated in olive in Turkey which were molecularly different from Spanish isolates did not exhibit significant differences in virulence in pathogenicity trials (Dervis *et al.*, 2010). Research in Mediterranean olive growing areas has shown that the D pathotypes have spread rapidly in Spain (Mercado-Blanco *et al.*, 2003; Navas-Cortés *et al.*, 2008). Greater virulence of isolates belonging to the

VCG 1A/D group/pathotype has been reported in Turkey (Dervis *et al*., 2010) whereas in Italy the ND pathotypes seem to be prevalent (Nigro *et al*., 2005; Vizzarri, 2005). It is not surprising, therefore, that resistance trials conducted in artificial conditions and field observations give discrepant results for varietal performance, which may be widely influenced by environmental conditions, the amount of pathogen inoculum in the soil, pathogen virulence and agricultural crop practices. More than 40 years' research conducted in several countries in natural environments and in experimental conditions has revealed that the best varieties from the agricultural standpoint are most susceptible to the D pathotype, for instance 'Picual', 'Hojiblanca', 'Arbequina', 'Manzanilla de Sevilla' (Spanish cvs), 'Kalamon', 'Amfissis', 'Konservolia' (Greek cvs), 'Ascolana', 'Leccino' (Italian cvs), while higher levels of resistance are reported for the 'Oblonga', 'Changlot Real', 'Empeltre' and 'Frantoio' cultivars (López-Escudero and Mercado-Blanco, 2011).

Another alternative is to use resistant rootstock. When grafted on to 'Oblonga', the 'Sevillano' (Hartman *et al*., 1971) and 'Conservolia' cultivars (Tjamos *et al*., 1985) showed greater resistance to *V. dahliae*; however, grafting of other cultivars of *Olea europea* or grafting on to species other than the *Olea* genus has given varying results. The response of a specific genotype in experimental conditions may in fact change in the field as a result of the environmental, biological and cultural factors already mentioned (Blanco-Lopez *et al*., 1998). Resistance in natural conditions may be modified by the phenomenon of natural recovery in infected plants in which the temporary remission of symptoms (even after more than 2–3 years) is observed along with the growth of new vegetation under the withered branches (Tosi and Zazzerini, 1998; 2000; Trapero and Blanco, 2001; Ciccarese, 2003). Hence, new infections are necessary for the disease to reoccur. Natural recovery is thought to be attributable to the activation of defence responses in the host plant, which lead to the production of phenolic compounds, and to the blockage of the infected xylem vessels and ensuing de-activation of *V. dahliae*. This phenomenon is dependent on pathogen virulence and varietal resistance and has been observed more frequently in olive orchards with ND pathotypes and resistant cultivars (Tosi and Zazzerini, 1998; 2000; Vizzarri, 2005; Lopez-Escudero *et al*., 2004; Martos-Moreno *et al*., 2001). It may be encouraged by the application of appropriate cultural practices such as balanced nitrogen fertilisation, weed removal to avoid soil tillage that might cause wounds and stimulate new infections, and soil solarisation around the trees in order to reduce the amount of *V. dahliae* inoculum (Tosi and Zazzerini, 1998; 2000; Ciccarese, 2003; Trapero and Blanco, 2001). However, a recent study conducted in Apulia (southern Italy) in orchards with young olive trees (5–12 years old) of the highly susceptible 'Bella di Cerignola' cultivar and a moderate density of *V. dahliae* inoculum in the soil (ND pathotype) revealed that natural recovery was quite low and limited to only one year (Bubici and Cirulli, 2014) when it was induced artificially by applying solarisation, calcium cyanamide and shoot removal, either singly or combined.

In short, owing to the polyetic nature of *V. dahliae*, the wider dissemination of the ND pathotypes and the impossibility of eradication, control strategies in the olive growing areas of Italy should be based on the application of agricultural crop practices that can ensure beneficial, if not synergic, effects in containing this disease by interacting with some biological characteristics of the pathogen as well as with the environmental conditions.

Epidemiological aspects of anthracnose and influence of phytosanitary practices

Anthracnose is found in many olive growing areas around the world. It has been widespread for some time in certain Mediterranean countries, albeit to differing degrees of intensity as a result of the mild, rainy autumns. In Italy, this disease is more frequent and more serious in some regions of the south (Apulia, Calabria) although it has also been observed in Sardinia and Sicily and occasionally in Umbria and Tuscany. After it was first reported in Apulia (Ciccarone, 1950), infections tended to be epidemic in regions of southern Italy for at least 20 years but then receded (Apulia, Sardinia, Sicily). This phenomenon was initially ascribed to a loss of virulence of the causal agent, but was later attributed to drier seasonal trends caused by the climatic changes of the last two decades. The exceptions are the Gioia Tauro Plain (Calabria) and Salento (Apulia) where the environmental conditions are still conducive to the outbreak of serious epidemics (Cacciola *et al*., 2012).

Different species of *Colletotrichum* have been reported to be the culprits of anthracnose in the countries where this disease is found; recent morphological and molecular studies (Faedda *et al*., 2011) have shown that *C. clavatum* (formerly known as genetic group A4 of *C. acutatum*) has caused serious epidemics in Greece, Montenegro, some limited areas of Portugal and Spain,

and Italy. More particularly, vegetative compatibility trials conducted in southern regions of Italy (Agosteo *et al*., 2002) to correlate geographical origin and molecular profiles (by RAPD) identified two VCG groups in the *Colletotrichum* population: one comprised isolates of Apulian origin while the other comprised isolates from Calabria, Sardinia and Umbria and the reference Greek isolate (CBS 193.32) registered by Petri in 1930. The presence of two different VCG groups in Apulia and Calabria led to the hypothesis that *C. clavatum* had been introduced to Italy from the Balkans at different times, probably during the 1930s/40s (Agosteo, 2010) and then via the spread of infected material from Calabria and other regions (Sardinia, Umbria). However, it cannot be excluded that the pathogen was introduced once only and that the different VCG groups in various countries may have appeared as a result of a process of diversification induced by geographical isolation. Hence, *C. simmondsii* (initially identified as group A2 of *C. acutatum sensu lato*), the species responsible for the anthracnose epidemic in Portugal (Talhinhas *et al*., 2011), has not been isolated from olive in Italy despite the fact that it is known to be the causal agent of anthracnose in strawberry in several regions including Apulia and Calabria. Similarly, *C. acutatum sensu stricto* (initially group A5) has not been isolated from olive in Italy yet it is the prevalent species in olive growing areas in the southern hemisphere (Faedda *et al*., 2011). In short, several species of *Colletotrichum* are responsible for anthracnose in olive and research has shown that, with few exceptions, a given species is prevalent in a specific olive growing area where its potential adaptation depends on the environmental conditions and the genetic characteristics of the host.

It emerges from this brief outline that pathogen virulence, cultivar susceptibility and environmental conditions are the predisposing factors to *Colletrotrichum* spp infection. The latest epidemiological findings reveal that in natural conditions, the flowers and young fruit are infected in spring when the temperature and humidity conditions are more favourable; during fruit growth in the summer months, which are characterised by warm, dry periods, the infections became latent and thus turn into the chief inoculum source for the autumn epidemics. The pathogen can penetrate directly by producing extracellular cutinase to perforate the fruit epicarp, but the severity of infection and colonisation rate are significantly higher when there are microlesions caused by the oviposition punctures or exit holes of the olive fruit fly (*Bactrocera oleae* Gmelin). Larval trophic activity encourages pathogen colonisation and causes earlier fruit ripening; the insect can also contribute to the spread of the conidia.

Olive susceptibility gradually increases with fruit ripening. In favourable conditions, the olives may be affected by rot (Fig. 2) and mummification, which cause heavy losses in terms of crop quality and quantity, especially because of the poor quality of the resultant oil. In addition, the pathogen sporulates on the surface of the infected olives, thus giving rise to secondary infection cycles if the favourable environmental conditions persist; the mummified olives that remain attached to the shoots enable the pathogen to survive in winter and are the source of inoculum for the next season.

Figure 2: Olive anthracnose. Typical symptoms of soft nose on olive fruits attacked by *Colletotrichum gloesporioides* with clear fungal acervuli.

The data on cultivar susceptibility/resistance are not altogether consistent owing to the fact that cultivar identification is not always clear and that there are discrepancies between laboratory and field results. Data are more consistent when artificial infections are performed in controlled conditions on green, unripe olives than when they are carried out on ripe olives because fruit susceptibility increases with ripening. 'Ottobratica', 'Corniola', 'Strana', 'Carolea', 'Picudo', 'Morona' and 'Manzanilla de Sevilla' are some of the most susceptible cultivars whereas 'Frantoio', 'Leccino', 'Santomauro', 'Picual', 'Blanqueta', 'Koroneiki' and 'Razzola' are resistant (Cacciola *et al*., 2012). Using resistant or less susceptible cultivars is therefore a valid approach to disease containment when establishing new orchards. Conversely, in existing olive orchards where susceptible cultivars are grown or the environmental conditions are conducive to the development of anthracnose, it is advisable to prune the trees to remove the infected shoots and branches and mummified fruits in order to reduce the amount of inoculum and increase canopy aeration and so lower the humidity inside the foliage and bring forward olive harvest. These control measures should nevertheless be combined with chemical treatments which, while not definitive, are necessary

to prevent serious epidemics. It is advisable to apply copper products, preferably low-dose cupric ion formulations which are more adherent and longer lasting. When these are applied during pre-flowering and post-fruit set to control olive leaf spot, their collateral action can be harnessed to control anthracnose. Cupric treatments should be repeated during fruit growth and prior to colour change; when it is a rainy year and the trees are centuries old, it is wise to check that the plant protection products have been applied properly and uniformly inside the tree canopy. Experimental trials have recently been conducted in olive orchards in Apulia to test the effectiveness of various plant protection strategies entailing the use of copper-based products, pyraclostrobin and a mix of trifloxystrobin + tebuconazole. The trials have shown that pyraclostrobin combined with copper and the trifloxystrobin + tebuconazole mix are very active in controlling the pathogen and limiting fruit infection. It is essential to apply them during pre-flowering in order to contain latent infections and to apply copper-based products during post-colour change in order to reduce secondary infections (Nigro *et al.*, 2011; Cacciola *et al.*, 2012). In Italy, since 2011 the Ministry of Health has temporarily authorised a single application of pyraclostrobin between July and August in olive orchards in Calabria under heavy pressure from the disease but this treatment has proved to have little effect (Cacciola *et al.*, 2012).

Newly emerging phytosanitary threats: Olive Quick Decline Syndrome

The first report of **O**live **Q**uick **D**ecline **S**yndrome (**OQDS**) in some olive orchards in Salento (Apulia, southern Italy) dates back to 2010 (Saponari *et al.*, 2013). Since then, this plant disease has spread rapidly, affecting approximately 10,000 ha in 2013. The symptoms are sudden withering of the shoots and branches, which spreads into the canopy and leads to the death of the tree; the tips and edges of the leaves are yellowed and then become scorched to the point of desiccation. Cross-sections of tree trunks, branches and shoots have revealed fairly extensive browning of the vascular elements. Ancient trees suffer the greatest damage. If they do not die, they look withered and abundant suckers grow from the collar due to drastic pruning by farmers in the hope of encouraging new growth (Fig. 3).

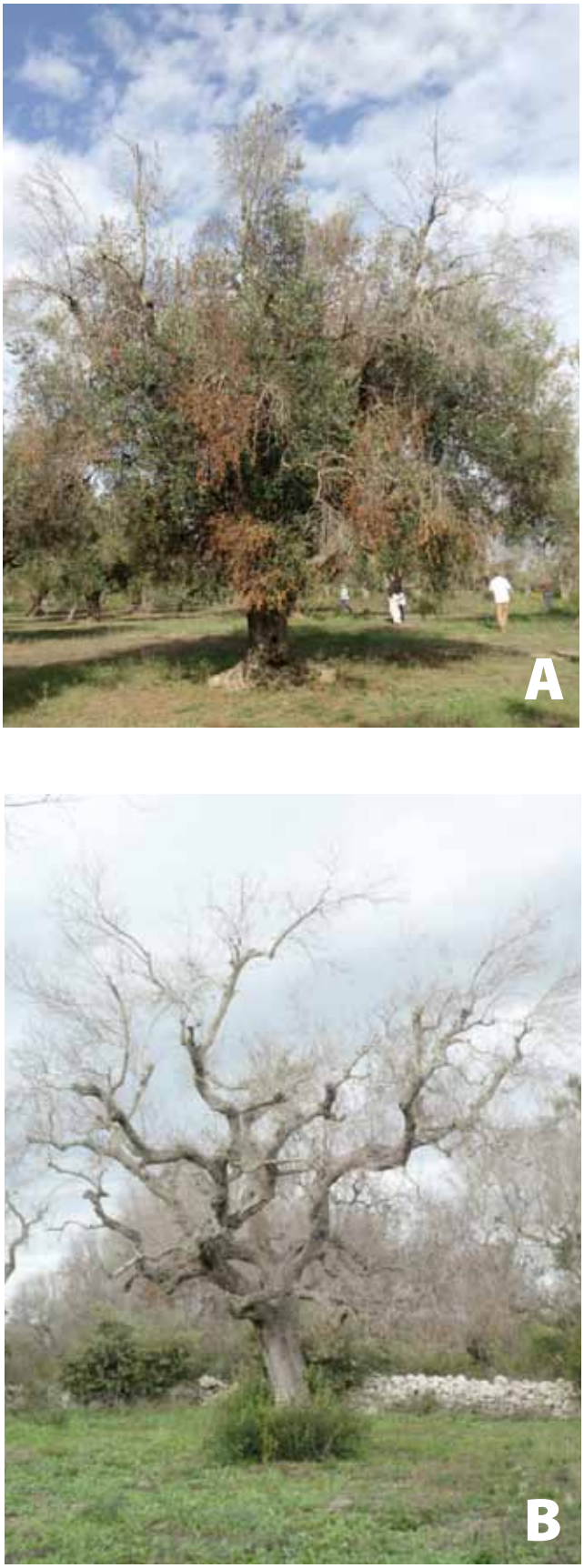

Figure 3: Olive quick decline syndrome (OQDS). Typical "leopard skin" withering of the canopy (A) and total tree decline due to blockage of the sap vessels (B).

Although not yet conclusive or exhaustive, lengthy complex etiological studies have enabled: (*i*) identification, by means of serological and molecular assays, of the Gram-negative bacterium *Xylella fastidiosa* (Well and Raju) subsp. *pauca* (Cariddi *et al*., 2014; Loconsole *et al*., 2014) in symptomatic olive trees as well as in oleander and almond suffering from leaf scorch and other tree and herbaceous species; *(ii)* pure culture isolation of the bacterium (Elbeaino *et al*., 2014a); (*iii)* sequencing of the bacterium genome (Giampetruzzi *et al*., 2015); (*iv*) proof that the only species that is able to transmit the bacterium is *Philaenus spumarius* L., known as the meadow spittlebug because it expels a whitish abdominal glandular secretion which envelopes the juvenile forms (nymphs) in a spittle-like substance, thus protecting them from desiccation and natural enemies, (Saponari *et al*., 2014). In Salento, this polyphagous, ecologically adaptable insect does not cause significant direct damage to agricultural crops; however, its ability to transmit bacteria such as *X. fastidiosa* makes it much more important from the epidemiological viewpoint. In point of fact, since *Xylella* is a xylem-restricted, asporogenous bacterium, it can only be transmitted by insects equipped with a piercing-sucking mouth apparatus (called xylem fluid feeding insects). Once they have the bacterium, the vector insects can inoculate it into spontaneous and cultivated species when they feed, but it will only be able to multiply in susceptible hosts, leading to latent infections or symptomatic plants. Field surveys have revealed other potential vector insects (Elbeaino *et al*., 2014b) but their infectivity has not yet been demonstrated.

X. fastidiosa is a quarantine pathogen included in List A1, Annex I, Part A, Section I of Directive 2000/29/EC. It is endemic in the American continent where it causes citrus variegated chlorosis (Costa Rica, Brazil) and Pierce's disease of grape (California). Its appearance in a previously *Xylella*-free area was attributed to the importation of infected plant material, a hypothesis that was later confirmed in October 2014 when the Dutch Phytosanitary Services intercepted a shipment of *Xylella*-infected ornamental coffee plants imported from Costa Rica; consequently, it is very plausible that the OQDS strain is of Costa Rican origin, as also appears to be confirmed by molecular assays (Giampetruzzi *et al*., 2015).

Current rules and regulations can be summed up as follows. A ministerial decree on emergency measures to prevent, control and contain *Xylella fastidiosa* (Well and Raju) in the territory of the Italian Republic (Official Gazette No 148 of 29 June 2015) was passed on 19 June 2015 to implement European Commission Decision No 2015/789 of 18 May 2015, aimed at reinforcing the protection measures to prevent the introduction and spread of the bacterium in the European Union. The decree entered into force on 30 June 2015 and rescinded the earlier decrees of 26 September 2014 and 17 March 2015 which implemented the far-sighted EU Decision No 2014/497. The most important aspects of the decree include intensified nationwide monitoring, the determination of demarcated areas comprising the infected zone (colonisation area) and buffer zone (area intended to restrict the spread of the epidemic), a ban on the introduction into the European Union of plants from Costa Rica and Honduras with the exception of coffee seeds and the determination of measures for the eradication and containment of the bacterium. In the light of experience so far, the most rational measures are based on the application of agricultural practices (weed removal in spring and soil tillage to eliminate the juvenile stages of the vector and lower the number of adult insects capable of spreading the bacterium), the use of registered insecticides authorised for olive cultivation to treat any vector insect adults that might have slipped through prior applications, and the uprooting and destruction of infected plants.

Decline caused by olive bark beetle [*Phloeotribus scarabaeoides* (Bernard)]

The olive bark beetle (Coleoptera, Scolytidae) is a small (2–2.4 mm long) but robust, dark coloured insect covered with blackish bristles. It is spread through all the olive growing regions as well as Morocco (Lozano *et al*., 1999) and attacks olive and other *Oleaceae*. It is xylophagous at both the larval and adult stages. There are between one and three generations per year depending on the susceptibility of the debilitated trees or other plant material (Russo, 1938 a; b). In springtime, the adults mine feeding galleries (Fig. 4) in the axil of growing inflorescences and shoots. Later, the females, followed by the males, dig egg-laying galleries in dry or pruned branches in which numerous small piles of whitish frass can be observed.

Figure 4: Olive bark beetle. Frass indicates the presence of the beetle in the area under the bark (A) and feeding galleries (B).

The adults cause the most damage, digging numerous galleries that lead to the death of the one-year-old fruiting shoots, with the ensuing crop losses; they can eliminate up to 73% of potential yields (González and Campos, 1994). Attacks can cause the death of the infected plants if they suffer from heavy decline or have been recently planted out in the field (Benazoun, 1992). This beetle also causes indirect damage by providing shelter for olive thrips (*Liothrips oleae*) to overwinter and lay its eggs in the beetle feeding galleries.

The bark beetle preferably attacks debilitated xylem with a low sap flow. Trunks, branches and fruiting shoots that are weak or that have suffered the effects of bad weather (frost, prolonged drought, etc.) are the most appetising organs for this insect. The infected parts are further debilitated by the trophic activity of the numerous larvae that develop under the bark. The bark beetle can also cause damage to olive trees located close to piles of olive wood or pruning debris, which are a focus of infection. In such cases, the larvae develop on the dead or debilitated wood (pruning debris, dry branches, etc.) and the new adults move on to healthy trees during the feeding stage where they mine feeding galleries, including on branches that are healthy from the vegetative point of view, so causing the death of small shoots, inflorescences and even young fruiting shoots, which considerably lowers the season's crop.

According to several reports, the dry hot summers of recent years have encouraged the development of the bark beetle in olive orchards in the Sibari Plain (Calabria), leading to the death of young olive plants and the failure of new orchards established with the 'Carolea' cultivar. The beetle overwinters as a larva and/or adult. Since winters have lately been particularly mild without prolonged frosts, this species had not been affected by the cold, and has thus bred to an excessive extent. Moreover, there are no known natural antagonists capable of keeping beetle populations within the limits of the economic treatment threshold.

Hence, it is vital for plant protection from olive bark beetle attacks to include preventive measures to keep the trees in good vegetative health by applying proper cultural care: soil tillage, balanced fertilisation, irrigation (where possible), necessary pesticide treatments against other pests and diseases and pruning of any

debilitated or declining parts of the tree (Furlong *et al*., 2004). Winter pruning debris is a potential site for insect development and should be removed as quickly as possible; likewise, piles of olive wood (for firewood) should not be left near crops. However, one effective control measure is to pile pruning debris in the shadiest parts of infested olive orchards in March in order to act as bait and attract the proliferating adults. This debris should then be removed and burned in April when the first frass starts to appear and before the first-generation adults start to disperse and move to the trees to breed. The fact that this small beetle develops deep in the olive wood makes it very difficult to reach it with external chemical treatments. Deltamethrin has given good results in this respect. As with other pyrethroids (Loch, 2005), one single application of this insecticide decreases the presence of the olive bark beetle; however, deltamethrin is a broad-spectrum active ingredient and therefore causes high mortality among non-target arthropods (Ruano *et al*., 2008). The application of deltamethrin to trunks already colonised by *P. scarabaeoides* is a good strategy for containing this insect for several reasons: (*i*) it prevents beetle dispersal due to the repellent effect of the insecticide; (*ii*) the application of the active ingredient to the trunk, which is not used for human consumption, does not have toxic effects for humans; (*iii*) the beetle population is depleted before the olive trees are attacked.

Concluding remarks and prospects

The phytopathological problems concisely outlined in this review show that proper protection should be based on accurate etiological insight, which is becoming more and more of a determinant in guaranteeing the success of phytosanitary measures. Integrated protection alone can offer the most effective strategy for counteracting the established and new threats to the olive pathosystem and can therefore stand as an example for other woody crops to follow. The poor effectiveness of fungicides and the increasing attention paid to toxicological and environmental aspects in EU and domestic rules and regulations call for an integrated approach that harnesses the potential and distinctive characteristics of the different control measures by taking preventive action in both established and new olive orchards. Prevention plays a significant role in the nursery industry where certification guarantees not only varietal compliance but also plant phytosanitary status. Diagnostic methods, particularly molecular tools such as real-time quantitative PCR (qRT-PCR), are essential in the case of verticillium wilt in order to check for the absence of the pathogen in asymptomatic parts of the plants as well as in the soil (Ceccherini *et al*., 2014). Confirmation of the existence of latent infections and/or the presence of *V.dahliae* propagules in the soil would make it possible to monitor the situation in olive orchards and to assess the effectiveness of the phytosanitary measures applied while the use of the same method in olive nurseries would ensure the production of healthy plants and would prevent the spread of the pathogen to new growing areas, including via contaminated soil.

The events associated with Olive Quick Decline Syndrome make it necessary to intensify EU and domestic surveillance of the introduction and circulation of plant material in order to guarantee its phytosanitary health. Continuing and constant orchard monitoring and the adoption of diagnostic approaches based on specific protocols are prerequisites for guaranteeing maximum success in containing old and new threats.

References

AGOSTEO G.E. (2010) – Avversità dell'olivo ed evoluzione dei relativi sistemi di difesa in Calabria fra XVIII e XX secolo. Petria 20 (Suppl.1), 41-70.

AGOSTEO G.E., MAGNANO DI SAN LIO G., CACCIOLA S.O., FRISULLO S. (2002) – Characterisation of the causal agent of olive anthracnose in Southern Italy. Acta Horticulturae 586, 713-716.

BENAZOUN A. (1992) - Contribución al estudio bioecológico del barrenillo *Phloeotribus scarabaeoides* Bern. (Col., Escolítidos) en el olivo en la región de Taroudant, Marruecos. Olivae 40, 26–35.

BLANCO- LÓPEZ M.A., HIEMSTRA J., HARRIS D., LÓPEZ-ESCUDERO F.J., ANTONIOU P. (1998) – Selection and screening for host resistance. In: Himenstra J., Harris D. (eds.) Compendium of *Verticillium* wilt in tree species. Ponsen & Loonijen, Wageningen, 51-54.

BUBICI G., CIRULLI M. (2014) – Natural recovery from Verticillium wilt in olive: can it be exploited in a control strategy? Plant and Soil 381, 85-94.

CACCIOLA S.O., FAEDDA R., SINATRA F., AGOSTEO G.E., SCHENA L., FRISULLO S., MAGNANO DI SAN LIO (2012) – Olive anthracnose. Journal of Plant Pathology 94, (1), 29-44.

CARIDDI C., SAPONARI M., BOSCIA D., DE STRADIS A., LOCONSOLE G., NIGRO F., PORCELLI

F., POTERE O., MARTELLI G.P. (2014) – Isolation of a *Xylella fastidiosa* strain infecting olive and oleander in Apulia, Italy. Journal of Plant Pathology 96, (2), 425-429.
CECCHERINI M.T., LUCHI N., PANTANI O.-L., ASCHER J., CAPRETTI P., PIETRAMELLARA G. (2014) – Upward movement of *Verticillium dahliae* from soil to olive plants detected by qPCR. World J. Microbiol. Biotechnol. 29, 1961-1967.
CICCARESE F. (2003) – Grave minaccia all'olivo: la verticilliosi. Informatore fitopatologico 53, (12), 17-21.
CICCARONE A. (1950) – Considerazioni biologiche e sistematiche sull'agente della "lebbra" delle olive, recentemente osservate nel Leccese. Bollettino della Regia Stazione di Patologia Vegetale di Roma S.III, 5, 143-165.
COLLADO-ROMERO M., MERCADO-BLANCO J., OLIVARES-GARCÍA C., JIMÉNEZ-DÍAZ R.M. (2008) – Phylogenetic analysis of *Verticillim dahliae* vegetative compatibility groups. Phytopathology 98, 1019-1028.
COLLADO-ROMERO M., MERCADO-BLANCO J., OLIVARES-GARCÍA C., VALVERDE-CORREDOR A., JIMÉNEZ-DÍAZ R.M. (2006) – Molecular variability within and among *Verticillium dahliae* vegetative compatibility groups determined by fluorescent amplified fragment length polymorphism and polymerase chain reaction markers. Phytopathology 96, 485-495.
DERVIS S., MERCADO-BLANCO J., ERTEN L., VALVERDE-CORREDOR A., PÉREZ-ARTÉS E. (2010) – *Verticillium* wilt of olive in Turkey: a survey on disease importance, pathogen diversity and susceptibility of relevant olive cultivars. Eur. J. Plant Pathol. 127, 287-301.
ELBEAINO T., VALENTINI F., KUBBA R.A., MOUBARAK P., YASEEN T., DI GIARO M. (2014a) – Multilocus sequences typing of *Xylella fastidios*a isolated from olive affected by "olive quick decline syndrome" in Italy. Phytopathologia Mediterranea 53, 3, 533-542.
ELBEAINO T., YASSEN T., VALENTINI F., BEN MOUSSA I.E., MAZZONI V., D'ONGHIA A.M. (2014b) – Identification of three potential vectors of *Xylella fastidiosa* in southern Italy. Phytopathologia Mediterranea 53, 126-130
FAEDDA R., AGOSTEO G.E., SCHENA L., MOSCA S., FRISULLO S. MAGNANO DI SAN LIO G., CACCIOLA S.O. (2011) – *Colletotrichum clavatum* sp. nov. identified as the causal agent of olive anthracnose in Italy. Phytopathologia Mediterranea 50, 283-302.
FURLONG M.J., ZU-HUA S., YIN-QUAN L., SIU-JIAN G., YAO-BIN L., SHU-SHENG L., ZALUCKI M.P. (2004) - Experimental analysis of the influence of pest management practice on the efficacy of an endemic arthropod natural enemy complex of the diamondback moth. J. Econ. Entomol. 97, 1814–1827.
GIAMPETRUZZI A., CHIUMENTI M., SAPONARI M., DONVITO G., ITALIANO A., LOCONSOLE G., BOSCIA D., CARIDDI C., MARTELLI G.P., SALDARELLI P. (2015) – Draft Genome Sequence of the *Xylella fastidiosa* CoDiRO Strain. Genome Announcements 3, 1, 1-2.
GONZÁLEZ R., CAMPOS M. (1994) - A preliminary study of the effect of attack by *Phloeotribus scarabaeoides* (Bern.) (Col., Scolytidae) on the productivity of the olive tree (*Olea europaea*). Bull. Soc. Entomol. Suisse 67, 67–75.
HARTMAN H., SCHNATHORST W.C., WHISLER J. (1971) – Oblonga, clonal olive rootstock resistant to *Verticillium* wilt. Calif. Agric. 25, 12-15.
LOCH A.D. (2005) - Mortality and recovery of eucalypt beetle pest and beneficial arthropod populations after commercial application of the insecticide alpha-cypermethrin. Forest Ecol. Manage. 217, 255–265.
LOCONSOLE G., POTERE O., BOSCIA D., ALTAMURA G., DIELOUAH K., ELBEAINO T., FRASHERI D., LORUSSO D., PALMISANO F., SILLETTI M.R., TRISCIUZZI N., VALENTINI F., SAVINO V., SAPONARI M. (2014) – Detection of *Xylella fastidiosa* in olive trees by molecular and serological methods. Journal of Plant Pathology 96, (1), 7-14.
LÓPEZ-ESCUDERO F.J., DEL RIO C., CABALLERI J.M., BLANCO-LÓPEZ M.A., (2004) – Evaluation of olive cultivars for resistance to *Verticillium dahliae*. Eur. J. Plant Pathol. 100, 79-85.
LÓPEZ-ESCUDERO F.J., MERCADO-BLANCO J. (2011) – *Verticillium* wilt of olive: a case study to implement an integrated strategy to control a soil-borne pathogen. Plant Soil 344, 1-50.
LOZANO C., BENAZOUN A., KIDD N., CAMPOS M. (1999) - Population dynamics of the bark beetle *Phloeotribus scarabaeoides* Bernard (Coleoptera: Scolytidae), a pest of African olive orchards in Morocco. African Entomology 7, (2), 271-275.
MADAU F.A. (2009) – Evoluzione del mercato internazionale e nazionale del comparto olivicolo. Convegno di studi "Un piano strategico per lo sviluppo dell'olivicoltura del Medio Campidano" Gonnosfanadiga (VS) 7 novembre 2009, 1-21.
MARTOS-MORENO C., CABALLERO J.M., DEL RÍO C, BLANCO-LÓPEZ M.A. (2001) – Epidemiological behaviour of olive cultivars in orchard infested with mixtures of defoliating and non-defoliating isolates of *Verticillium dahliae*. 8th Int. *Verticillium* Symposium, Book of abstracts, Córdoba, Spain, 67.
MARTOS-MORENO C., LÓPEZ-ESCUDERO F.J., BLANCO-LÓPEZ M.A. (2006) – Resistance of olive cultivars to the defoliating pathotype of *Verticillium dahliae*. HortScience 41, 1313-1316.

MERCADO-BLANCO J., RODRÍGUEZ-JURADO D., PARRILLA-ARAUJO S., JIMÉNEZ-DÍAZ R.M. (2003) – Simultaneous detection of the defoliating and non-defoliating *Verticillium dahliae* pathotypes in infected olive plants by duplex, nested polymerase chain reaction. Plant Dis. 87, 1487-1494.

NAVARRO C., PARRA MA. (2008) – Plantación. In: Barranco D., Fernández-Escobar R., Rallo L., (eds) El cultivo del olivo, 6th ed. Junta de Andalucía and Ediciones Mundi-prensa, Madrid, 189-238.

NAVAS-CORTÉS J.A., LANDA B.B., MERCADO-BLANCO J., TRAPERO-CASA J.L., RODRÍGUEZ-JURADO D., JIMÉNEZ-DÍAZ R.M. (2008) – Spatiotemporal analysis of spreads of infections by *Verticillium dahliae* pathotypes within a high tree-density olive orchard in Southern Spain. Phytopathology 98, 167-180.

NIGRO F., GALLONE P., ROMANAZZI G., SCHENA L., IPPOLITO A., SALERNO M.G. (2005) – Incidence of Verticillim wilt on olive in Apulia and genetic diversity of *Verticillium dahliae* isolates from infected trees. J. Plant Pathol. 87, 13-23.

NIGRO F., GUARIO A., LACCONE G. (2011) – Ultime acquisizioni sull'epidemiologia e sulla protezione della lebbra dell'olivo. 23° Forum Medicina vegetale, Bari 15 Dicembre 2011.

PÉREZ-ARTÉS E., GARCIA-PEDRAJAS M.D., BEJARANO-ALCÁZAR J., JIMÉNEZ-DÍAZ R.M. (2000) – Differentiation of cotton-defoliating and nondefoliating pathotypes of *Verticillium dahliae* by RAPD and specific PCR analyses. Eur. J. Plant Pathol. 106, 507-517.

PETRI L. (1930) – Azione tossica dell'arseniato sodico sopra le spore del *Gloeosporium olivarum* Alm. Bollettino della Regia Stazione di Patologia Vegetale di Roma 10, 359-361.

RODRÍGUEZ-JURADO D., BLANCO-LÓPEZ M.A., RAPOPORT H., JIMÉNEZ-DÍAZ R.M. (1993) – Present status of *Verticillium* wilt of olive in Andalucia (southern Spain) Bull OEPP/EPPO Bull 23, 513-516.

RUANO F., CAMPOS M., SÁNCHEZ - RAYA A.J., PEÑA A. (2008) - Deltamethrin application on colonized olive logs: effect on the emergence of the olive bark beetle *Phloeotribus scarabaeoides* Bernard 1788 (Coleoptera: Scolytidae) and its associated parasitoids. Crop Protection 27, 614 – 621.

RUSSO G., (1938a) - V. Contributo alla conoscenza dei coleoteri scolitidi, fleotribo: *Phloeotribus scarabaeoides* (Bern.). Fauv. Parte prima: Morfologia, anatomia e istologia. Boll. R. Lab. Ent. Agr. Portici 1, 1–260.

RUSSO G., (1938b) - VI. Contributo alla conoscenza dei coleoteri scolitidi, fleotribo: *Phloeotribus scarabaeoides* (Bern.). Fauv. Parte seconda: Biografia, simbionti, danni e lotta. Boll. R. Lab. Ent. Agr. Portici 2, 1–419.

SAPONARI M., BOSCIA D., NIGRO F., MARTELLI G.P. (2013) – Identification of DNA sequence related to *Xylella fastidiosa* in oleander, almond and olive tree exhibiting leaf scorch symptoms in Apulia (southern Italy). Journal of Plant Pathology 95, 668.

SAPONARI M., LOCONSOLE G., CORNARA D., YOKOMI R. K., DE STRADIS A., BOSCIA D., BOSCO D., MARTELLI G.P., KRUGNER R., PORCELLI F. (2014) – Infectivity and trasmission of *Xylella fastidiosa* by *Philaenus spumarious* L. (Hemiptera: Aphrophridae) in Apulia, Italy. Journal of Economic Entomology 107, (4), 1316-1319.

TALHINHAS P., MOTA-CAPITÃO, MARTINS S., RAMOS A.P., NEVES-MARTINS J., GUERRA_GUIMARÃES L., VÁRZEA V., SILVA M.C., SREENIVASAPRASAD S., OLIVEIRA H. (2011) – Epidemiology, histopathology, and etiology of olive anthracnose caused by *Colletotrichum acutatum* and *C. gloeosporioides* in Portugal. Plant Pathology 60, 483-495.

TANATOUI A., LACHQER K., SEDRA M.H. (2002) – Vegetative compatibility and pathogenicity of *Verticillium dahliae* isolates from olive (*Olea europaea*). Phytopathologia Mediterranea 41, 19-27.

TJAMOS E.C., THANASSOULOPOULOS C.C., BIRIS D.A. (1985) – Resistance evaluation to *Verticillium dahliae* olive rootstocks. Proc. 3rd Nat. Phytopathol. Conf. Hellenic Phytopathol. Soc., 18-19.

TOSI L., ZAZZERINI A. (1998) – Indagini epidemiologiche sulla verticilliosi dell'olivo nell'Italia centrale. Olivae 71, 50-55.

TOSI L., ZAZZERINI A. (2000) – Ulteriori indagini su alcuni aspetti epidemiologici della verticilliosi dell'olivo in Umbria. Atti Giornate Fitopatologiche 2, 81-86.

TOSI L., ZAZZERINI A. (2005) – La difesa integrata dell'olivo dai patogeni vegetali. Informatore fitopatologico LV, 11, 12-18.

TRAPERO A., BLANCO M.A. (2001) – Enfermedades. In: Barranco D., Fernández-Escobar R., Rallo L., (eds) El cultivo del olivo, 4th ed. Junta de Andalucía and Ediciones Mundi-prensa, Madrid, 495-550.

VIZZARRI V. (2005) – Aspetti epidemiologici di *Verticillium dahliae* Kleb. in oliveti umbri, *screening* per la resistenza e caratterizzazione molecolare degli isolati. Selezione di microrganismi fungini, isolati dal filloplano, per possibili applicazioni in lotta biologica. Tesi di Dottorato, XIX Ciclo, Università degli Studi di Perugia, A.A. 2005/2006, 122 pp.

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