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Advanced solutions for authenticity and quality of virgin olive oils

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Abstract

This Ph.D. project aimed to the development and improvement of analytical solutions for control of quality and authenticity of virgin olive oils. According to this main objective, different research activities were carried out: concerning the quality control of olive oil, two of the official parameters defined by regulations (free acidity and fatty acid ethyl esters) were taken into account, and more sustainable and easier analytical solutions were developed and validated in-house. Regarding authenticity, two different issues were faced: verification of the geographical origin of extra virgin (EVOOs) and virgin olive oils (VOOs), and assessment of soft-deodorized oils illegally mixed with EVOOs.

About fatty acid ethyl esters, a revised method based on the application of off-line HPLC-GC-FID (with PTV injector), revising both the preparative phase and the GC injector required in the official method, was developed. Next, the method was in-house validated evaluating several figures of merit (repeatability, linearity, limit of detection and quantification, robustness, recovery, precision and accuracy).

Concerning free acidity, a portable system suitable for in-situ measurements of olive oil free acidity was developed and in-house validated. Its working principle is based on the estimation of the olive oil free acidity by measuring the conductance of an emulsion between a hydro-alcoholic solution and the sample to be tested. The procedure is very quick and easy and, therefore, suitable for people without specific training.

Another study developed during the Ph.D. was about the application of flash gas chromatography for volatile compounds analysis, combined with untargeted chemometric data elaborations, for discrimination of EVOOs and VOOs of different geographical origin. A set of 210 samples coming from different EU member states and extra-EU countries were collected and analyzed. Data were elaborated applying two different classification techniques, one linear (Partial Least Square - Discriminant Analysis, PLS-DA) and one non-linear (Artificial Neural Network, ANN).

Finally, a preliminary study about the application of GC-IMS (Gas Chromatograph - Ion Mobility Spectrometer) for assessment of soft-deodorized olive oils was carried out. This study was realized at Instituto de la Grasa (Seville, Spain) under the supervision of Dr. Wenceslao Moreda during an abroad period (4 months) financially supported by the Marco Polo programme.

All the activities of this Ph.D. project were developed in the context of the project OLEUM "Advanced solutions for assuring authenticity and quality of olive oil at global scale", funded by the European Commission within the Horizon 2020 Programme (2014–2020, GA no. 635690).

Sommario

Questo progetto di dottorato è stato focalizzato sullo sviluppo e miglioramento di soluzioni analitiche per il controllo della qualità e genuinità degli oli di oliva vergini.
A tale scopo, sono state svolte diverse attività di ricerca: per quanto riguarda il controllo della qualità, due dei parametri ufficiali definiti dai regolamenti (acidità libera ed etil esteri degli acidi grassi) sono stati considerati per lo sviluppo e validazione interna di soluzioni analitiche più semplici e sostenibili. Relativamente alla genuinità, invece, sono stati affrontati due diversi aspetti: la verifica dell'origine geografica di oli extra vergini e vergini di oliva e la valutazione di oli soft deodorati miscelati illegalmente con oli extra vergini di oliva.
Per quanto riguarda la determinazione degli etil esteri degli acidi grassi, è stato messo a punto un protocollo analitico basato sull'applicazione di un sistema off-line HPLC-GC-FID (con iniettore PTV), revisionando la fase preparativa e proponendo un iniettore GC alternativo a quello on-column richiesto dal metodo ufficiale. Successivamente, il metodo è stato validato internamente valutando diversi parametri (ripetibilità, linearità, limite di rilevazione e quantificazione, robustezza, recupero, precisione e accuratezza).
In merito alla valutazione dell'acidità libera, è stato sviluppato e validato internamente un sistema portatile per la misura in-situ di tale parametro. L'acidità libera del campione viene stimata misurando la conduttività di un'emulsione creata tra una soluzione idroalcolica e l'olio da analizzare. La procedura è molto rapida e di semplice realizzazione, caratteristiche che rendono questo sistema adatto ad essere utilizzato anche da persone che non abbiano specifiche competenze analitiche.
Un altro studio sviluppato durante il dottorato ha riguardato l'analisi dei composti volatili mediante flash-gascromatografia combinata con elaborazioni chemiometriche dei dati, seguendo un approccio non-targeted, al fine di discriminare oli extra vergini e vergini di oliva caratterizzati da diversa origine geografica.
Un set di 210 campioni provenienti da diversi stati membri dell'Unione Europea (Spagna, Italia, Grecia, Croazia, Slovenia e Portogallo) e paesi esterni all'Unione Europea (Tunisia, Turchia, Marocco e Cile) sono stati raccolti ed analizzati. I dati sono stati elaborati applicando due diverse tecniche statistiche di classificazione, una lineare (Partial Least Square-Discriminant Analysis, PLS-DA) ed una non lineare (Artificial Neural Network, ANN).
Infine, è stato realizzato uno studio preliminare relativo all'applicazione di un GC-IMS (Gas Chromatograph - Ion Mobility Spectrometer) per la valutazione di oli soft deodorati.

Questa sperimentazione è stata svolta presso l'Istituto de la Grasa (Siviglia, Spagna) sotto la supervisione del Dott. Wenceslao Moreda durante un periodo (4 mesi) di ricerca all'estero finanziato mediante il programma Marco Polo per l'erogazione di incentivi alla mobilità per la ricerca.

Tutte le attività di questo progetto di dottorato sono state realizzate nel contesto del progetto OLEUM "Advanced solutions for assuring authenticity and quality of olive oil at global scale", finanziato dalla Commissione Europea nell'ambito del programma Horizon 2020 (2014–2020, GA no. 635690).

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Chapter 1

Aim of the work

Chapter 1. Aim of the work

Over the last decades, the control of quality and authenticity of olive oil has become an issue of great importance to consumers, suppliers, retailers, and regulators in both traditional and emerging olive oil producing countries, mainly due to the increasing worldwide popularity and trade globalization of this product (Bajoub et al., 2018). Interest in this product has also been accentuated thanks to its beneficial effects on human health (reduces risk of coronary heart disease, positive effects on plasma lipids, oxidative damage, inflammation platelet and cellular function, and antimicrobial activity), and to its role as one of the main components of the Mediterranean diet (Boskou, 2011). These factors bestow extraordinary quality and high commercial value to extra virgin olive oil (EVOO), but at the same time the limited production, high price, and large demand of this healthy and palatable product make it susceptible for deliberate adulteration with lower quality oils by fraudsters (Meenu et al., 2019).

Despite European Union Commission, International Olive Council, and Codex Committee on Fats and Oils are all dealing with the regulation and supervision of EVOO, specifying similar although not identical permissible limits for quality and purity parameters, legislation continuously chases after the emerging frauds (Conte et al., 2019).

The frauds affecting olive oil are actually highly diversified. Those reported in the literature more frequently include (Gallina Toschi et al., 2013; Fang et al., 2015; Azadmard-Damirchi et al., 2015; Tsimidou et al., 2016; Cugat et al., 2016; Aparicio-Ruiz et al., 2017; Bajoub et al., 2018): adulteration of EVOO with other vegetable oils (refined seeds oil, high oleic or desterolized sunflower oil, artificially dyed sunflower oil, hazelnut oil) or lower quality olive oils (soft-deodorized olive oil, olive pomace oil); addition of coloring additives; sale of lower quality oils labeled as EVOO or with other false declarations on the label (mention of organic and integrated production in oils obtained from conventional systems, false declaration of origin for olives or oils, false declaration of the olive cultivar).

Since the early 1970s, many investigations have extensively focused on the establishment of reliable analytical methods to detect frauds in the olive oil sector, allowing to define those parameters, officially recognized by the control bodies, that must be considered to evaluate the quality and authenticity of olive oils. Over the years, these methods have undergone several updates, taking account of improvements in analytical instrumentation, considerations about solvent toxicity, or through improvements in the method (Conte et al., 2019). However, there are still several critical aspects: the need for simple, rapid, and environmentally friendly techniques for the control of quality

and authenticity of virgin olive oils, and the lack of proper analytical methods for identification of specific frauds and markers.

In this context, this Ph.D. project had the main aim of the development and improvement of analytical solutions for control of quality and authenticity of virgin olive oils.

To achieve this main objective, the project was developed through different research activities. Concerning the quality control of olive oil, two of the official parameters defined by regulations (free acidity and fatty acid ethyl esters) were taken into account, and more sustainable and easier analytical solutions were developed and validated in-house. Regarding authenticity, two different issues were faced: verification of the geographical origin of EVOOs and virgin olive oils (VOOs) and assessment of soft-deodorized oils illegally mixed with EVOOs.

In particular, the following was carried out:

- Development and in-house validation of a revised method, based on off-line HPLC-GC-FID, for determination of fatty acid ethyl esters;
- Development and in-house validation of a portable system for rapid determination of free acidity;
- The application of untargeted flash gas chromatography, combined with chemometric data elaborations, for discrimination of EVOOs and VOOs of different geographical origin;
- The application of untargeted GC-IMS for evaluation of soft-deodorized oils.

All the activities of this Ph.D. project were developed in the context of the project OLEUM "Advanced solutions for assuring authenticity and quality of olive oil at global scale", funded by the European Commission within the Horizon 2020 Programme (2014–2020, GA no. 635690).

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Chapter 2

Thesis structure

Chapter 2. Thesis structure

This dissertation reports the activities carried out and the results achieved within the Ph.D. project entitled *"Advanced solutions for authenticity and quality of virgin olive oils"*.

The project was developed through different research activities, some of which related to quality control (Chapter 3 and 4) and others to authentication issues (Chapter 5 and 6) of virgin olive oils.

After the description of the aim of the work (**Chapter 1**), the experimental part of the project is presented in Chapters 3-6, and for each, when available, the details of the relative publication in peer-reviewed journals are reported.

In particular:

- **Chapter 3** focuses on the development and in-house validation of a revised method for determination of fatty acid ethyl esters, whose content is the latest parameter adopted by EU regulations among the quality criteria to define if an olive oil can be classified as extra virgin. The method proposed is based on an off-line combination of HPLC-GC-FID that allows to reduce the time and volume of solvents needed for each analytical determination compared to the official method (defined by EU Reg. 61/2011). After optimization, the method was validated in-house by analyzing several parameters (repeatability, linearity, LOD, LOQ, robustness, recovery, precision and accuracy).
- **Chapter 4** presents a portable battery-operated electronic system for rapid determination of free acidity in virgin olive oils: this quality parameter gives information about the quality of the olives used to produce the oil as well as the hydrolytic state of oil just produced and stored. The working principle of the system is based on the creation of an emulsion between oil and a hydroalcoholic solution: the free acidity is estimated on the value of the electrical conductance of the emulsion. The system was developed, calibrated, and validated in-house (according to the main validation parameters as repeatability, LOD, LOQ, precision and accuracy).
- **Chapter 5** reports the results obtained from the application of a flash gas chromatography untargeted approach for determination of volatile compounds, followed by chemometric data elaborations (PLS-DA and ANN), for discrimination of extra virgin and virgin olive oils according to geographical origin. At present, an official analytical procedure to verify the conformity of the label-declared geographical origin does not exist, representing one of the possible frauds for which a specific analytical method is lacking.
- **Chapter 6** deals with the application of a GC-IMS (Gas Chromatograph - Ion Mobility Spectrometer) as a new analytical approach for evaluation of soft deodorized oils. Soft

deodorization is a technological process applied to virgin olive oils with weak organoleptic defects in order to remove or reduce their intensity. The illegal blending of treated oils with extra virgin ones, and possible commercialization of this blend labelled as top-quality grade (EVOO), represents a fraud that is very difficult to detect since the technological conditions of temperature and pressure applied during the treatment are “mild” and avoid the formation of typical markers of refining.

The conclusions and outlooks of this Ph.D. project are presented in **Chapter 7**, while in **Chapter 8** the details of other scientific papers realized during the Ph.D. thesis, even if they are not strictly related to the project, are reported.

Chapter 3

Development and in-house validation
of a revised method for determination
of fatty acid ethyl esters in virgin olive oils

3.0 Details of the publication based on Chapter 3

Title: Development and in-house validation of a revised method for determination of fatty acid ethyl esters in virgin olive oils

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Abstract

The content of fatty acid ethyl esters (FAEEs) is one of the quality parameters to define if an olive oil can be classified as extra virgin since these compounds are considered markers for virgin olive oils obtained from poor quality olives, but can be also indirect markers to detect soft deodorization treatment. The official protocol for determination (EU Reg. 61/2011) can be improved in terms of solvents amount and time of analysis.
In this study, an off-line HPLC-GC-FID method for FAEEs determination is presented, revising both the preparative step and the GC injector required by the official method. After optimization, the method was in-house validated by analyzing several parameters to determine its effectiveness. The main advantages of this revised protocol are: *i)* a significant reduction of time and solvents needed for each analytical determination *ii)* the application of HPLC as an alternative to traditional LC, carried with manually packed glass columns, thus simplifying the separation step.

Key words: Fatty acids ethyl esters; Virgin olive oil; Off-line HPLC-GC-FID; In-house validation.

3.1 Introduction

Considering world production of vegetable oils, olive oil is at the ninth position (after palm, soybean, rapeseed, sunflower, palm kernel, peanut, cottonseed and coconut oil) with about 3 million metric tons produced during the 2018/19 harvest (USDA, 2019). Nevertheless, virgin olive oil (VOO) is a product with high commercial value, mainly linked to the presence of healthy components and its sensory characteristics, rendering control of its quality a highly complex and evolving issue (Clodoveo et al., 2014).

At present, European regulations (EEC Reg. 2568/91 and following amendments) define five parameters to determine and verify the quality of a VOO: free acidity, peroxide index, specific extinction in UV, sensory evaluation and content of fatty acid ethyl esters (FAEEs). Even if this latter parameter was officially introduced by the International Olive Council (IOC) in 2010 (COI/T.20/Doc. No 28) and by the European Union in 2011 (EU Reg. 61/2011), the study of these compounds started many years ago leading to evidence a double meaning of their evaluation.

Fatty acid alkyl esters (FAAEs, which include fatty acid methyl and ethyl esters) are formed by degradation and fermentation processes in low-quality olives, which can be overripe, damaged, or stored in poor conditions before they are processed (Biedermann et al., 2008). As a consequence, the degradation of pectins by endogenous pectin methylesterases and aerobic metabolism of microorganisms can lead to production of short chain alcohols, namely methanol and ethanol, respectively (Conte et al., 2019). The esterification of these compounds with free fatty acids, produced by lipolysis of triacylglycerols, leads to the development of methyl and ethyl esters of fatty acids. It is a typical second order reaction that takes place in an acid medium and catalyzed by enzymes (Pérez-Camino et al., 2008). Moreover, the formation of these molecules can continue during storage of the oil, but this phenomenon is strictly linked to the presence of free alcohols and fatty acids formed as consequence of fermentation processes of olives (Conte et al., 2014).

In 2002 (Pérez-Camino et al., 2002), an analytical method to determine the content of FAAEs in vegetable oils was proposed for the first time, showing a relation between their content and olive oil quality: in fact, FAAEs are more abundant in VOOs obtained from damaged olive fruits. This result, subsequently confirmed by other studies (Mariani et al., 2008; Biedermann et al., 2008; Pérez-Camino et al., 2008), suggested the possibility to introduce this parameter for quality control of VOOs, since it represents a direct indicator of the degradation and fermentation of olives, and is thus linked to the presence of fermentative sensory defects, such as fusty/muddy sediment, musty/humid/earthy, and winey/vinegary (Gomez-Coca et al., 2012; Di Serio et al., 2017). However, since fermentation processes are linked only to the content of FAEEs, in 2013 (EU Reg. 1348/2013), the legal limit for

this parameter was changed to exclude evaluation of the methyl esters. At present (EU Reg. 2019/1604), the limit fixed by IOC and EU for extra virgin olive oil is 35 mg/kg of oil (Table 3.1).

Year	*International Olive Council - IOC*	*European Union - EU*
2010	**COI/T.20/Doc. No 28 -** Description of the analytical protocol for FAAEs determination **COI/T.15/ Doc. No 3/Rev. 5 -** Introduction of FAAEs content as quality parameter **Legal limit for EVOO category:** Σ FAME + FAEE < 75 mg/kg or 150 mg/kg > Σ FAME + FAEE > 75 mg/kg and FAEE/FAME ratio < 1.5	
2011		**EU Reg. 61/2011 -** Introduction of FAAEs content as quality parameter and description of the analytical protocol for their determination **Legal limit for EVOO category:** Σ FAME + FAEE < 75 mg/kg or 150 mg/kg > Σ FAME + FAEE > 75 mg/kg and FAEE/FAME ratio < 1.5
2012	**COI/T.20/Doc. No 31 -** Description of a simplified analytical protocol for FAAEs determination **COI/T.15/ Doc. No 3/Rev. 7 -** Revision of the **legal limit for EVOO category**: Σ FAME + FAEE < 75 mg/kg	
2013	**COI/T.15/ Doc. No 3/Rev. 8 -** Revision of the **legal limit for EVOO category**: FAEEs < 40 mg/kg (2013-2014 crop year)	**EU Reg. 1348/2013 -** Revision of the **legal limit for EVOO category**: FAEEs < 40 mg/kg (2013-2014 crop year)
2014	**COI/T.15/ Doc. No 3/Rev. 9 -** Revision of the **legal limit for EVOO category**: FAEEs ≤ 35 mg/kg (2014-2015 crop year)	**EU Reg. 1348/2013 -** Revision of the **legal limit for EVOO category**: FAEEs ≤ 35 mg/kg (2014-2015 crop year)
2015	**COI/T.15/ Doc. No 3/Rev. 10 -** Revision of the **legal limit for EVOO category**: FAEEs ≤ 35 mg/kg (2015-2016 crop year)	**EU Reg. 1348/2013 -** Revision of the **legal limit for EVOO category**: FAEEs ≤ 35 mg/kg < 30 mg/kg (after 2015 crop year)
2016	**COI/T.15/ Doc. No 3/Rev. 11 -** Revision of the **legal limit for EVOO category**: FAEEs ≤ 35 mg/kg	**EU Reg. 2016/2095 -** Revision of the **legal limit for EVOO category**: FAEEs ≤ 35 mg/kg
2017	**DECISION No DEC-III-6/106-VI/2017 -** Revision of the analytical protocol (possibility to replace *n*-hexane with iso-octane)	
2019		**EU Reg. 2019/1604 -** Revision of the analytical protocol (possibility to replace *n*-hexane with iso-octane) **Legal limit for EVOO category**: FAEEs ≤ 35 mg/kg

Table 3.1. Timeline of regulations and revisions applied by IOC and EU about the analytical method for determination of fatty acid methyl (FAMEs) and ethyl (FAEEs) esters and their legal limit.

The second relevance of FAEEs content is related to soft deodorization, a technological process applied to VOOs with weak organoleptic defects in order to remove or reduce their intensity. The blending of soft deodorized oil with extra virgin and possible commercialization of this blend labelled as top-quality grade (EVOO) represents fraud: a soft deodorized oil cannot be considered 'virgin' any more since it has undergone a thermal process that is outside of the legal definition of VOO (Aparicio-Ruiz et al., 2017). Since the technological conditions of temperature and pressure applied during soft deodorization are "mild" and avoid the formation of typical markers of refining (such as stigmastadienes or trans isomers of fatty acids), in treated oils, it is very difficult to detect this type of fraud (Valli et al., 2013). Many studies (Pérez-Camino et al., 2008; Bendini et al., 2009; Jabeur et al., 2015) have demonstrated that the FAAEs content is not affected by the soft deodorization process, but allows for indirect detection of this illegal blend, since oils subjected to soft deodorization, and in particular those derived from oils defected by fermentative processes, are usually rich in FAEEs that are not removed or altered by this treatment.

The official method for determination of FAEEs content (EU Reg. 61/2011), which also allows the determination of waxes, is based on the addition of suitable internal standards to the oil followed by solid-liquid chromatography in a traditional glass column to isolate the fraction containing alkyl esters. After recovery of the eluted fraction, this is analyzed by capillary gas chromatography. However, it should be pointed out that the official method requires a large volume of solvents and a very long and complex preparative procedure. For these reasons, the method has been revised by the IOC (COI/T.20/Doc. No 31), reducing the amount of silica (from 15 g to 3 g) and eluent mixture, and including the use of *n*-hexane to remove any *n*-alkanes naturally present in the sample. More recently, EU Reg. 2019/1604 introduced the possibility to replace *n*-hexane with iso-octane.

A further revision described in the literature involves the injection technique used for GC analysis: a programmed temperature vaporizer (PTV) injector can be used as an alternative to the on-column one required by the regulation and gives comparable results for analysis of real-world samples (Purcaro et al., 2015).

Moreover, other analytical techniques have been tested as alternatives to the official method. Regarding the chromatographic ones, the first method proposed in 2002 (Pérez-Camino et al., 2002), and later slightly modified by the same research group (Pérez-Camino et al., 2008; Gomez-Coca et al., 2012), was based on isolation of the fraction from the oil by solid phase extraction (SPE) and subsequently analyzed by GC-FID. In 2008 (Biedermann et al., 2008), an on-line LC-GC-FID method was optimized for analysis of methyl/ethyl oleate and selected straight chain wax esters. Subsequently, the same approach was used by Küchler and coworkers (Küchler et al., 2014), reducing the manual sample preparation effort by 90%. More recently, a GC-(EI)MS with a PTV injector

coupled by a capillary transfer line with an external thermal extraction unit was optimized and applied to a large set of olive oil samples with different quality (Boggia et al., 2014).

Finally, rapid and non-destructive techniques based on FT-IR (Valli et al., 2013; Squeo et al., 2019; Uncu et al., 2019), Time Domain Reflectometry (TDR, Berardinelli et al., 2013) and NIR (Garrido-Varo et al., 2017; Cayuela, 2017), coupled to chemometric data elaboration, have also been applied with good results.

The aim of the present investigation was to evaluate the application of possible improvements, recently highlighted (Conte et al., 2019), to the official method for FAEEs determination. In particular, the use of HPLC-UV-Vis as an alternative to traditional liquid chromatography applied in the preparative phase for the determination of FAEEs, as well as the use of a PTV injector as an alternative to the required OC were investigated. Next, the revised method (off-line HPLC-GC-FID with PTV injector) was in-house validated by evaluating selected figure of merits (linearity, LOD and LOQ, robustness, intra-day and inter-day precision, accuracy, and recovery). The use of an off-line HPLC-GC approach could represent a good compromise between the traditional liquid chromatography foreseen in the official IOC and EC methods and the application of an on-line LC-GC system that requires expensive instrumentation, which is not affordable for most laboratories.

3.2 Materials and methods

3.2.1 Reagents and chemicals

Diethyl ether (purity ≥99.8%, CAS Number 60-29-7), *n*-hexane (purity ≥95%, CAS Number 110-54-3), *n*-heptane (purity ≥99%, CAS Number 142-82-5), and *tert*-butyl methyl ether (MTBE, purity ≥99.8%, CAS Number 1634-04-4) were supplied by Sigma-Aldrich, Inc. (St. Louis, MO, USA). Sudan I (1-Phenylazo-2-naphthol, CAS Number 842-07-9), methyl heptadecanoate (analytical standard, CAS Number 1731-92-6), ethyl palmitate (analytical standard, CAS Number 628-97-7), ethyl oleate (analytical standard, CAS Number 111-62-6), ethyl linoleate (analytical standard, CAS Number 544-354), ethyl stearate (analytical standard, CAS Number 111-61-5), and silica gel (high-purity grade, Davisil Grade 62, particle size 60-200 mesh, CAS Number 112926-00-8) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA).

3.2.2 Equipment and instrumentations

Usual laboratory glassware; plastic syringes and filters (polyamide, 0.20 μm); glass column for liquid chromatography (internal diameter 15 mm, length 40 cm, fitted with a stopcock); analytical balance for weighing to an accuracy of ± 0.1 mg; system to dry by a flow of nitrogen at room temperature; rotary evaporator; muffle oven; HPLC-UV-Vis 1260 Infinity II (Agilent) equipped with a 100 μL loop injector; silica column for HPLC Luna® (Phenomenex, length 25 cm; internal diameter 4.6 mm; particle sizes 5 μm) with guard column; GC-FID Trace 1300 (Thermo Fisher Scientific, Waltham, MA, USA) equipped with both PTV and OC injector; capillary column for GC TG-5SILMS (length 15 m, internal diameter 0.32 mm, film thickness 0.25 μm, stationary phase 5% diphenyl/95% dimethyl polysiloxane; Thermo Fisher Scientific, Waltham, MA, USA).

3.2.3 Samples

For the evaluation of almost all parameters considered for in-house validation of the revised method, refined olive oil was used as the fat matrix.
To investigate the suitability of a PTV injector in place of the OC one and to measure the accuracy and intra-day precision of the revised method (off-line HPLC-GC-FID with PTV injector), three samples with a different FAEEs content were analyzed:

- Sample **LC – Low Content**: extra virgin olive oil with a content of FAEEs under the legal limit;
- Sample **MC – Medium Content**: non-extra virgin olive oil with a content of FAEEs slightly beyond the legal limit;
- Sample **HC – High Content**: non-extra virgin olive oil with a content of FAEEs much higher than the legal limit.

3.2.4 Determination of FAEEs by official method

The reference value for FAEEs content, considered for the evaluation of the accuracy of the revised method, was determined according to the analytical protocol defined in EU Reg. 61/2011.
Sample preparation: 500 mg of oil sample were weighed and dissolved in 0.25 mL of internal standard solution (methyl heptadecanoate C17:0, 0.02% in *n*-heptane), 2 mL of *n*-hexane and 0.1 mL of Sudan I solution (1% in *n*-hexane/ethyl ether 99:1 v/v).

Chromatography column preparation: 15 g of silica gel, previously placed in the muffle oven at 500 °C for at least 4 h and then added with 2% of water, were suspended in 50 mL of *n*-hexane and introduced into a glass column for liquid chromatography. The solvent was allowed to flow in order to facilitate the deposit of silica. Next, 30 mL of *n*-hexane are percolated through the column to remove interfering compounds.

Collection and separation of the FAEEs fraction: the prepared sample was transferred into the chromatography column with the aid of two 2 mL portions of *n*-hexane and the solvent was allowed to flow to 1 mm above the upper level of the absorbent. For elution of analytes, a mixture of *n*-hexane/ethyl ether (99:1 v/v) was used, at a rate of about 15 drops/10 sec until the dye (Sudan I) reached the bottom of the chromatography column (Figure 3.1). About 250 mL of solution containing FAEEs were collected and evaporated, and the residue was re-dissolved in 5 mL of *n*-heptane for the following GC-FID analysis.

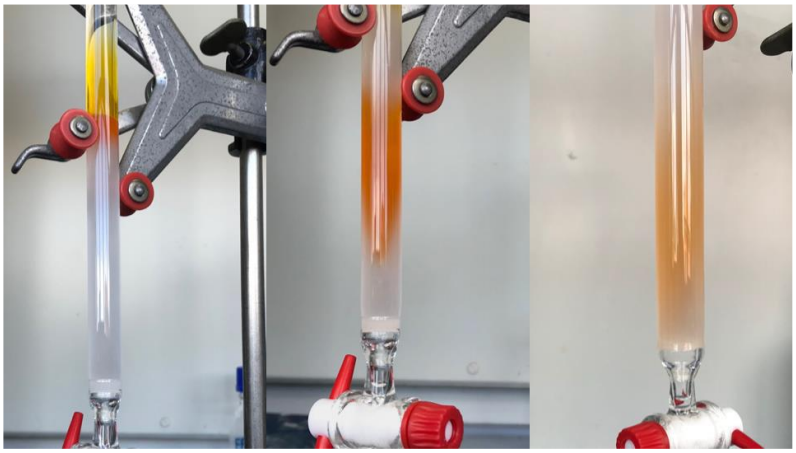


Figure 3.1. Phases of the elution process of the fraction containing FAEEs.

GC-FID analysis: the oven temperature was programmed as follows: 80 °C for 1 min; temperature increased by 20 °C/min until reaching 140 °C; temperature increased by 5 °C/min until 335 °C and maintained for 20 min. The detector temperature was 350 °C and 1 µL was injected with on-column injector. Helium was used as carrier gas with a flow of 1 mL/min.

For PTV injection conditions, the splitless mode with an injection temperature of 70 °C, increasing by 3°C/s until 300 °C (maintained for 1 min) during the transfer phase, was applied.

FAEEs identification and quantification: peak identification was carried out by injecting directly a solution of a mix of standards (ethyl palmitate, ethyl oleate, ethyl linoleate, ethyl stearate) in the GC-FID and comparing the retention times of each compound vs. known standards. Relative retention times were also calculated by measuring the ratio between the retention time of each compound and of the internal standard. FID response is assumed to be equal for all compounds and no correction for

response was applied. The quantification step was carried out applying the formula described in the EU Reg. 61/2011:

$$\frac{A_x \times m_s \times 1000}{A_s \times m}$$

where:

- A_x = area corresponding to the peak for the individual ester, in computer counts;
- A_s = area corresponding to the peak for the methyl heptadecanoate (used as internal standard), in computer counts;
- m_s = mass of the methyl heptadecanoate (used as internal standard) added, in milligrams;
- m = mass of the oil sample taken for determination, in grams.

3.2.5 Determination of FAEEs by off-line HPLC-GC-FID

The scheme and the analytical conditions of the revised method developed for the FAEEs determination are presented in Figure 3.2 and detailed below.

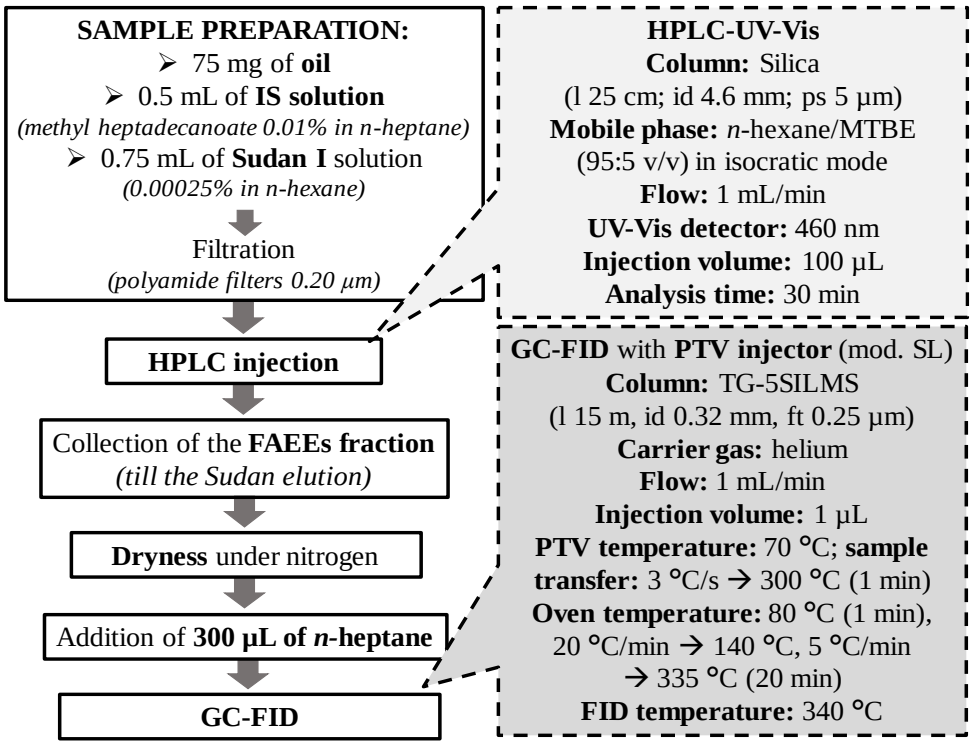


Figure 3.2. Scheme and analytical conditions of the revised method for FAEEs determination.

Sample preparation: 75 mg of oil sample were weighed and dissolved in 0.50 mL of internal standard solution (methyl heptadecanoate C17:0, 0.01% in *n*-heptane) and 0.75 mL of Sudan I solution

(0.00025% in *n*-hexane). The sample was then filtered with a polyamide filter (0.20 μm) and transferred in a vial for HPLC.

Collection and separation of the FAEEs fraction: the separation and collection of the fraction containing FAEEs were carried out using an HPLC-UV-Vis. The isocratic mode is applied with *n*-hexane/MTBE (95:5 v/v). The flow was set at 1 mL/min and the UV detector at 460 nm to monitor the dye (Sudan I) elution. 100 μL of the sample were injected and the FAEEs fraction was manually collected until the beginning of elution of Sudan (by monitoring the elution from the real time window of the HPLC software). Next, the solution collected (about 8-9 mL) was dried under a gentle nitrogen flow and recovered with 300 μL of *n*-heptane for subsequent GC-FID analysis.

GC-FID analysis: this step was carried out by applying the same analytical conditions described in the previous paragraph (section 3.2.4). In this case, only the PTV injector was used.

FAEEs identification and quantification: this step is carried out following what described in the section 3.2.4.

3.2.6 In-house validation of the revised method

The parameters considered for in-house validation of the method were linearity, limit of detection (LOD) and quantification (LOQ), recovery, robustness, accuracy, and intra-day and inter-day precision (Table 3.2).

Parameter	***Definition***
Linearity	The ability of the method to obtain test results proportional to the concentration of analyte within a given range.
Limit of detection (LOD)	The lowest concentration or amount of analyte that can be reliably distinguished from zero or that can be detected with reasonable certainty.
Limit of quantification (LOQ)	The lowest concentration or amount of analyte that can be determined quantitatively with an acceptable level of repeatability, precision and trueness.
Recovery	The fraction of analyte added to the test sample (fortified or spiked) prior to analysis, which is measured by the method.
Robustness	The measure of the capacity of the analytical procedure to remain unaffected by small, but deliberate variations in method-performance parameters.
Accuracy	The closeness of agreement between a test result and the accepted reference value.
Precision	The closeness of agreement between independent test results obtained under stipulated conditions.
Intra-day precision	Precision under conditions where independent test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment within short intervals of time.
Inter-day precision	Precision under conditions where independent test results are obtained with the same method in identical test items in the same laboratory but by different analysts, using different equipment over an extended period of time.

Table 3.2. Definition of parameters measured for in-house validation of the revised method (Taverniers et al., 2004).

For evaluation of these parameters (except for accuracy and intra-day precision), samples were prepared following the steps described above (see section 3.2.5), but using refined olive oil as the fat matrix and replacing the internal standard solution with 0.50 mL of a mix of standards solution in *n*-hexane. The standards used, chosen according to the usual FAEEs composition in olive oil, were ethyl palmitate, ethyl stearate, ethyl oleate and ethyl linoleate dissolved in *n*-hexane at different approximate concentrations (Table 3.3).

Code	***Approximate concentration****
C1	0.0025 mg/mL
C2	0.005 mg/mL
C3	0.010 mg/mL
C4	0.025 mg/mL
C5	0.050 mg/mL

Table 3.3. Code and approximate concentration of the solutions of mix of standard used for the evaluation of some validation parameters. * The concentration of each solution should be considered as approximate since the initial weight, and therefore the concentration, of each compound was slightly different.

Linearity

To evaluate the linearity of the method, all the solutions of the mixture of standards (C1-C5) were analyzed and the correlation (expressed as R^2) between the areas measured for each compound and their concentration was determined.

Limit of detection (LOD) and quantification (LOQ)

LOD and LOQ for each compound were determined considering the standard deviation of the lowest acceptable concentration of the analyte (in this case C1) and the slope of the calibration function of each molecule (González et al., 2007).

Recovery

For recovery, the solutions of the mix of standards C3, C4, and C5 were analyzed by applying the revised procedure (off-line HPLC-GC-FID with PTV injector) and by direct gas chromatographic on-column injection. The results obtained for ethyl esters and methyl heptadecanoate (used as internal standard) were then compared and recovery percentages were calculated.

Robustness

For robustness, the standard solution C4 was analyzed with the revised protocol changing the mobile phase flow for the HPLC analysis from 1 mL/min to 0.7 mL/min.

The data obtained for each standard analyzed applying the different mobile phase flow were compared (Student's test, $p \leq 0.05$).

Inter-day precision

To measure the inter-day precision, the solution of mix of standards C4 was analyzed applying the proposed method on three different days and by different operator (it was not possible realize the tests using different equipment). Next, the area values of each compound measured in the three different days were statistically evaluated (One-way ANOVA, $p \leq 0.05$).

Accuracy and intra-day precision

To study the accuracy and intra-day precision, the samples LC – Low Content, MC – Medium Content and HC – High Content were analyzed by both the official EU method and the revised one. The results measured with the two methods were compared (Student's test, $p \leq 0.05$) to verify the effectiveness of the proposed protocol. The intra-day precision was expressed as RSD (Relative Standard Deviation).

3.2.7 Data elaboration

Elaboration of results (One-way ANOVA and Student's test) were carried out using the software XLSTAT (Addinsoft, New York, USA, version 2018.1).

3.3 Results and discussion

3.3.1 Development of the revised method for FAEEs determination

The starting point of the present research was the need to find an analytical solution for determination of FAEEs in VOO that can overcome some of the drawbacks of the official method and, at the same time, would be applicable by the majority of laboratories and industries. In fact, on-line approaches, already reported in the literature and discussed above, require expensive instrumentation and specific analytical skills that are not always available especially outside the academic contest.

In this study, an off-line combination of HPLC-GC-FID as an alternative to the official method for determination of FAEEs in VOOs was developed. The preparative phase, i.e. extraction and separation of the fraction containing FAEEs, was carried out using HPLC-UV-Vis in place of

traditional liquid chromatography applied in the official method (Reg. EU 61/2011). The specific analytical conditions adopted were chosen in order to apply the same analytical principle of the official method: normal phase liquid chromatography in isocratic mode. For this reason and following information available in the literature concerning the on-line approaches (Biedermann et al., 2008; Küchler et al., 2014), a silica column and a slightly polar mixture of solvents (*n*-hexane/MTBE 95:5 v/v) as the mobile phase were chosen. The analytical conditions applied herein lead to elution of both methyl and ethyl esters of fatty acids, even though a legal limit is defined only for the latter. For this reason, only the results on FAEEs will be presented herein.

As in the official method, in this case Sudan I was used as a dye to visually check the elution of the fraction containing FAAEs and waxes since it is characterized by an elution time between that of waxes and triglycerides. Therefore, elution of Sudan I indicates the end of elution of the analytes of interest avoiding the undesired elution of triglycerides (Pérez-Camino et al., 2002). Consequently, to detect dye elution, a UV-Vis detector was selected and used at a wavelength of 460 nm, which is in the absorption range of this compound. Some attempts to find an alternative to this compound were carried out since it is known to be both toxic and carcinogenic and its addition to food as a colorant is not permitted (Genualdi et al., 2016). In particular, *α*-tocopherol and bixin were tested, but satisfactory results were not obtained due to a different retention time and low solubility in the solvent used for the analysis, respectively. Despite the elution of Sudan I, and then the elution of the fraction of interest containing the FAEEs, ends after about 8-9 minutes of analysis (Figure 3.3), the total duration of each HPLC analysis was set at 30 min to allow the complete elution of TAGs prior to the next injection, avoiding their residency in the column.

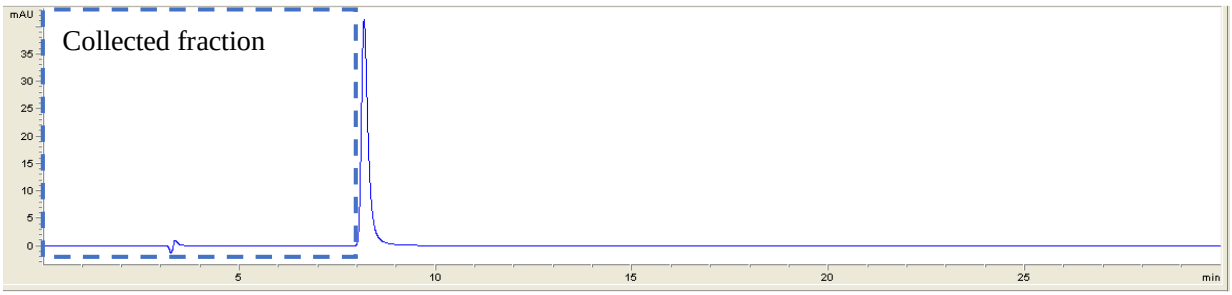


Figure 3.3. HPLC-UV chromatogram of Sudan I.

Following this, the fraction containing the FAEEs was analyzed by capillary GC-FID with PTV injector. This represents the only difference of the gas chromatographic step between the proposed method and the official one for which an OC injector is needed. The use of an OC injector is required in the official method as well as in many other applications in the analysis of fats and oils to prevent discrimination effects (e.g. for waxes determination) and reduce degradation of labile compounds

(Purcaro et al., 2015), but is not widely applied in quality control laboratories, and thus an alternative is needed. The PTV injector represents a more versatile option since the temperature control is time-programmed and the injection mode (split/splitless) can be optimized according to the specific application (Engewald et al., 1999).

Before introducing this modification in the revised method, the use of the PTV injector in place of the OC one was tested on the official method: the samples LC, MC and HC were analyzed by applying the official method and injected using both the PTV and OC injector.

Comparing the total content of FAEEs measured employing the two injectors (Table 3.4) no significant differences were found (Student's test, $p \leq 0.05$).

Sample	***LC - Low Content***		***MC - Medium Content***		***HC - High Content***	
Method	OC	PTV	OC	PTV	OC	PTV
FAEEs *(mg/kg oil)*	18.4 ± 1.1	20.3 ± 1.0	39.8 ± 0.7	40.6 ± 0.3	82.1 ± 2.8	80.8 ± 2.7

Table 3.4. Mean values and standard deviation (calculated on three replicates) of the FAEEs content measured applying the official method and using the on-column injector (OC) and the PTV (Programmed Temperature Vaporizer) one.

Considering these preliminary results, the PTV injector was introduced in place of the OC one also in the revised method.

Identification of compounds was carried out by injecting the analytical standard of each molecule, and the relative retention time (RRT) was calculated (Table 3.5) considering the solutions of mix of standards (C1-C5).

Compound	**Relative Retention Time (RRT)**
Ethyl palmitate	0.95
Ethyl linoleate	1.21
Ethyl oleate	1.22
Ethyl stearate	1.28
Methyl heptadecanoate	1.00

Table 3.5. Relative Retention Time (RRT) measured for the FAEEs considered in relation to methyl heptadecanoate (used as internal standard).

Applying the revised method, the total time required for each analytical determination is reduced from about 6 h to roughly 2.5 h (more than 50%). Considering that the duration of the chromatographic run is the same for the official and revised method, the reduced time of analysis is

mainly due to speeding up of the preparative phase realized by HPLC that does not require drying almost 250 mL in a rotary evaporator. In fact, the volume of solvents needed for each analytical measurement was reduced more than 80% (Table 3.6). Another advantage of the application of an HPLC is that the manual packing of the glass column for the liquid chromatography is not required, thus simplifying this step.

	Official method *(off-line LC-GC-FID)*	**Revised method** *(off-line HPLC-GC-FID)*
Volume of solvents	~ 350 mL	~ 40 mL
Time of analysis	~ 6 hours	~ 2.5 hours

Table 3.6. Volume of solvents and time of analysis required for each analytical determination by the official method (Reg. EU 61/2011) and the revised one.

3.3.2 Method performance determined with in-house validation

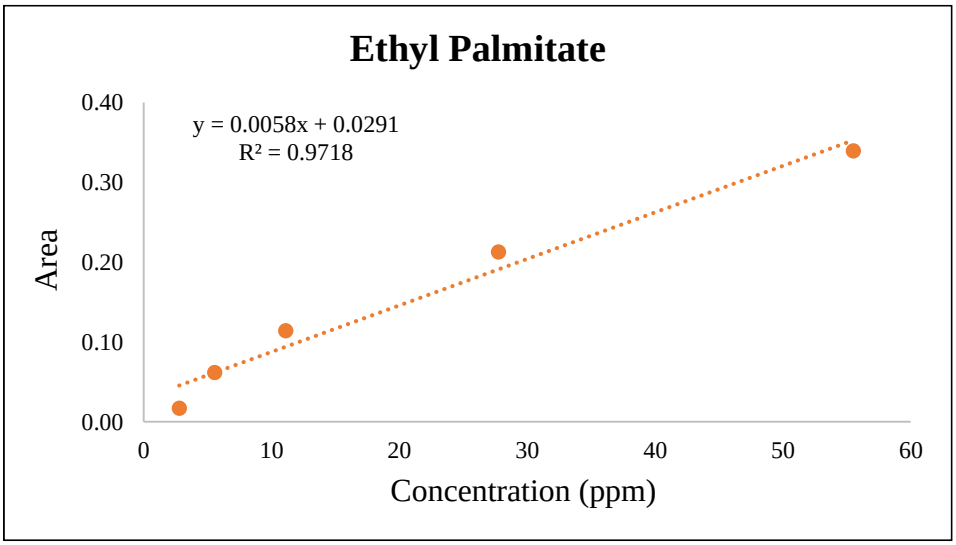
After establishing the analytical conditions, the proposed method was in-house validated using specific parameters: linearity, LOD and LOQ, recovery, robustness, accuracy, inter-day and intra-day precision.

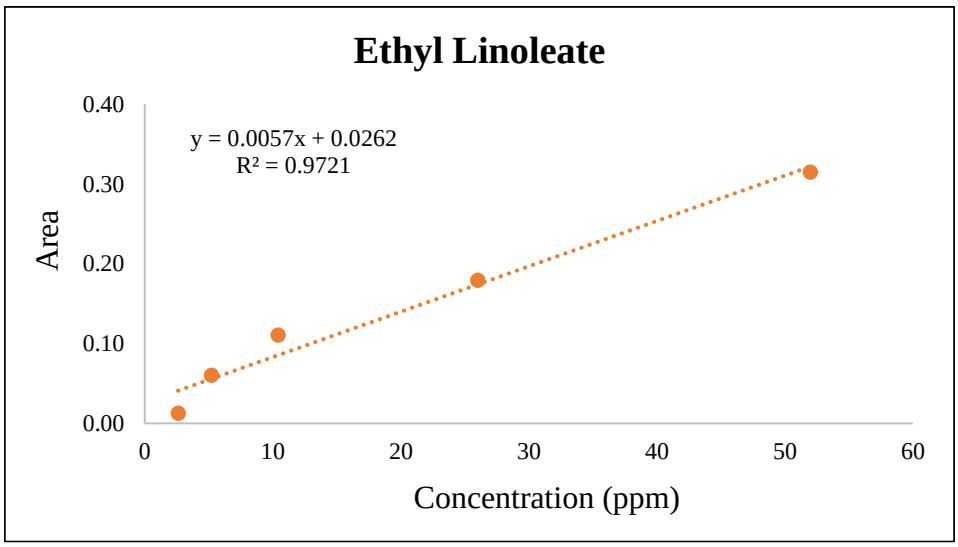
Linearity

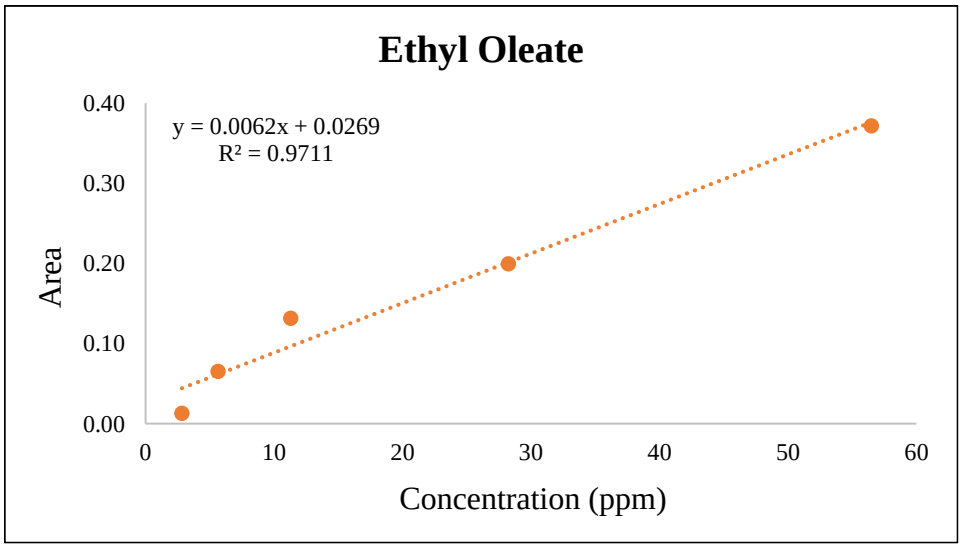
For linearity, the mixture of standards was analyzed at 5 different concentrations: considering peak area vs. analytes concentration, good correlation coefficients (expressed as R^2) were obtained for all compounds (Table 3.7; Figure 3.4). The method showed a linear response between 2.5 mg/L and 50 mg/L (Figure 3.5), which is a reasonable range in view of the legal limit established for extra virgin olive oil (EU Reg. 2019/1604).

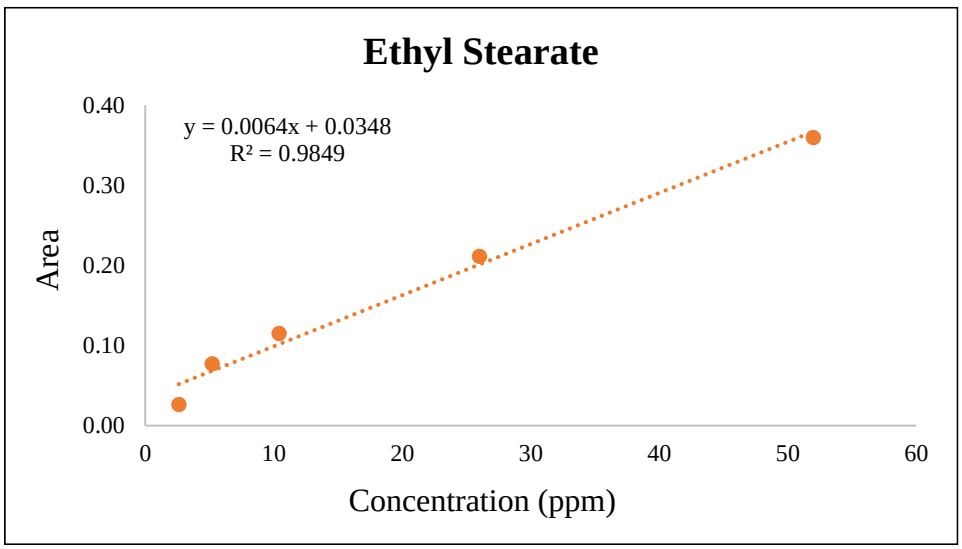
Compound	Linearity (R^2)
Ethyl palmitate	0.97
Ethyl linoleate	0.97
Ethyl oleate	0.97
Ethyl stearate	0.98
Methyl heptadecanoate	0.98

Table 3.7. Linearity (R^2 value), examined considering the mixture of standards at 5 different concentrations (C1: 0.0025 mg/mL, C2: 0.005 mg/mL, C3: 0.010 mg/mL, C4: 0.025 mg/ml, C5: 0.050 mg/mL), for the fatty acid ethyl esters considered and methyl heptadecanoate (used as internal standard).









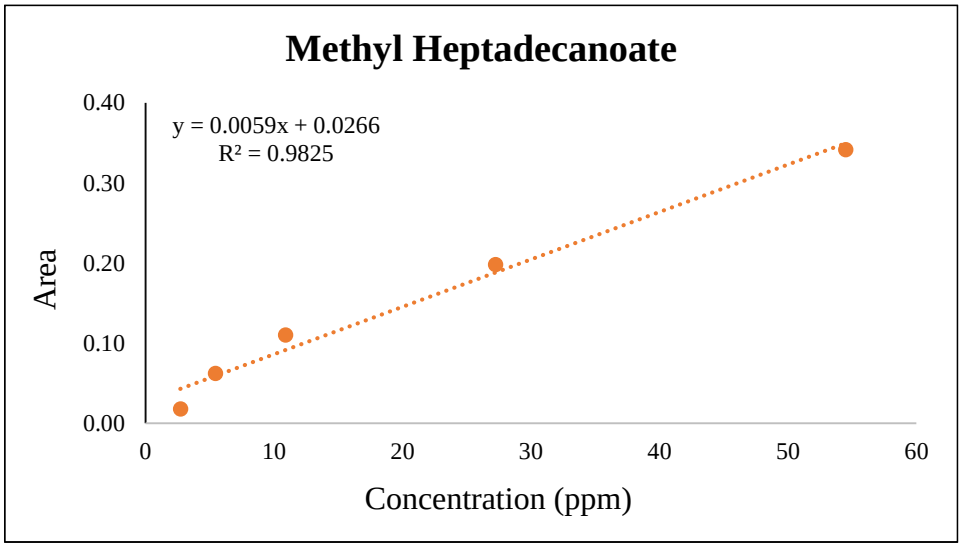


Figure 3.4. Correlation curves between area and concentration for each FAEE considered (ethyl palmitate, ethyl linoleate, ethyl oleate, ethyl stearate) and methyl heptadecanoate (used as internal standard).

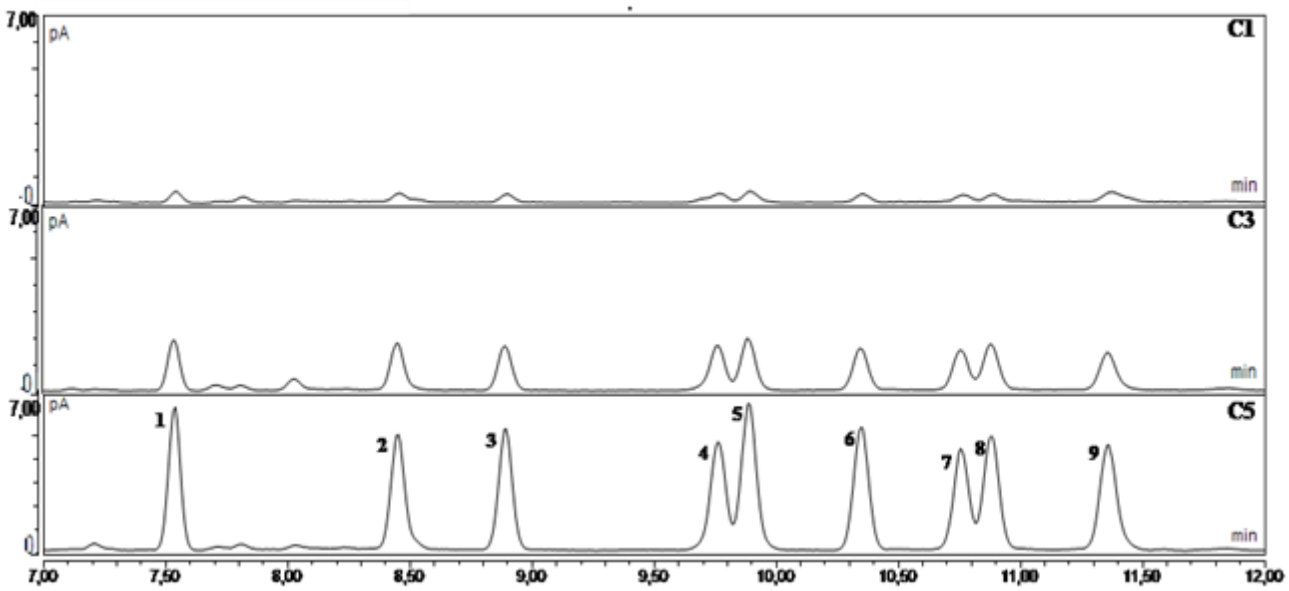


Figure 3.5. Overlay of chromatograms obtained from the analysis of the solutions of standards C1, C3 and C5 applying the revised method.

Compounds identification: 1) Methyl Palmitate, 2) Ethyl Palmitate, 3) Methyl Heptadecanoate (internal standard), 4) Methyl Linoleate, 5) Methyl Oleate, 6) Methyl Stearate, 7) Ethyl Linoleate, 8) Ethyl Oleate, 9) Ethyl Stearate.

LOD and LOQ

LOD and LOQ values for each examined FAEE were calculated by multiplying the standard deviation of each compound area, at the lowest concentration level (in this case C1), 3 and 10 times, respectively, and then by dividing the result by the slope of the specific calibration curve. All the values obtained for LOD were lower than 1 mg/kg and those measured for LOQ were lower than 1.5 mg/kg, in accordance with what previously reported (Biedermann et al., 2008).

Recovery

Recovery percentages for ethyl esters and methyl heptadecanoate (used as internal standard) were calculated considering the mixture of standards at the concentrations C3, C4 and C5 and satisfactory results were obtained (Table 3.8).

Compound	C3 (recovery %)	C4 (recovery %)	C5 (recovery %)
Ethyl palmitate	70.8	100.9	88.9
Ethyl linoleate	67.5	92.6	84.3
Ethyl oleate	71.1	93.2	87.2
Ethyl stearate	83.0	100.8	93.3
Methyl heptadecanoate	72.7	97.5	86.7

Table 3.8. Recovery percentages calculated analyzing the mix of standards at 3 concentrations (C3, C4, C5) for the FAEEs considered and methyl heptadecanoate (used as internal standard).

Robustness

Regarding the robustness of the method, standards solution C4 was injected in HPLC at two different mobile phase flows, namely 1 mL/min and 0.7 mL/min. The data obtained for all the compounds was elaborated and no significant differences between the two methods (Student's test, $p \leq 0.05$) were seen, even though a reduction in the flow rate led to a decrease of the pressure and an increase in the elution time of the dye (Figure 3.6).

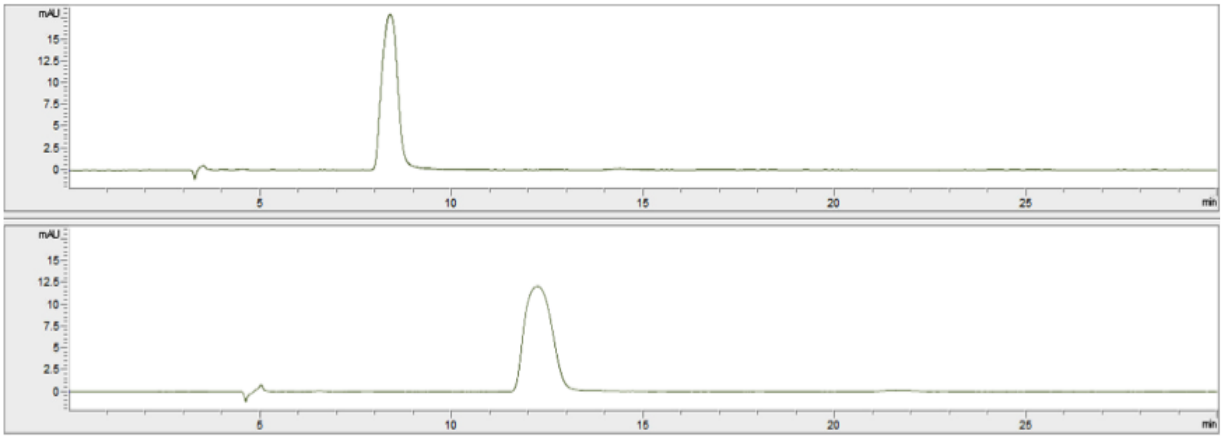


Figure 3.6. HPLC-UV chromatogram of the Sudan I (0.0025 mg/mL in *n*-hexane) analyzed at a flow of 1 mL/min (upper) and 0.7 mL/min (lower).

Inter-day precision

To evaluate the inter-day precision, a sample prepared with the solution of standards C4 added to a refined olive oil was analyzed in three different days and by different operators. Comparing the areas of each compound in the three different days, no significant differences (One-way ANOVA, $p \leq 0.05$) were found.

Accuracy and intra-day precision

For evaluation of accuracy and intra-day precision, and in order to test the revised method on real-world oil samples, 3 VOOs with a different FAEEs content (low, medium and high) were analyzed by both the official method and the revised one (off-line HPLC-GC-FID with PTV injector). In Table 3.9 the total FAEEs contents are presented, since this is the parameter considered by the official method, together with the amount of ethyl oleate, which is the FAEE most present in VOOs. Next, the total content of FAEEs was compared and the revised method did not show significant differences (Student's test, $p \leq 0.05$) compared to the official one for any sample. Considering these results, the three samples were classified in the same commercial category by both methods: sample LC was an extra virgin olive oil, while samples MC and HC were not. Finally, intra-day precision was expressed as RSD and all the values obtained were lower than 15%, which is considered acceptable for validation of a new method (Peters et al., 2007).

Sample	***LC - Low Content***		***MC - Medium Content***		***HC - High Content***	
Compound	*Official method*	*Revised method*	*Official method*	*Revised method*	*Official method*	*Revised method*
Ethyl Oleate	12.0 ± 0.3	9.9 ± 0.5	29.6 ± 0.7	28.4 ± 1.2	63.4 ± 2.1	60.8 ± 4.0
Total FAEEs	18.4 ± 1.1	17.8 ± 1.0	39.8 ± 0.7	38.7 ± 0.4	82.1 ± 2.8	85.1 ± 0.5

Table 3.9. Mean value and standard deviation (calculated on three replicates) of the content of ethyl oleate and fatty acids ethyl esters measured by both official method (EU Reg. 61/2011) and revised one (off-line HPLC-GC-FID) on three olive oil samples. Data are expressed as mg/kg of oil.

Sample LC – Low Content: extra virgin olive oil with a content of FAEEs under the legal limit; sample MC – Medium Content: non-extra virgin olive oil with a medium content of FAEES beyond the legal limit; sample HC – High Content: non-extra virgin olive oil with a content of FAEEs much higher than the legal limit.

3.4 Conclusions

A revised protocol, based on an off-line HPLC-GC-FID approach, for FAEEs determination in VOOs is presented. The method is based on the application of HPLC-UV-Vis as an alternative to traditional liquid chromatography applied in the preparative phase for FAEEs determination, and the use of a PTV injector in place of the OC one required by the official method.

After the optimization of the working conditions, the method was in-house validated. The data obtained showed good performance in terms of linearity, LOD, LOQ, robustness, intra-day precision,

and recovery. No significant differences were found when the proposed method was tested on VOOs with a different amount of FAEEs and the results were comparable to those obtained using the official protocol.

The main advantages of this revised protocol are: *i)* significant reduction of time (more than 50%) and solvents (more than 80%) required for each analytical determination, representing a more environmentally sustainable and rapid alternative to the official method; *ii)* use of HPLC as an alternative to traditional liquid chromatography carried out in manually packed glass columns, allowing for simplification of the technique.

Moreover, the adoption of an off-line HPLC-GC approach could represent a good compromise between what required by the EC and IOC official methods (traditional liquid chromatography) and the application of an on-line LC-GC system that needs expensive instrumentation, which is not affordable for most laboratories.

These characteristics make the method proposed herein exploitable by both control laboratories and industry, satisfying the needs of cost reduction and work optimization.

In order to confirm and strengthen the reliability and good performances of the approach presented herein, and in view of its proposal to normative bodies for possible adoption, an inter-lab validation of this method, involving several laboratories (also from private industries), is being carried out within the OLEUM project.

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Chapter 4

Development and in-house validation of a portable system for determination of free acidity in virgin olive oil

4.0 Details of the publication based on Chapter 4

Title: Design and in-house validation of a portable system for the determination of free acidity in virgin olive oil

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Abstract

Nutritional and healthy values are well known properties of virgin olive oil (VOO). The product quality, in terms of belonging to a specific quality grade (extra virgin, virgin, lampante), is defined by a set of chemical-physical and sensory measurements. According to the official regulation of the European Union (EU Reg. 1348/2013) the free acidity is the first parameter that has to be determined by analysts; it gives information about the quality of the olives used to produce the VOO as well as the hydrolytic state of VOO just produced and stored. The official procedure is based on an acid-base titration that needs to be carried out in a chemical laboratory.

In this paper a portable battery-operated electronic system to measure olive oil free acidity is presented: the system can be used for quick "in situ" tests in a production environment (olive oil mills or packaging centers) by people without particular training. The working principle of the system is based on the creation of an emulsion between oil and a hydroalcoholic solution: the free acidity is estimated on the value of the emulsion electrical conductance.

The proposed system has been calibrated and in-house validated showing good results in terms of limit of detection and quantification, precision and accuracy. Moreover, a good correlation (R^2_{adj} =

0.97) with free acidity data obtained applying the official method on 30 olive oil samples belonging to different commercial categories (extra virgin, virgin and lampante olive oil) has been evidenced.

Keywords: Portable system; Free acidity; Virgin olive oil; Impedance spectroscopy; In-house validation; Electrical conductance.

4.1 Introduction

Virgin olive oil (VOO) is obtained from olives (the fruits of *Olea europaea* L.) applying only a mechanical-physical extraction process and represents a product that is highly appreciated for its beneficial effects on human health, mainly due to the high content of oleic acid and presence of minor components such as phytosterols, carotenoids, tocopherols, and hydrophilic phenols (Bendini et al., 2007). European Commission Implementing Regulation 2019/1604 (EU Reg. 2019/1604) defines a decision tree for verifying whether a VOO is consistent with the category declared, and the quality criteria that have to be checked by analysts are: free acidity (FA), peroxide value, specific extinctions in UV, sensory characteristics and ethyl esters of fatty acids. The first quality parameter in the above cited decision tree is, therefore, determination of free acidity; this is defined as the amount of free fatty acids that are no longer linked to their parent triglyceride molecules (TAGs) and measured as a percentage of oleic acid. Specifically, the top-quality product, extra virgin olive oil (EVOO), features a maximum FA of 0.8 g oleic acid/100 g oil, then the VOO presents a maximum FA value of 2.0 g oleic acid/100 g oil and, finally, lampante olive oil (LOO), which is not suitable for the commercialization, is characterized by a FA higher than 2.0 g oleic acid/100 g oil.

This parameter is particularly affected by the quality of the olives used to produce the oil since the separation of free fatty acids from TAGs is due to the enzymatic reaction of lipases. Given that FA increases with enzymatic reactions, it is important to avoid contact between enzymes and oil. In the fruit, the oil is protected in the vacuoles of cells and rupture of vacuoles before the mill can raise the free acidity because it brings lipase and olive oil in contact. There are different circumstances that lead to a fracture of vacuoles to increase the enzymatic activity, such as mold and bacterial attack of olives, infestation of the olive fly (*Bactrocera oleae*), storage time and unsuitable conditions before the milling (Angerosa et al., 2006). Thus, FA is an indicator of how fresh, healthy, and how well handled the olives were before being milled (Tena et al., 2015). Moreover, several studies have confirmed that geographic and environmental factors (Bustan et al., 2014), and the application of specific technological processes (such as filtration or a cooling treatment of olive paste) (Veneziani et al., 2018 a-b) do significantly not affect this parameter.

The official procedure to measure the FA in oil is defined by European regulations (EEC Reg. 2568/1991 and following amendments) and consists of an acid-base titration that, albeit simple and quick, must be carried out in a laboratory with trained personnel due to the need for specific solvents and lab equipment.

However, in addition to official methods for quality control of VOOs, there is a strong need for simple, rapid and environmental friendly techniques, also suitable for on-site quality control even for

new users who are "non-professional analytical skilled" (Inajeros-García et al., 2013; Valli et al., 2016). This represents an important issue, especially for small oil mills and packaging centers that cannot afford the cost of external laboratory analysis.

The possibility of simple, quick and in-situ analysis for food quality control (often implemented in the form of portable electronic systems) has been widely investigated in recent years. Some examples in the food quality field are an opto-electronic system for in-situ determination of peroxide value and total phenol content in olive oil (Grossi et al., 2015), a 3D machine video system for quality grading of Atlantic salmon (Sture et al., 2016), and an embedded sensor for rapid freshness prediction and identification of beef meat (Arsalane et al., 2018). Concerning olive oil free acidity determination, different rapid and innovative techniques have been presented in literature, some of which also allow measurement of other quality parameters (Table 4.1).

Technique	***Reference(s)***
UV-VIS-NIR	Mailer, 2004
Impedance spectroscopy	Grossi et al., 2014 a-b
Electrical conductivity	Yu et al., 2012
Hyperspectral imaging	Bendini et al., 2007
Fluorescence	Poulli et al., 2005 Guzmán et al., 2015
Voltammetry	Baldo et al., 2016 Baldo et al., 2019
Dielectric spectroscopy	Cataldo et al., 2010 Sanaeifar et al., 2018
NIR	Manley et al., 2006 Armenta et al., 2007 Inarejos-García et al., 2013 Martínez Gila et al., 2015
Raman	Muik et al., 2003 Zou et al., 2009 El-Abassy et al., 2009 Gouvinhas et al., 2015

Table 4.1. Techniques, and relative references, for determination of free acidity in virgin olive oil reported in the literature.

The aim of this work is to present a portable electronic system to measure free acidity in olive oil samples: the instrument is battery-operated and can be used for quick "in-situ" measurements in the oil production or bottling site. The working principle is based on estimation of FA in oil using

electrical conductance measured by electrical impedance spectroscopy (EIS) of the oil emulsion with a hydroalcoholic solution. EIS is used in a wide range of applications (Grossi et al., 2017a), such as to estimate the ripening degree of fruits (Harker et al., 1994), characterize plant tissues (Lin et al., 2012; Ben Hamed et al., 2016), define and detect the freezing end point of ice cream mixes (Grossi et al., 2012a), estimate the water content in EVOO (Ragni et al., 2013), analyze the composition of the human body (Khalil et al., 2014; Bera, 2014), detect the end-point in a titration assay (Grossi et al., 2017b).

The system described in this paper represents a considerable improvement over previous studies (Grossi et al., 2014 a-b) since it was completely re-designed with a focus on low-cost electronics and small dimensions. To make the system completely portable and reliable for on-site measurements, a temperature sensor was included to compensate for conductance variations linked to changes in environmental temperature. Moreover, the presented instrument was in-house validated evaluating its performances in terms of correlation between concentration of analyte and instrument response, limit of detection (LOD) and quantification (LOQ), precision, and accuracy. The validation step, in fact, is one of the measures that is universally recognized as a necessary part of a comprehensive system of quality assurance in analytical chemistry and is an essential component of the measures that a laboratory should implement to produce reliable analytical data (Thompson et al., 2002).

4.2 Materials and methods

4.2.1 Reagents and chemicals

Diethyl ether (purity ≥99.8%, CAS Number 60-29-7), ethanol (purity ≥96%, CAS Number 64-17-5), and phenolphthalein solution (indicator, 1% in ethanol, CAS Number 77-09-8) were supplied from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Sodium hydroxide 0.1 mol/L (N/10, CAS Number 1310-73-2) was purchased from Carlo Erba Reagents S.r.l. (Milan, Italy).

Distilled water was produced by Elix Essential system (Millipore, Molsheim, France). Oleic acid (assay 90%, CAS Number 112-80-1) used as standard for building the calibration curve was supplied from Sigma-Aldrich, Inc. (St. Louis, MO, USA).

4.2.2 Equipment and instrumentations

Usual laboratory glassware; analytical balance for weighing to an accuracy of ± 0.1 mg; magnetic stirrer; stir bar; 3 mL disposable pipettes (graduated in 0.5 mL); portable system for determination of free acidity and 50 mL tubes featuring a couple of stainless-steel electrodes (described below).

4.2.3 Samples

In order to calibrate the system, 5 samples of refined sunflower oil (with free acidity and peroxide value under the limits for vegetables oils according to CODEX STAN 210-1999) added with oleic acid were prepared. To cover the range of FA in the different quality grades (EVOO, VOO, LOO) different concentrations of oleic acid (from 0.25% to 3.75%) were added and properly mixed (Table 4.2).

Sample code	*Sample description*
A	Refined sunflower oil (without addition of oleic acid)
B	Refined sunflower oil + 0.25% oleic acid
C	Refined sunflower oil + 1.25% oleic acid
D	Refined sunflower oil + 2.50% oleic acid
E	Refined sunflower oil + 3.75% oleic acid

Table 4.2. Codes and description of the samples used for the calibration of the system.

The in-house validation study (except for LOD and LOQ) was carried out on a set of 30 olive oils with a different quality grade: 20 EVOOs, 7 VOOs and 3 LOOs.
An aliquot of 50 mL for each sample was stored in PET dark bottles at 11-12 °C before the analysis.

4.2.4 Determination of free acidity by official method

The determination of FA by titration was applied according to the EU Reg. 2016/1227. An aliquot of oil was dissolved in 100 mL of a mixture of diethyl ether and ethanol (1:1 v/v, previously neutralized) and the free fatty acids present were neutralized using sodium hydroxide solution (0.1 mol/L). Phenolphthalein (solution 1% in ethanol) was used as indicator.

4.2.5 Determination of free acidity by portable system

The working principle of the portable system (Figure 4.1) is based on the measure of the electrical conductance of an emulsion between a hydroalcoholic solution and the oil sample. In presence of the hydroalcoholic solution, the free fatty acid molecules RCOOH, where R is the hydrocarbon chain, dissociate in the ionic compounds H_3O^+ and RCO_2^- that contribute to the increase of electrical conductance in the emulsion. Consequently, the higher the concentration of free fatty acids, the higher ion concentration and the higher the electrical conductance.

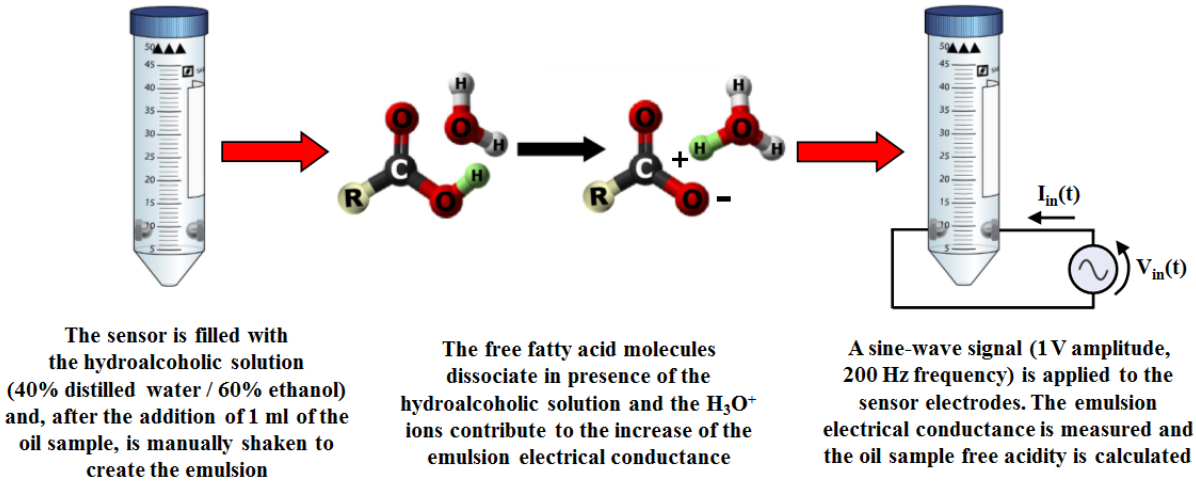

Figure 4.1. Scheme of the working principle of the proposed electronic system for the determination of free acidity in virgin olive oil.

A 50 mL round bottom polypropylene tube (Falcon) modified to feature a couple of cap-shaped stainless-steel electrodes (6 mm in diameter, spaced by 12 mm one from the other) required for the electrical characterization is used as a sensor to carry out the measurement.

As shown in Figure 4.2, the first step in making the measurement is a "reagent test" to check the electrical properties (electrical conductance) of the hydroalcoholic solution in order to avoid overestimation of the oil FA linked to the conductance of the solution. For this purpose, the tube (sensor) is filled with 9 mL of ethanol and 6 mL of distilled water and its conductance is measured. If the reagent passes the test and is suitable for the measure (conductance not higher than 0.6 µS), 1 mL of the oil sample to be tested is added using a plastic graduated Pasteur pipette and the obtained mixture is manually shaken vigorously for about 20 sec to create an emulsion, whose conductance is measured to estimate the oil acidity. In order to avoid error due to the eventual instability of the emulsion, the measurement should be made within 30 min after agitation.

The instrument is also equipped with a temperature sensor to measure environmental temperature during the analysis. The emulsion electrical conductance and room temperature are measured, and these values are used to calculate the emulsion conductance at the calibration temperature (23.5 °C). The free acidity of the sample is then estimated using the calibration function stored inside the non-volatile memory of the microcontroller.

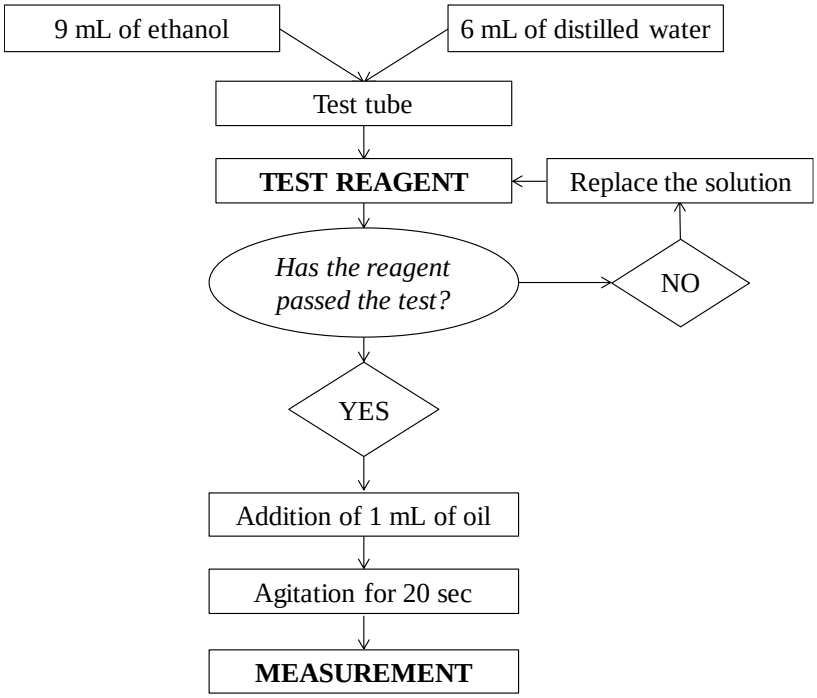

Figure 4.2. Scheme of the method for determination of free acidity using the portable system.

4.2.6 Calibration and in-house validation

After calibration of the system, the following parameters were evaluated in order to ensure the quality of the method: limit of detection (LOD) and quantification (LOQ), intra-day and inter-day precision, and accuracy.

Calibration

Before application of the portable system on olive oil samples and in order to verify the correlation between concentration of the analyte (oleic acid) and response of the instrument, calibration using the samples of refined sunflower oil added with oleic acid previously described (see paragraph 4.2.3) was carried out. All samples were analyzed in triplicate by both the official method, to determine the

reference value of FA, and with the portable system, to measure the conductance value. Next, the values were plotted to study the correlation between electrical conductance and FA of the samples.

Limit of detection (LOD) and quantification (LOQ)

Among the different formulas available for the evaluation of LOD and LOQ (González et al., 2007), the one that considers the standard deviation (σ) of the sample with the lowest acceptable concentration of the analyte and slope of the calibration function (m) was applied. The formulas used are:

$$LOD = 3\sigma/m \quad (1)$$

$$LOQ = 10\sigma/m \quad (2)$$

Accuracy and intra-day precision

Accuracy and intra-day precision were studied by analyzing the set of 30 olive oils of a different quality grade (EVOO, VOO, LOO). Precision was expressed in terms of RSD (Relative Standard Deviation) measured for the free acidity values obtained with the portable system. For accuracy, all the samples were also analyzed following the official method (acid-base titration) and the values obtained by the two methods were compared (One-way ANOVA, $p \leq 0.05$).

Inter-day precision

To evaluate the inter-day precision of the method, two oil samples for each quality grade (samples EVOO_2 and EVOO_19 for EVOOs, samples VOO_1 and VOO_6 for VOOs and samples LOO_1 and LOO_3 for LOOs) were analyzed in triplicate on three different days and the values were evaluated with Student's Test *($p \leq 0.05$)*.

4.3 Results and discussion

4.3.1 Design of the portable system

A portable battery-operated electronic system for in-situ measurements of olive oil FA was built to allow olive oil quality assurance directly at oil mills or packaging centers.

The dimensions of the instrument are 11 x 15 x 5 cm and some pictures are presented in Figure 4.3a.

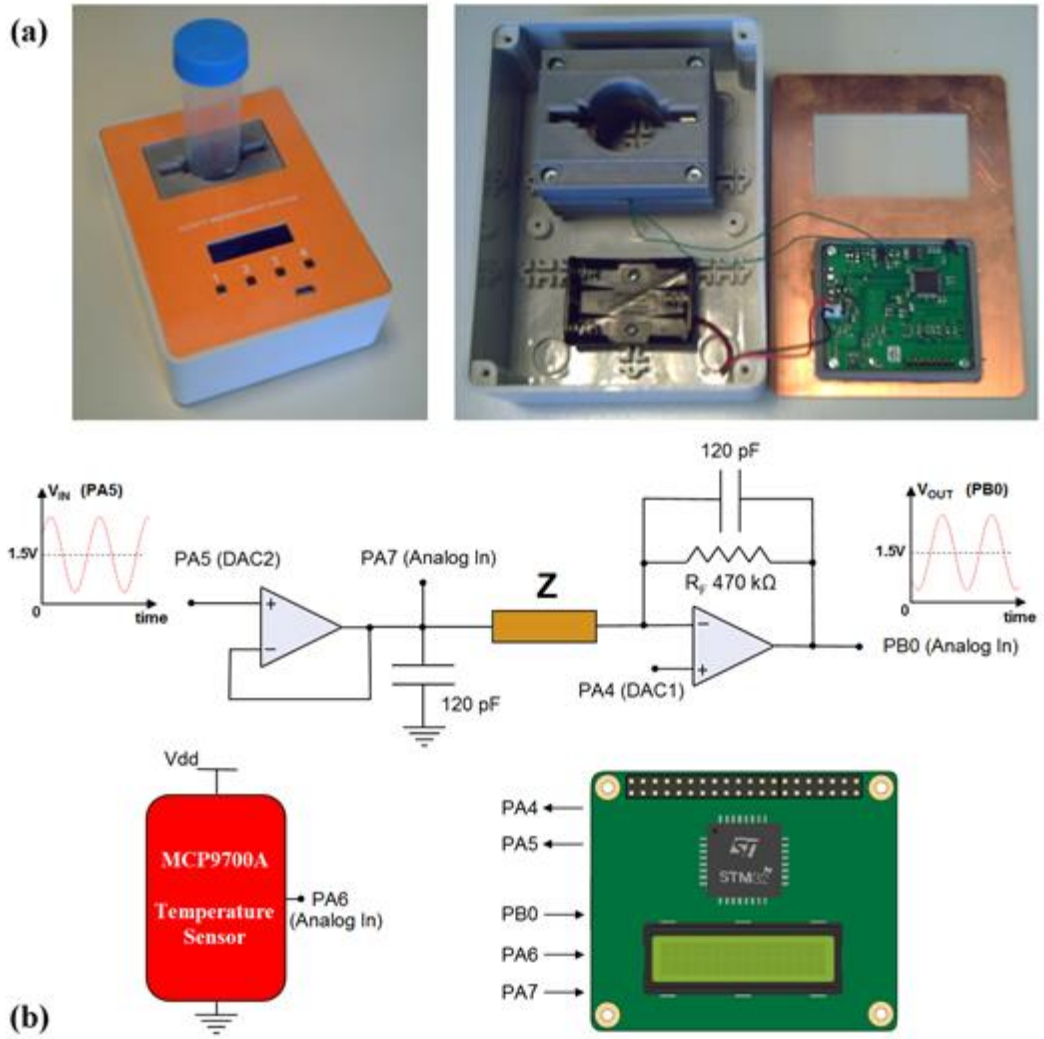

Figure 4.3. Pictures of the outside and inside of the electronic system and the sensor for olive oil free acidity analysis (a); system hardware and electrical scheme (b).

The instrument can be powered by USB port or using batteries (3 AAA alkaline batteries 1.5 V). The system is composed of an electronic board designed ad-hoc that performs all the operations to measure free acidity, a 2 rows 16 columns LCD screen to output the results, four buttons for user interaction and the sensor. The electronic board is based on the microcontroller STM32L152RCT6A and its scheme is presented in Figure 4.3b. The sensor used for the measurements is made of stainless steel electrodes, a material less affected by passivation than other materials. The same sensor can be used many times for the analysis since it can be easily washed with water and soap, dried with paper, and rinsed with distilled water. Before each analysis, the reagent test allows to confirm that no water/soap residues, which could affect conductance, remain in the sensor.

To determine the FA of an oil sample the electrical conductance of the emulsion is measured by Electrical Impedance Spectroscopy (EIS). In the proposed approach the sample under investigation

is stimulated with a 1 V 200 Hz sine-wave voltage signal $V_{in}(t)$ generated by the built-in 12-bits DAC inside the microcontroller and applied to the sensor electrodes.

The current drawn by the sensor is converted to a sine-wave voltage $V_{out}(t)$ by means of a current-to-voltage converter. Given

$$V_{in}(t) = V_{M,in} \times sen(2\pi ft) \quad (3)$$

it is

$$V_{out}(t) = V_{M,out} \times sen(2\pi ft + \varphi) \quad (4)$$

where $V_{M,in}$ and $V_{M,out}$ are the amplitude of the corresponding signals, φ is the phase difference between the current through the sample and $V_{in}(t)$, while f is the frequency of the test signal (200 Hz). Both $V_{in}(t)$ and $V_{out}(t)$ are acquired by the built-in 12-bits ADC inside the microcontroller using a sampling frequency of 50 kHz and the sine-wave parameters are calculated using the algorithm previously presented by Grossi et al. (Grossi et al., 2012b).

The emulsion in direct contact with the electrodes can be modeled as the parallel of an electrical conductance (accounting for the conductance of the emulsion) and a capacitance (accounting for the emulsion dielectric properties): while the emulsion conductance (that dominates at low frequency) is affected by the sample acidity due to the variation of the ions concentration, the dielectric properties are almost independent.

The electrical conductance (G_m) is thus calculated as:

$$G_m = \frac{1}{R_F} \times \frac{V_{M,out}}{V_{M,in}} \times cos(\varphi) \quad (5)$$

where R_F is the feedback resistance (470 kΩ) of the current-to-voltage converter.

However, the relation between G_m and free acidity is non-linear and can be modelled with the function:

$$G_m = \alpha + \beta \times \sqrt{FA} \quad (6)$$

where α, β are empirical parameters that must be determined by a suitable calibration procedure and are also function of the calibration temperature.

Next, free acidity can be estimated from the electrical conductance of the emulsion with the following formula:

$$FA = ((G_m - \alpha) / \beta)^2 \quad (7)$$

Since the system must be operated "in the field" and environmental temperature (T) is not a parameter that can be controlled, it is measured using a MCP9700A analog temperature sensor integrated on the electronic board: the output voltage (that has a sensitivity of 10 mV/°C) is acquired by a channel of the microcontroller ADC and converted to the temperature value.

The system works as follows: the emulsion electrical conductance registered at a certain temperature ($G_{m,T}$) and T are measured, and these values are used to calculate the emulsion conductance at the calibration temperature ($G_{m,Tcalib}$). The free acidity of the oil sample is estimated from the calculated value of $G_{m,Tcalib}$ using the calibration function stored inside the non-volatile memory of the microcontroller.

A more detailed description of the system from an electronical and mathematical point of view is presented in Appendix A (paragraph 4.7), while a discussion on the influence of the environmental temperature on the conductance measured and the method used for compensation is presented in Appendix B (paragraph 4.8).

4.3.2 In-house method validation

Calibration

Before being used with real olive oil samples, the portable instrument was calibrated. For this purpose, all the samples of refined sunflower oil added with oleic acid (samples A-E) were analyzed by both the official method to determine the reference value of free acidity and the portable system to measure the conductance value. The data obtained are shown in Table 4.3.

Sample code	**Free acidity** *(% oleic acid)*	**Conductance** *(µS)*
A	0.10 ± 0.01	0.79 ± 0.03
B	0.36 ± 0.03	1.58 ± 0.06
C	1.4 ± 0.04	3.36 ± 0.04
D	2.6 ± 0.05	4.35 ± 0.10
E	3.8 ± 0.04	5.40 ± 0.05

Table 4.3. Data (mean of three replicates) of free acidity determined by the official method and conductance measured with the portable system for the samples used to calibrate the system.
According to the EU Reg. 2016/1227, the FA values from 0 up to 1 (including 1) are reported with two decimal places and the FA values higher than 1 are reported with one decimal place.

The values were then plotted (Figure 4.4): in all cases the electrical conductance measured for the samples increased with free acidity with a non-linear relation, confirming what previously presented (Grossi et al., 2014b).

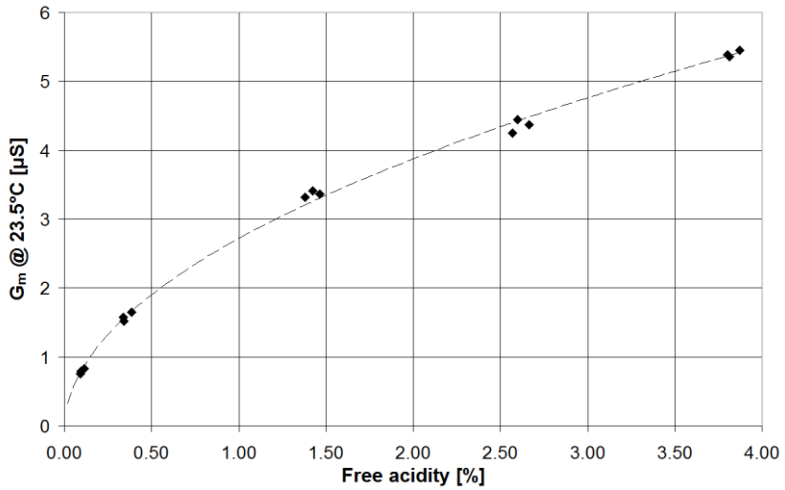

Figure 4.4. Measured electrical conductance (G_m) at 23.5°C vs. free acidity for the sunflower oil calibration set.

Starting from these data and applying a non-linear regression, the equation of the calibration curve was defined as follow:

$$FA = \left(\frac{G_{m,23.5°C} + 0.0678}{2.7877} \right)^2 \quad (8)$$

where $G_{m,23.5°C}$ is the emulsion electrical conductance at the calibration temperature of 23.5 °C.
It allows obtaining, directly on the display of the portable system, the free acidity value of the sample estimated starting from its electrical properties. Since this procedure is necessary to define the relation between the oil FA and the conductance, and, consequently, the mathematical function that has to be stored in the non-volatile memory of the microcontroller of the instrument, it needs to be realized only when the instrument is built.

Limit of detection (LOD) and quantification (LOQ)

LOD and LOQ were measured applying formulas (1) and (2). Since the relation between the conductance and the FA of the sample is non-linear, and to simplify the LOD and LOQ calculation, the linear correlation between the free acidity square root and conductance is considered. In this way, σ is the standard deviation of the square root of free acidity measured for the refined sunflower oil without addition of oleic acid (sample A) and m is the slope of the curve. The values obtained were 0.02% and 0.07% of oleic acid for LOD and LOQ, respectively.

Accuracy and intra-day precision

The set of 30 olive oil samples with a different quality grade (20 EVOOs; 7 VOOs; 3 LOOs) was analyzed in triplicate using this new approach to study its accuracy and intra-day precision (expressed in terms of RSD).

The portable instrument showed good precision since all the values obtained were under 15%, which is considered as acceptable for the validation of a new method (Peters et al., 2007). All samples were also analyzed using the official method (acid-base titration) and the values obtained by the two methods were compared to measure the accuracy of the system (Table 4.4). The differences between the two series of results (official method vs. portable system) were evaluated by One-way ANOVA ($p \leq 0.05$). The two approaches did not give statistically significant differences.

Sample code	Free acidity (portable system)	Free acidity (official method)
EVOO_1	0.18 ± 0.01	0.25 ± 0.01
EVOO_2	0.31 ± 0.03	0.34 ± 0.02
EVOO_3	0.25 ± 0.01	0.28 ± 0.01
EVOO_4	0.43 ± 0.01	0.27 ± 0.01
EVOO_5	0.30 ± 0.02	0.25 ± 0.01
EVOO_6	0.22 ± 0.01	0.25 ± 0.01
EVOO_7	0.18 ± 0.02	0.25 ± 0.01
EVOO_8	0.29 ± 0.04	0.37 ± 0.02
EVOO_9	0.29 ± 0.01	0.34 ± 0.01
EVOO_10	0.47 ± 0.01	0.27 ± 0.01
EVOO_11	0.32 ± 0.02	0.33 ± 0.01
EVOO_12	0.41 ± 0.02	0.42 ± 0.02
EVOO_13	0.24 ± 0.02	0.28 ± 0.01
EVOO_14	0.27 ± 0.03	0.28 ± 0.01
EVOO_15	0.32 ± 0.02	0.44 ± 0.02
EVOO_16	0.35 ± 0.02	0.35 ± 0.01
EVOO_17	0.18 ± 0.02	0.28 ± 0.01
EVOO_18	0.15 ± 0.01	0.22 ± 0.01
EVOO_19	0.27 ± 0.02	0.39 ± 0.02
EVOO_20	0.24 ± 0.00	0.31 ± 0.01
VOO_1	1.3 ± 0.09	1.4 ± 0.02
VOO_2	1.3 ± 0.02	1.4 ± 0.03
VOO_3	0.88 ± 0.07	1.1 ± 0.01
VOO_4	1.1 ± 0.05	1.3 ± 0.00
VOO_5	1.2 ± 0.05	0.99 ± 0.00
VOO_6	1.8 ± 0.12	1.9 ± 0.01
VOO_7	1.9 ± 0.07	2.8 ± 0.02
LOO_1	2.4 ± 0.32	2.6 ± 0.00
LOO_2	4.4 ± 0.04	6.6 ± 0.05
LOO_3	4.5 ± 0.17	5.7 ± 0.01

Table 4.4 Values of free acidity (mean and standard deviation) for all samples measured by the portable system and the official method. Results are expressed as % of oleic acid. According to the EU Reg. 2016/1227, the FA values from 0 up to 1 (including 1) are reported with two decimal places and the FA values higher than 1 are reported with one decimal place.

Moreover, the regression between the two series of values provided a coefficient R^2 of 0.97 (Figure 4.5) in agreement with data previously presented by Grossi et al. (Grossi et al., 2014). Considering the commercial categories of the samples analyzed, all of them, with the only exception of sample VOO_7, were classified in the same way by both approaches.

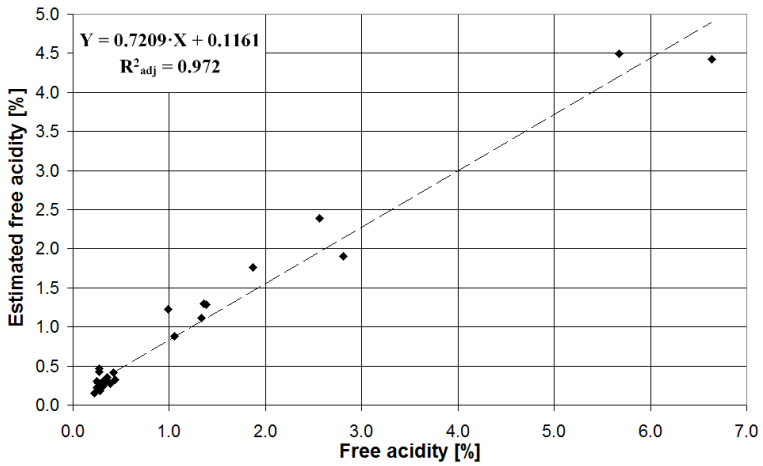

Figure 4.5 Scatter plot of the estimated free acidity vs. the free acidity measured by titration for a set of 30 olive oil samples.

Inter-day precision

Considering the inter-day precision of the instrument (Table 4.5), no significant differences (Student's Test, $p \leq 0.05$) were found among the results obtained for each sample on the three different days.

Sample	Day 1	Day 2	Day 3
EVOO_2	0.31 ± 0.03	0.34 ± 0.01	0.32 ± 0.02
EVOO_19	0.27 ± 0.02	0.25 ± 0.01	0.27 ± 0.03
VOO_1	1.3 ± 0.09	1.3 ± 0.02	1.3 ± 0.01
VOO_6	1.8 ± 0.12	1.8 ± 0.08	1.8 ± 0.05
LOO_1	2.4 ± 0.32	2.5 ± 0.18	2.5 ± 0.11
LOO_3	4.5 ± 0.17	4.6 ± 0.25	4.4 ± 0.04

Table 4.5 Values of free acidity (mean and standard deviation) measured in three different days to evaluate the inter-day precision. EVOO_2 and EVOO_19: extra virgin olive oils; VOO_1 and VOO_6: virgin olive oils; LOO_1 and LOO_3: lampante olive oils. According to the EU Reg. 2016/1227, the FA values from 0 up to 1 (including 1) are reported with two decimal places and the FA values higher than 1 are reported with one decimal place.

4.4 Conclusions

The design and in-house validation of a portable battery-operated electronic system suitable for in-situ measurements of olive oil free acidity is presented. The system is built with low cost electronics and embeds a temperature sensor to compensate for variations in electrical parameters with environmental temperature, thus making it suitable for on-site free acidity measurements outside a laboratory. Its working principle is based on the estimation of the olive oil FA by measuring the conductance of an emulsion between a hydro-alcoholic solution and the sample to be tested. When the free fatty acids present in the sample come into contact with the hydroalcoholic solution, dissociation occurs, leading to the formation of ions that produce an increase in electrical conductance.

The system has been calibrated and in-house validated. The data obtained showed good performances of the instrument in terms of LOD and LOQ, intra-day and inter-day precision. Moreover, it showed good correlation (R^2= 0.97) with the FA values obtained using the official method, thus demonstrating satisfactory accuracy.

The procedure is very quick and easy. This makes the system suitable for people without specific training. The application of this analytical device is addressed, in particular, to estimation of free acidity of newly produced VOOs in oil mills.

4.5 Appendix A

According to the working principle of the portable system, a 50 mL polypropylene tube (Falcon) modified to feature a couple of stainless-steel electrodes to measure the emulsion conductance (hereafter the sensor) is filled with 15 mL of hydro-alcoholic solution (40% distilled water/60% ethanol), then 1 mL of the olive oil sample is added and all is stirred to create an emulsion.

In presence of the hydroalcoholic solution, the free fatty acid molecule RCOOH, where R is the hydrocarbon chain, dissociates in the ionic compounds H_3O^+ and RCO_2^- that contribute to the increase of the emulsion electrical conductance. In the end, the higher the free fatty acid molecules concentration, the higher ions concentration and the higher the electrical conductance.

The emulsion electrical conductance is measured by Electrical Impedance Spectroscopy (EIS).

In the proposed approach the sample under investigation is stimulated with a sine-wave voltage signal $V_{in}(t)$:

$$V_{in}(t) = V_{M,in} \times sin\ (2\pi ft) \quad \text{(A1)}$$

and the current $I_{in}(t)$ through the sample is measured:

$$I_{in}(t) = I_{M,in} \times sin\ (2\pi ft + \varphi) \quad \text{(A2)}$$

where $V_{M,in}$ and $I_{M,in}$ are the amplitudes of the corresponding signals, f is the frequency of the test signal and φ is the phase difference between $I_{in}(t)$ and $V_{in}(t)$.

The sample electrical admittance is then expressed as:

$$Y = \frac{I_{in}(j2\pi f)}{V_{in}(j2\pi f)} = \frac{I_{M,in}}{V_{M,in}} \times (cos\ \varphi + j \times sin\ \varphi) = Re(Y) + j \times Im(Y) \quad \text{(A3)}$$

The emulsion in direct contact with the electrodes can be modeled as the parallel of an electrical conductance (accounting for the conductance of the emulsion) and a capacitance (accounting for the emulsion dielectric properties): while the emulsion conductance (that dominates at low frequency) is affected by the sample acidity due to the variation of the ions concentration, the dielectric properties are almost independent. Thus, the emulsion electrical conductance G_m can be estimated with the real component of the emulsion admittance $Re(Y) = |Y| \cdot cos(\varphi)$ where $|Y|$ is the admittance modulus.

In Figure A1-a the admittance modulus (measured with the commercial LCR meter Agilent E4980A) is plotted vs. the frequency of the applied test signal for different samples featuring different acidity in the frequency range 20 Hz – 2 MHz. As can be seen, $|Y|$ increases with sample acidity for test signals with a frequency lower than 20 kHz, while it is almost independent on sample acidity for higher frequencies. This is the reason why the designed electronic system measures G_m with a single frequency measurement at 200 Hz.

The relation between G_m and the free acidity is non-linear and it can be modelled with the function:

$$G_m = \alpha + \beta \times \sqrt{FA} + \gamma \times \sqrt[4]{FA^3} \quad \text{(A4)}$$

where α, β and γ are empirical parameters that must be determined by a suitable calibration procedure and are also function of the calibration temperature.

In Figure A1-b the qualitative plot of G_m vs. FA is shown: as can be seen, the non-linear function results in better accuracy for the estimated free acidity for lower acidity levels. This has been taken into account by using a suitable amount of sample to create the emulsion to obtain good accuracy in the acidity range of interest.

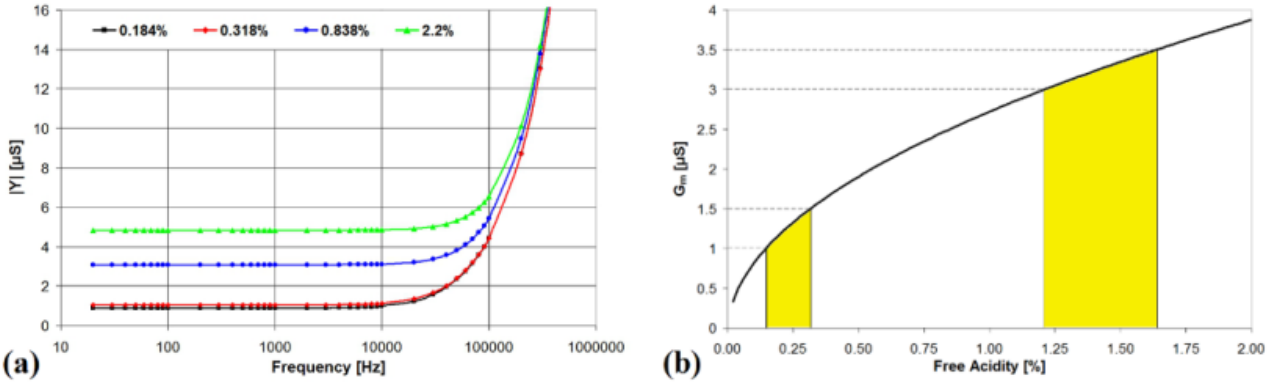

Figure A1 (a) admittance modulus plotted vs. frequency for olive oil samples featuring different free acidity; (b) qualitative plot of the electrical conductance as function of sample acidity.
(permission for Figure A1a obtained from Grossi et al., 2014. Microelectronics Journal, 45, 1701-1707)

However, eq. A4 needs a computation intensive iterative algorithm to extract the estimated acidity from the measured value of G_m as well as manual input of starting point to avoid failing in algorithm convergence. Thus, a simpler model has been chosen (obtained by neglecting the molar conductivity dependence on the H_3O^+ ions concentration) that is much more suitable to be implemented in a low-cost microcontroller and can be described by the following function:

$$G_m = \alpha + \beta \times \sqrt{FA} \quad \text{(A5)}$$

The two models of eq. A4 and A5 have been tested with the data set of Grossi et al. (Grossi et al., 2014b) and the results have shown how the accuracy in estimating free acidity is only marginal higher for model A4 than model A5.

The sample free acidity can thus be estimated from the measured electrical conductance of the emulsion with the following formula:

$$FA = \left(\frac{G_m - \alpha}{\beta}\right)^2 \quad \text{(A6)}$$

4.6 Appendix B

The oil sample free acidity can be estimated by measuring the emulsion electrical conductance at the temperature of calibration and then calculating the FA using equation A6. However, since the system must be operated "in the field" and the environmental temperature is not a parameter under control, there is the need to investigate how the emulsion conductance varies with the temperature so that the oil free acidity can be estimated by the measure of the emulsion conductance and the temperature.

Four different olive oil samples featuring different free acidity values (0.06% sample A, 0.37% sample B, 0.81% sample C and 3.7% sample D) were tested inside a Binder APT KB 53 thermal incubator for different temperatures between 15°C and 35°C.

In Figure B1a the measured emulsion conductance is plotted vs. the incubation temperature for each sample. In all cases the G_m is a linear function of the temperature with determination coefficients R^2 >0.99. The calculated linear regression lines allow to determine the conductance variation with temperature (i.e. $\partial G_m/\partial T$) for all samples: 0.0196 for sample A, 0.0495 for sample B, 0.0579 for sample C and 0.1417 for sample D. $\partial G_m/\partial T$ is thus found to increase with the sample free acidity. Since the sample FA is also a function of the emulsion electrical conductance, the relation between $\partial G_m/\partial T$ and $G_{m,23.5°C}$ is plotted in Figure B1b.

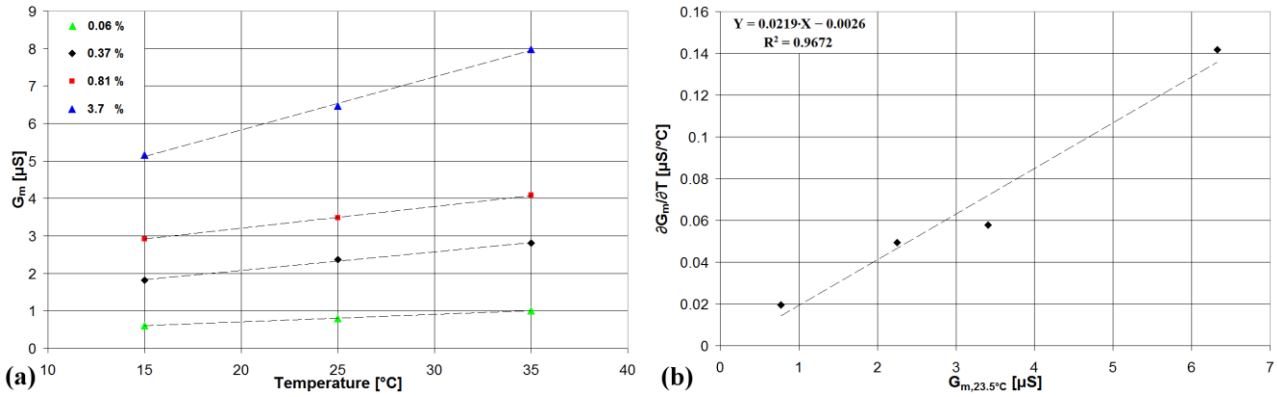

Figure B1 (a) measured emulsion conductance vs the incubation temperature for olive oil samples featuring different free acidity; (b) $\partial G_m/\partial T$ plotted vs. the electrical conductance at 23.5 °C.

As can be seen, a linear relation gives a good approximation (R^2 = 0.967) of the function between $\partial G_m/\partial T$ and $G_{m,23.5°C}$ thus:

$$\frac{\partial G_m}{\partial T} = 0.0219 \times G_{m,23.5°C} - 0.0026 \qquad \text{(B1)}$$

where $\partial G_m/\partial T$ and $G_{m,23.5°C}$ are expressed as µS/°C and µS, respectively. The emulsion electrical conductance at the environmental temperature T can thus be expressed as:

$$G_{m,T} = G_{m,23.5°C} + \frac{\partial G_m}{\partial T} \times (T\text{-}23.5) \quad \text{(B2)}$$

and

$$G_{m,T} = G_{m,23.5°C} + \left(0.0219 \times G_{m,23.5°C}\text{-}0.0026\right) \times (T\text{-}23.5) \quad \text{(B3)}$$

The emulsion electrical conductance at $T_{calib} = 23.5°C$ can be estimated from the electrical conductance measured at temperature T and the value of T using the following formula:

$$G_{m,23.5°C} = \frac{G_{m,T}+0.0026 \times (T\text{-}23.5)}{1+0.0219 \times (T\text{-}23.5)} \quad \text{(B4)}$$

Thus, by measuring $G_{m,T}$ and T, the value of $G_{m,23.5°C}$ can be calculated using equation B4 and, from this value, the sample acidity can be estimated using equation A6.

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Chapter 5

Discrimination of virgin olive oils with different geographical origin: a rapid untargeted chromatographic approach based on volatile compounds

5.0 Details of the publication based on Chapter 5

Title: Discrimination of virgin olive oils with different geographical origin: a rapid untargeted chromatographic approach based on volatile compounds

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The paper will soon be submitted to: LWT – Food Science and Technology

Abstract

Many studies have shown that the geographic origin is one of the most influencing factors in consumers' choice of olive oil. To avoid misleading, European regulation has established specific rules to report the geographical origin of extra virgin (EVOOs) and virgin olive oils (VOOs) on the product label, but an official analytical procedure to verify this information has not been yet defined. In this work, a flash gas chromatography untargeted approach for determination of volatile compounds, followed by a chemometric data elaboration, is proposed for discrimination of EVOOs and VOOs according to their geographical origin (EU and Extra-EU). A set of 210 samples was analyzed and two different classification techniques were used, one linear (Partial Least Square-Discriminant Analysis, PLS-DA) and one non-linear (Artificial Neural Network, ANN). The two models were also validated using an external data set. Satisfactory results were obtained for both chemometric approaches: considering the PLS-DA, 89% and 81% of EU and Extra-EU samples, respectively, were correctly classified; for ANN the percentages were 93% and 89%, respectively. These results confirm the reliability of the method as a rapid approach to discriminate EVOOs and VOOs according to their geographical provenance.

Key words: Virgin olive oil; Geographical origin; PLS-DA; Untargeted approach; Volatile compounds; ANN.

5.1 Introduction

Over the last 40 years many investigations have been focused on understanding what attributes are important determinants in consumers' choice, which have highlighted that the geographic origin is one of the most influencing factors for olive oil (Dekhili et al., 2011; Del Giudice et al., 2015).
In order to ensure that consumers are not misled, the fourth article of the EU Reg. 29/2012 establishes that *"Extra virgin and virgin olive oil shall bear a designation of origin on the labelling"*. This means that for extra virgin (EVOOs) and virgin olive oils (VOOs) commercialized within the EU, it is mandatory to specify the geographical provenance on the label of the product following specific rules. If an oil comes from an EU Member State or third country, a reference to the EU Member State, to the EU, or to the third country must be reported. In the case of blends of oils originating from more than one EU Member State or third country, one of the following mentions must be used: 'blend of olive oils of European Union origin' or a reference to the EU; 'blend of olive oils not of European Union origin' or a reference to origin outside the EU; 'blend of olive oils of European Union origin and not of European Union origin' or a reference to origin within the EU and outside the EU. An exception is the case where the olives were harvested in an EU Member State or third country other than that in which the mill where the oil was extracted is located. In this case, the designation of origin shall contain the following wording: *'(extra) virgin olive oil obtained in (the Union or the name of the Member State concerned) from olives harvested in (the Union or the name of the Member State or third country concerned)'*.
However, the regulation does not specify an official analytical procedure to verify the conformity of the label-declared geographical origin, and this has raised the interest of researchers to develop a reliable and effective method for purposes of authentication (Conte et al., 2019). During the last years, different analytical techniques have been applied in order to find potentially useful markers and efficient instrumental approaches that are able to discriminate olive oils according to their geographical origin.
In this regard, traditional chromatographic techniques, analyzing both major and minor compounds either individually or in a combined way, coupled or not with specific statistical chemometric data elaboration, have been investigated. A study in 2009 (García-González et al., 2009) proposed the application of artificial neural network (ANN) models for different levels of geographical classification (country, region, province, PDO) on a set of 687 EVOOs and VOOs from Spain, Italy, and Portugal, which were chemically characterized for the content of fatty acids, hydrocarbons, sterols, and alcohols. Other researchers evaluated the triacylglycerol (TAG) content and composition to discriminate Moroccan oils (Bajoub et al., 2016) and Croatian samples (Peršurić et al., 2018). In

addition, the stereospecific distribution of fatty acids in TAGs was reported to be useful in discriminating olive oils from different areas of North-Eastern Italy (Vichi et al., 2007). Specific metabolites such as sterols and phenolic compounds have been investigated to identify the optimal markers, and may be a promising approach to discriminate oils according to geographical origin (Giacalone et al., 2015; Ben Mohamed et al., 2018; Ghisoni et al., 2019). Interesting findings have also been recently reported on sesquiterpene hydrocarbons as geographical markers (Quintanilla-Casas et al., 2018). Moreover, volatile compounds have been amply studied by applying different instrumental techniques combined with chemometric data elaborations (Kosma et al., 2017; Bajoub et al., 2018; Lukić et al., 2019).

Furthermore, rapid and innovative instrumental approaches have been developed and tested in order to deal with the need for simple, rapid, and environmentally friendly techniques (Valli et al., 2016) (Table 5.1).

In addition to these approaches, other promising techniques include stable isotopes analysis (Angerosa et al., 1999; Chiocchini et al., 2016; Bontempo et al., 2019), multi-element fingerprint (Sayago et al., 2018), differential scanning calorimetry (Mallamace et al., 2017), and GC-IMS (Gerhardt et al., 2017).

Melucci and co-workers (Melucci et al., 2016) proposed the application of a Flash Gas Chromatography Electronic Nose (Heracles II) and a multivariate data analysis to control the compliance of information on geographic origin declared in the label ("100% Italian" vs. "non-100% Italian") for the first time. This instrumental approach allows to realize the headspace analysis in short time and the results are processed by chemometric tools following an untargeted approach. For this reason, it can be considered as a fingerprint method, since the data can be elaborated for sample classification that is not aimed towards identification and quantification of specific analytes. Following these preliminary results and the actual need for a rapid and effective method for geographical authentication of VOOs, the aim of this work was the application of flash gas chromatography (Heracles II) for rapid discrimination of 210 EVOOs and VOOs according to geographical provenance. In this case, the categories considered for samples classification were EU member states vs. third countries, and the data obtained were elaborated by applying two different classification techniques, one linear (Partial Least Square-Discriminant Analysis, PLS-DA) and one non-linear (ANN). Finally, also technical repeatability and sensitivity of the method were evaluated as validation parameters to verify that a repeatable and reproducible signal, with enough sensitivity to collect the valuable information from the samples, was obtained.

Technique	***Statistics***	***Reference***
UV-VIS	Classification (LDA-SIMCA)	Casale et al., 2007
	Classification (LDA-PLS-DA)	Pizarro et al., 2013
UV-VIS-NIR	Classification (PLS-FDA-k-NN)	Downey et al., 2003
	Classification (SIMCA-UNEQ-QDA)	Casale et al., 2010
NIR	Classification (ANN-LR)	Bertran et al., 2000
	Classification (PLS-DA)	Galtier et al., 2007
	Classification (PLS-DA)	Woodcock et al., 2008
	Classification (SIMCA)	Laroussi-Mezghani et al., 2015
	Classification (PLS-DA)	Jiménez-Carvelo et al., 2019
NIR-MIR	Classification (LDA-PLS-DA-SIMCA)	Sinelli et al., 2008
MIR	Classification (CART-SVM)	Caetano et al., 2007
	Classification (FDA-PLS-DA)	Hennessy et al., 2009
	Classification (PLS-DA)	De Luca et al., 2011
	Classification (PLS-DA-SIMCA)	Bevilacqua et al., 2012
Terahertz spectroscopy	Classification (LS-SVM)	Liu et al., 2018
Hyperspectral imaging	Discrimination (CM)	Mignani et al., 2007
Raman	Classification (PLS-DA)	Korifi et al., 2011
	Classification (DF)	Sánchez-López et al., 2016
E-nose	Classification (ANN)	Cosio et al., 2006
	Classification (LDA)	Haddi et al., 2011
E-tongue	Classification (LDA)	Dias et al., 2014
	Classification (LDA-SA)	Souayah et al., 2017
E-nose + E-tongue	Classification (SVM)	Haddi et al., 2013
PTR-MS	Classification (PLS-DA)	Araghipour et al., 2008

Table 5.1. Rapid and innovative techniques, and relative references, developed and tested for the verification of the geographical origin of EVOOs and VOOs.

5.2 Materials and methods

5.2.1 Reagents and chemicals

Ethanol (assay ≥99%, CAS Number 64-17-5), hexanal (assay ≥95%, CAS Number 66-25-1), and (*E*)-2-Hexen-1-al (assay ≥97%, CAS Number 6728-26-3) were supplied from Sigma-Aldrich, Inc. (St. Louis, MO, USA).

5.2.2 Equipment and instrumentations

Usual laboratory glassware; analytical balance for weighing to an accuracy of ± 0.1 mg; Flash Gas Chromatography E-Nose Heracles II (Alpha MOS, Toulouse, France) equipped with two metal capillary columns (MXT-5: 5% diphenyl, 95% methylpolysiloxane, and MXT-1701: 14% cyanopropylphenyl, 86% methylpolysiloxane, for both columns: 10 m length, 180 µm internal diameter, 0.4 µm film thickness).

5.2.3 Samples

A total of 210 EVOOs and VOOs with different geographical origin were collected directly from companies that were also asked to provide, when available, information about location of the mill, type of plant used, olive variety, and commercial category (Table 5.2). Considering that the indication of the geographical origin on the product label is mandatory for EVOOs and VOOs, samples belonging to both these two categories were included in this study.

Code	*Origin class*	*Country of origin*	*Olive variety*	*Commercial category*
1	Blend EU	GRC - ITA	Manaki - Coratina	EVOO [b]
2	Blend EU	GRC - ITA	Manaki - Moraiolo	EVOO [b]
3	Blend EU	GRC - ITA	Manaki - NA	EVOO [b]
4	Blend EU	GRC - ITA - ESP	Manaki - Arbequina - NA	EVOO [b]
5	Blend EU	GRC - ITA - ESP	Manaki - Arbequina - Coratina	EVOO [b]
6	Blend EU	GRC - ITA - ESP	Manaki - Arbequina - Moraiolo	EVOO [b]
7	Blend EU	GRC - ESP	Manaki - Arbequina	EVOO [b]
8	Blend EU	GRC - ESP	Koroneiki - Arbequina - Picual - Cornicabra	EVOO [a]
9	Blend EU	ESP - ITA	Arbequina - NA	EVOO [b]
10	Blend EU	ESP - ITA	Arbequina - Coratina	EVOO [b]
11	Blend EU	ESP - ITA	Arbequina - Moraiolo	EVOO [b]
12	Blend EU	ESP - PRT	Arbequina - Arbosana	EVOO [b]
13	Blend Extra-EU	GRC - TUN	Manaki - Sahli	VOO [b]
14	Blend Extra-EU	ITA - TUN	NA - Sahli	VOO [b]
15	Blend Extra-EU	ITA - TUN	Sahli - Coratina	VOO [b]
16	Blend Extra-EU	ITA - TUN	Sahli - Moraiolo	VOO [b]
17	Blend Extra-EU	ESP - MAR	Picual - Moroccan Picholine	EVOO [b]

18	Blend Extra-EU	ESP - MAR	Picual - Languedoc Picholine	EVOO [b]
19	Blend Extra-EU	ESP - MAR	Picual - Moroccan Picholine - Koroneiki	EVOO [b]
20	Blend Extra-EU	ESP - TUN	Arbequina - Sahli	VOO [b]
21	Blend Extra-EU	ESP - TUN	Picual - Chemlali - Chetoui	EVOO [b]
22	Blend Extra-EU	ESP - TUN	Picual - Chemlali	EVOO [b]
23	Blend Extra-EU	ESP - TUN	Picual - Chemlali	EVOO [b]
24	Blend Extra-EU	ESP - TUN	Picual - Chetoui	EVOO [b]
25	EU	HRV	Picholine	EVOO [a]
26	EU	HRV	Leccio del Corno	EVOO [a]
27	EU	HRV	Istarska Bjelica	EVOO [a]
28	EU	HRV	Rosinjola	EVOO [a]
29	EU	HRV	Leccino - Pendolino	EVOO [a]
30	EU	HRV	Leccino - Pendolino	EVOO [a]
31	EU	HRV	Picholine - Leccio del Corno	EVOO [a]
32	EU	HRV	Istarska Bjelica	EVOO [a]
33	EU	HRV	Oblica	EVOO [a]
34	EU	HRV	Istarska Bjelica - Leccino - Buža	EVOO [a]
35	EU	HRV	Istarska Bjelica - Leccino - Buža	EVOO [a]
36	EU	HRV	Leccino - Pendolino	EVOO [a]
37	EU	HRV	Picholine - Leccio del Corno	EVOO [a]
38	EU	HRV	Ascolana Tenera - Itrana - Frantoio	EVOO [a]
39	EU	HRV	Buža Puntoža - Rosinjola - Bova	EVOO [a]
40	EU	HRV	Istarska Bjelica	EVOO [a]
41	EU	HRV	Ascolana Tenera - Itrana - Frantoio	EVOO [a]
42	EU	HRV	Buža Puntoža	EVOO [a]
43	EU	HRV	Buža Puntoža	EVOO [a]
44	EU	HRV	Picholine	EVOO [a]
45	EU	HRV	Plominka - Simjaca	EVOO [a]
46	EU	HRV	Oblica	EVOO [a]
47	EU	GRC	Koroneiki	VOO [a]
48	EU	GRC	Koroneiki	EVOO [a]
49	EU	GRC	Koroneiki	EVOO [b]
50	EU	GRC	Manaki	EVOO [b]
51	EU	GRC	Koroneiki	EVOO [b]
52	EU	GRC	Koroneiki	EVOO [b]

53	EU	GRC	Manaki	EVOO [a]
54	EU	GRC	Koroneiki	EVOO [a]
55	EU	GRC	Koroneiki	EVOO [a]
56	EU	GRC	NA	VOO [a]
57	EU	GRC	Koroneiki	EVOO [a]
58	EU	GRC	Koroneiki	EVOO [a]
59	EU	GRC	Koroneiki	EVOO [b]
60	EU	GRC	Koroneiki	EVOO [a]
61	EU	GRC	Koroneiki	EVOO [a]
62	EU	GRC	NA	EVOO [a]
63	EU	ITA	Coratina	EVOO [a]
64	EU	ITA	Coratina	VOO [a]
65	EU	ITA	Frantoio	EVOO [a]
66	EU	ITA	Castiglionese	VOO [a]
67	EU	ITA	Leccino - Frantoio - Pendolino	EVOO [a]
68	EU	ITA	Leccino - Frantoio - Pendolino	VOO [a]
69	EU	ITA	Arbequina	EVOO [a]
70	EU	ITA	Coratina - Ogliarola	EVOO [a]
71	EU	ITA	Nocellara del Belice	EVOO [a]
72	EU	ITA	Biancolilla	EVOO [a]
73	EU	ITA	Nocellara del Belice	EVOO [a]
74	EU	ITA	Leccino - Frantoio - Moraiolo	EVOO [a]
75	EU	ITA	Coratina	EVOO [a]
76	EU	ITA	Coratina	VOO [a]
77	EU	ITA	Nostrana di Brisighella	EVOO [a]
78	EU	ITA	Leccino - Frantoio - Moraiolo	EVOO [a]
79	EU	ITA	Nostrana di Brisighella	EVOO [a]
80	EU	ITA	NA	EVOO [a]
81	EU	ITA	Coratina	EVOO [a]
82	EU	ITA	Moraiolo	EVOO [a]
83	EU	ITA	Carolea	EVOO [a]
84	EU	ITA	Dritta - Leccino	EVOO [a]
85	EU	ITA	Frantoio	EVOO [a]
86	EU	ITA	Peranzana	EVOO [a]
87	EU	ITA	Peranzana	EVOO [a]

88	EU	PRT	Arbequina - Koroneiki	EVOO [b]
89	EU	PRT	Arbosana	EVOO [b]
90	EU	PRT	Arbosana	EVOO [b]
91	EU	PRT	Arbosana	EVOO [b]
92	EU	PRT	Koroneiki	EVOO [b]
93	EU	PRT	Arbequina	EVOO [b]
94	EU	PRT	Arbequina	EVOO [b]
95	EU	PRT	Arbequina	EVOO [b]
96	EU	PRT	Sikitita	EVOO [b]
97	EU	PRT	Arbosana	EVOO [b]
98	EU	PRT	NA	EVOO [b]
99	EU	PRT	NA	EVOO [b]
100	EU	SVN	Istarska Bjelica - Leccino - Others	EVOO [a]
101	EU	SVN	Istarska Bjelica - Leccino - Others	EVOO [a]
102	EU	SVN	Istarska Bjelica - Leccino - Others	EVOO [a]
103	EU	SVN	Istarska Bjelica - Leccino - Others	EVOO [a]
104	EU	SVN	Istarska Bjelica - Leccino - Others	EVOO [a]
105	EU	SVN	Istarska Bjelica - Leccino - Maurino	EVOO [a]
106	EU	SVN	Istarska Bjelica - Leccino - Maurino	EVOO [a]
107	EU	SVN	Istarska Bjelica	EVOO [a]
108	EU	SVN	Istarska Bjelica - Leccino - Others	EVOO [a]
109	EU	SVN	Istarska Bjelica	EVOO [a]
110	EU	SVN	Istarska Bjelica - Leccino - Others	EVOO [a]
111	EU	SVN	Istarska Bjelica - Leccino - Others	EVOO [a]
112	EU	ESP	NA	EVOO [a]
113	EU	ESP	NA	VOO [a]
114	EU	ESP	NA	VOO [a]
115	EU	ESP	NA	VOO [a]
116	EU	ESP	NA	EVOO [a]
117	EU	ESP	NA	VOO [a]
118	EU	ESP	NA	EVOO [a]
119	EU	ESP	NA	VOO [a]
120	EU	ESP	NA	VOO [a]
121	EU	ESP	NA	EVOO [a]
122	EU	ESP	Hojiblanca	EVOO [a]

123	EU	ESP	Arbequina	EVOO [a]
124	EU	ESP	Picual	EVOO [a]
125	EU	ESP	Arbequina - Hojiblanca	EVOO [a]
126	EU	ESP	Arbequina - Hojiblanca	VOO [a]
127	EU	ESP	Manzanilla	EVOO [a]
128	EU	ESP	Manzanilla	EVOO [a]
129	EU	ESP	Arbequina	EVOO [a]
130	EU	ESP	Hojiblanca	EVOO [a]
131	EU	ESP	Koroneiki	EVOO [b]
132	EU	ESP	Hojiblanca	EVOO [b]
133	EU	ESP	Manzanilla - Hojiblanca - Picual	EVOO [a]
134	EU	ESP	NA	EVOO [a]
135	EU	ESP	NA	VOO [a]
136	EU	ESP	Hojiblanca	EVOO [a]
137	EU	ESP	Hojiblanca	EVOO [a]
138	EU	ESP	Picual	VOO [a]
139	EU	ESP	NA	EVOO [a]
140	EU	ESP	Arbequina	EVOO [a]
141	Extra-EU	CHL	NA	EVOO [a]
142	Extra-EU	MAR	Arbequina	EVOO [a]
143	Extra-EU	MAR	Arbequina	EVOO [b]
144	Extra-EU	MAR	Koroneiki	EVOO [b]
145	Extra-EU	MAR	Arbosana	EVOO [b]
146	Extra-EU	MAR	Arbequina	EVOO [b]
147	Extra-EU	MAR	Arbequina	EVOO [b]
148	Extra-EU	MAR	Moroccan Picholine	EVOO [b]
149	Extra-EU	MAR	Arbosana	EVOO [b]
150	Extra-EU	MAR	Koroneiki	EVOO [b]
151	Extra-EU	MAR	Moroccan Picholine	EVOO [b]
152	Extra-EU	MAR	Arbequina	EVOO [b]
153	Extra-EU	MAR	Arbosana	EVOO [b]
154	Extra-EU	MAR	Koroneiki	EVOO [b]
155	Extra-EU	MAR	Moroccan Picholine - Hojiblanca	EVOO [b]
156	Extra-EU	MAR	Moroccan Picholine	EVOO [b]
157	Extra-EU	MAR	Moroccan Picholine	EVOO [b]

158	Extra-EU	MAR	Moroccan Picholine	EVOO [b]
159	Extra-EU	MAR	Moroccan Picholine	EVOO [b]
160	Extra-EU	MAR	Moroccan Picholine	EVOO [b]
161	Extra-EU	MAR	Moroccan Picholine	EVOO [b]
162	Extra-EU	MAR	Moroccan Picholine	EVOO [b]
163	Extra-EU	MAR	Moroccan Picholine	EVOO [b]
164	Extra-EU	MAR	Moroccan Picholine	EVOO [b]
165	Extra-EU	MAR	Arbequina	EVOO [b]
166	Extra-EU	MAR	Moroccan Picholine	EVOO [b]
167	Extra-EU	MAR	Moroccan Picholine	EVOO [b]
168	Extra-EU	MAR	Moroccan Picholine	EVOO [b]
169	Extra-EU	MAR	Picholine - Arbosana	EVOO [b]
170	Extra-EU	MAR	Moroccan Picholine	EVOO [b]
171	Extra-EU	MAR	Moroccan Picholine	EVOO [b]
172	Extra-EU	MAR	Arbequina - Koroneiki	EVOO [b]
173	Extra-EU	MAR	Moroccan Picholine	EVOO [b]
174	Extra-EU	MAR	Moroccan Picholine - Koroneiki	EVOO [b]
175	Extra-EU	MAR	Moroccan Picholine	EVOO [b]
176	Extra-EU	MAR	Arbequina	EVOO [b]
177	Extra-EU	MAR	Moroccan Picholine	EVOO [b]
178	Extra-EU	MAR	Arbequina - Koroneiki	VOO [b]
179	Extra-EU	MAR	Arbequina	EVOO [b]
180	Extra-EU	MAR	Moroccan Picholine - Arbequina	EVOO [b]
181	Extra-EU	MAR	Moroccan Picholine	VOO [b]
182	Extra-EU	MAR	Moroccan Picholine	EVOO [b]
183	Extra-EU	MAR	Arbequina	VOO [b]
184	Extra-EU	TUN	Chetoui - Chemlali	VOO [a]
185	Extra-EU	TUN	Sahli	VOO [a]
186	Extra-EU	TUN	Sahli - Chemlali	EVOO [b]
187	Extra-EU	TUN	Chemlali	EVOO [b]
188	Extra-EU	TUN	Chemlali	EVOO [b]
189	Extra-EU	TUN	Chetoui	EVOO [b]
190	Extra-EU	TUR	Ayvalik - Domat	EVOO [a]
191	Extra-EU	TUR	Memecik - Gemlik	EVOO [a]
192	Extra-EU	TUR	Memecik	EVOO [a]

193	Extra-EU	TUR	Ayvalik	VOO [a]
194	Extra-EU	TUR	Ayvalik	VOO [a]
195	Extra-EU	TUR	Ayvalik	EVOO [a]
196	Extra-EU	TUR	Ayvalik	VOO [a]
197	Extra-EU	TUR	Domat	EVOO [a]
198	Extra-EU	TUR	Memecik	EVOO [a]
199	Extra-EU	TUR	Karamani - Hasebi	VOO [a]
200	Extra-EU	TUR	Memecik	EVOO [a]
201	Extra-EU	TUR	Gemlik - Memecik	VOO [a]
202	Extra-EU	TUR	Memecik	EVOO [a]
203	Extra-EU	TUR	Gemlik	EVOO [a]
204	Extra-EU	TUR	Memecik	VOO [a]
205	Extra-EU	TUR	Memecik	EVOO [a]
206	Extra-EU	TUR	Saurani - Halhali - Karamani	VOO [a]
207	Extra-EU	TUR	Edremit - Domat - Gemlik	VOO [a]
208	Extra-EU	TUR	Memecik	VOO [a]
209	Extra-EU	TUR	Ayvalik - Edremit	VOO [a]
210	Extra-EU	TUR	NA	EVOO [a]

Table 5.2. Information about country of origin, olive variety and commercial category for all the samples collected and analyzed. NA: information not available; EVOO: extra virgin olive oil; VOO: virgin olive oil. EU: oils from EU state members; Extra-EU: oils from countries outside the European Union; Blends: oils obtained mixing EU oils or EU and Extra-EU oils. CHL: Chile; ESP: Spain; GRC: Greece; HRV: Croatia; ITA: Italy; MAR: Morocco; PRT: Portugal; SVN: Slovenia; TUN: Tunisia; TUR: Turkey.

[a] Commercial category defined by Panel Test realized in the framework of the OLEUM project.

[b] Commercial category declared by the company that provided the sample.

According to geographical provenance, samples were distributed in 3 classes (Table 5.3): “EU” for oils coming from EU member states; “Extra-EU” for oils coming from third countries (outside EU); “Blends” for samples obtained by mixing oils coming from different EU state members or oils coming from EU state members and third countries.

Aliquots of each sample (50 mL) were stored at -18 °C in plastic dark bottles. Oil were defrosted for at least 12 h and stored at 12°C before analysis.

Origin class	N	Origin country
EU	116	29 Spain, 25 Italy, 22 Croatia, 16 Greece, 12 Portugal, 12 Slovenia
Extra-EU	70	42 Morocco, 21 Turkey, 6 Tunisia, 1 Chile
Blends	24	12 EU blends, 12 EU/Extra-EU blends

Table 5.3. Number of samples for each origin class considered and detail of their geographical origin. EU: oils from EU state members; Extra-EU: oils from countries outside the European Union; Blends: oils obtained mixing EU oils or EU and Extra-EU oils.

5.2.4 Volatile compounds analysis by Flash Gas Chromatography

The analysis of volatile compounds was carried out by the Flash Gas Chromatography Electronic Nose Heracles II (Figure 5.1).

Figure 5.1. HERACLES II Electronic Nose apparatus (image from Alpha MOS).

The instrument was equipped with two metal capillary columns (MXT-5 and MXT-1701) working in parallel mode and different in polarity of the stationary phase. This permits slight differences in the separation capability of molecules detected by a FID applied at the end of each column.

Each sample was analysed in triplicate, weighing 2 ± 0.1 g of oil in a 20 mL vial sealed with a magnetic plug. For analysis, the vial was placed in a shaker oven for 20 min at 40 °C and 500 rpm. Next, 5 mL of the headspace were collected, introduced in a splitless injector (injector temperature 200 °C, injection speed 100 µL/sec, carrier gas flow, to ensure a fast transfer of the sample from the inlet to the trap, 30 mL/min), and adsorbed on a Tenax® TA trap maintained at 40 °C for 60 sec to concentrate the analytes. The syringe temperature was set at 70 °C. Subsequently, desorption was obtained by increasing the trap temperature to 240 °C in 93 sec and the sample was injected (pressure

of the carrier gas at columns' head 40 kPa.) and split (split flow 5 mL/min) into the two columns. The thermal program started at 40 °C (held for 2 sec), increased up to 80 °C at 1 °C/sec, and then to 250 °C at 3 °C/sec. Hydrogen was used as the carrier gas with a pressure from 40 kPa to 64 kPa, increasing with a rate of 0.2 kPa/sec. At the end of each column, a FID detector (detector temperature 260 °C) was placed and the acquired signal was digitalized every 0.01 sec. The software used to control the instrument was AlphaSoft version 14.5.

5.2.5 Data processing: PLS-DA and ANN

For the data analysis, the full chromatograms were processed by applying chemometric elaborations with an untargeted approach. The raw data of each chromatogram (intensity values for each point of the chromatogram considering that the signal was digitalized every 0.01 sec) were exported from the software of the instrument and the data set with all the samples was imported into MatlabR2018a®. As data pre-treatment, chromatograms were aligned by COW (Correlation Optimized Warping) algorithm (Tomasi et al., 2004) and autoscaled (mean-centering followed by division of each column (variable) by the standard deviation of that column). Preliminary tests showed that chromatograms obtained from the MXT-5 column had a discriminant power higher than the other one (MXT-1701) and for this reason the classification models were developed considering only this column. Considering the reduced number of samples for the classes "Blend EU" and "Blend EU-Extra EU", these oils were grouped together with "EU" and "Extra-EU" samples, respectively. This means that for the data elaboration only two sample categories were considered: "EU" and "Extra-EU".

Two different statistical techniques were used to classify samples according to their geographical origin, the first (PLS-DA) based on a linear approach, and the second (ANN) on a non-linear approach.

In particular, the PLS-DA model was built using the PLS Toolbox for Matlab2018a®: intensity values of each point of the chromatogram, for a total of 19,900 data points, were used as variables X (matrix X), while the origin ("EU" and "Extra-EU") was implemented as variable Y (binary variables, 0 - 1). The sample data set was split into a calibration/full-cross validation set (75% of the sample) and an external validation set (25% of the sample) using the Kennard-Stone method (selects samples that best span the same range as the original data, but with an even distribution of samples across the same range) (Daszykowski et al., 2002). The threshold value useful to define the category of each sample was defined using a probabilistic approach based on Bayes's rule.

The ANN model was performed by using the Neural Net Pattern Recognition tool for Matlab2018a®. Specifically, a Multi-Layer Perceptron (MLP) neural network was built to predict the specific class

to which samples belong using a non-linear method. For input and hidden layers, linear and logistic activation functions, respectively, were used, while for output layer the SoftMax function was applied. From a statistical point of view, with the SoftMax activation function and cross entropy error, the output is interpretable as posterior probabilities for categorical target variables (Bishop, 1995). One nominal output variable is returned, assuming that the target output is 1.0 in the correct class output, and 0.0 in the non-correct class. Looking for the best classification ability, different node numbers in the hidden layer and combinations were tested. The convergence of ANN was ruled by a back propagation algorithm. The original data set was randomly divided into a training set (60%), verification set (20%), and test set (20%). The training set was used to calculate the transfer function parameters of the network, the verification set to indicate possible over-learning, and the test set was treated as an unknown, the correct classification of which indicates that the neural network is performing well. It was checked that samples from both classes were contained in the test set.

5.2.6 Validation of the analytical method

Fingerprinting methods intended for sample classification are not aimed to the identification and quantification of specific analytes, but to find distinctive patterns for a given category (in this case "EU" and "Extra-EU") in raw analytical signals (chromatograms). Conversely to what generally applied for in-house validation of targeted methods, the main constraint of the fingerprinting approaches is providing a repeatable and reproducible signal with the enough sensitivity to collect the valuable information from the samples. The parameters considered were technical repeatability (inter-day and intra-day repeatability) and sensitivity.

Technical repeatability

To assess the repeatability of the chromatographic signal, RSD (Relative Standard Deviation) value of each chromatogram data point, with intensity above noise signal, was measured (Allwood et al., 2009). Before evaluating technical intra-day and inter-day repeatability, chromatograms were pre-treated as described in section 5.2.5. Moreover, the noise was excluded to avoid considering non relevant RSD values.

For the evaluation of intra-day repeatability, a sample was analyzed within the same day, by the same operator, with the same equipment, and in the same instrument operative conditions. The inter-day repeatability was measured analyzing the same sample on 3 different days, with the same equipment but in different environmental conditions. In both cases 7 replicates were analyzed.

Sensitivity

As the fingerprinting approach is not aimed to measure the concentration of specific analytes, limits of detection or quantification cannot be calculated for the analytical outcome. However, the analytical method needs to be sufficiently sensitive to allow the detection of even minor constituents to avoid missing any valuable information about the sample. In this case, a target-type strategy was applied: three standard solutions were analyzed according to section 5.2.4 and the S/N (signal/noise) ratio for each compound considered was calculated.

The standard solutions used were:

- Solution of ethanol (0.05 mg/kg) in refined olive oil;
- Solution of hexanal (0.1 mg/kg) in refined olive oil;
- Solution of (*E*)-2-Hexen-1-al (0.75 mg/kg) in refined olive oil.

These compounds were chosen among the most representative of the qualitative and quantitative VOOs volatile composition and considering their retention time to cover the whole chromatogram and avoid overlap. The difference in terms of concentration used for each compound is due to the different response of the FID detector to molecules as function of their structure.

5.3 Results and discussion

5.3.1 Chemometric elaborations

A set of 210 EVOOs and VOOs were analyzed for their volatile profile by flash gas chromatography. Considering the large amount of data and the aim of this work, chemometric elaborations following an untargeted approach were carried out.

For elaborations, samples were grouped into two categories: “EU”, that included oils from single EU state members and blends of oils from different EU countries, “Extra-EU” that consisted of oils from single countries outside the European Union and blends of oils from EU and third countries.

In Figure 5.2a the mean chromatogram of “EU” and “Extra-EU” categories, obtained averaging the intensity of each variable for all “EU” or “Extra-EU” samples, is reported: even if almost all peaks are concentrated in the initial part of the chromatogram (between 2000 and 10000 variables), a clear difference, in terms of variable intensities, exists between the two groups, thus confirming the discriminating power of the volatile profile with respect to the geographical origin (Melucci et al., 2016; Lukić et al., 2019).

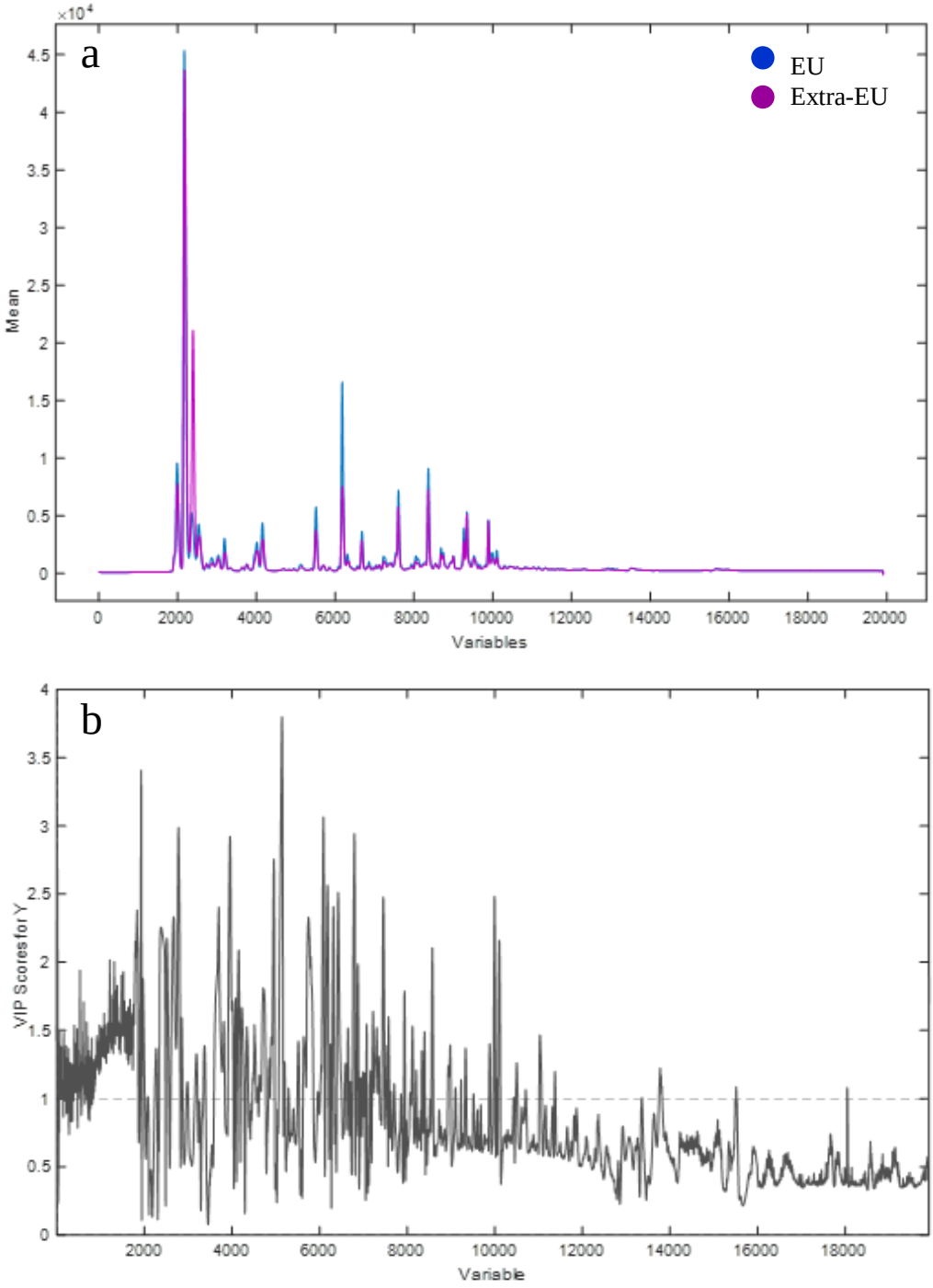

Figure 5.2. a) Mean chromatogram (mean intensity value for each variable) of EU (blue line) and Extra-EU (violet line) categories; b) VIP score for each variable.

Concerning the PLS-DA results, the values of the estimated Y variable (geographical category) by the model in cross and external validations are shown in Figure 5.3. The dotted line identifies the threshold value used to define the attribution of samples to different classes. Regarding the location of each sample, a greater distance from the threshold line can be interpreted as a better classification capacity of the model.

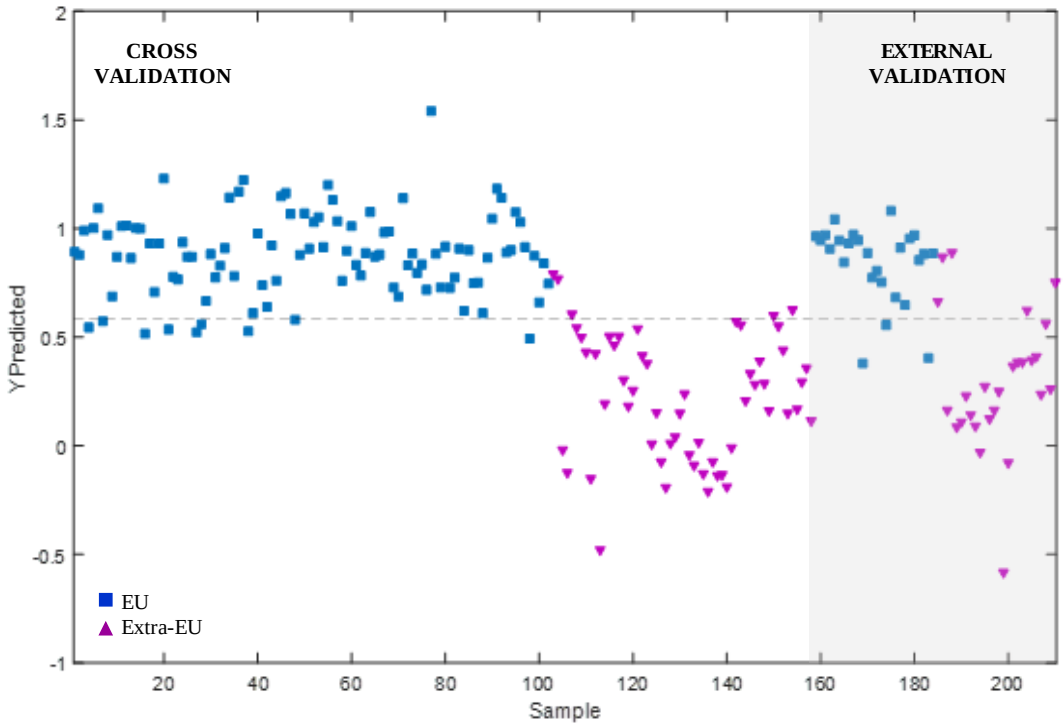

Figure 5.3. Graph of the values of the Y variable estimated by the PLS-DA model, in cross and external validation (grey area). Blue squares: EU; violet triangle: Extra-EU.

The results, in terms of percentage and number of samples correctly classified, are reported in Table 5.4. The percentage ranged from 80.8% to 91.2%. The values obtained for the "EU" category were higher, likely because of the greater number and variability of samples used to build the model. The external validation percentages were lower compared to those obtained for the cross-validation, but the results can be considered more robust since they were obtained considering the 25% of samples that were not used to build the model.

The VIP (Variable Importance in Projection) score obtained by the PLS-DA confirmed that the section of the chromatogram ranging from 2000 to 10000 variables has a major contribution to the sample discrimination (VIP values greater than 1) according to geographical origin (Figure 5.2b).

Category	Cross validation	External validation
EU	91.2% (93/102)	88.5% (23/26)
Extra-EU	91.1% (51/56)	80.8% (21/26)

Table 5.4. Numbers and percentages of samples correctly classified for each category using the PLS-DA model. EU: oils from a single state member of European Union and oils obtained mixing EU oils; Extra-EU: oils from a single country outside the European Union and oils obtained mixing EU and Extra-EU oils.

Focusing on those incorrectly classified samples, a specific trend as a function of characteristics that could usually affect the volatile profile of the oil (such as the commercial category, olive cultivar, or country of origin) was not seen.

Results related to the probabilistic approach are shown in Figure 5.4. The graph refers to the category "EU": this means that higher a sample is located, the higher the probability for which it is classified as member of the "EU" category. As a consequence, oils classified as members of the other category (Extra-EU) are located in the bottom area of the graph. In this case, the threshold value is fixed at 0.5, corresponding to a probability of 50%: a sample classified with a probability lower than this is considered as not correctly grouped. It is also interesting to note that most of samples were correctly classified with a probability between 90% and 100%.

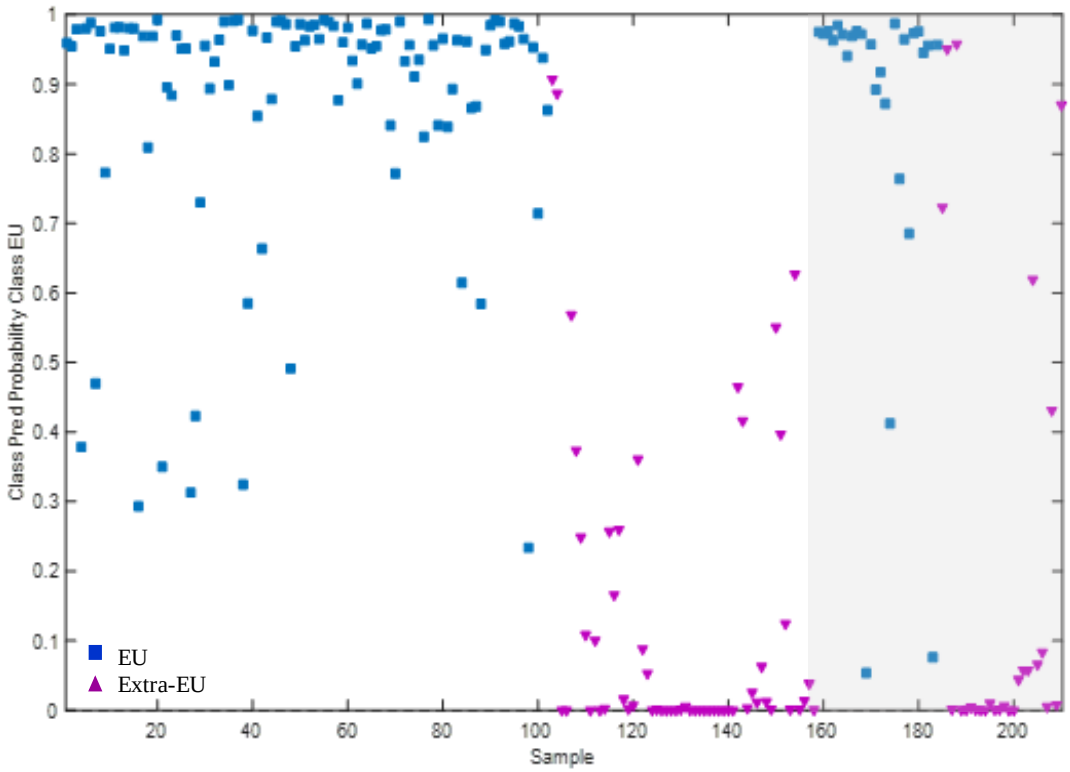

Figure 5.4. Graph of the values of the probability of belonging to the "EU" category. 1 = probability of 100%; 0 = probability of 0%. Grey area: test set used for the external validation of the PLS-DA model. Blue squares: EU; violet triangles: Extra-EU.

Regarding ANN, an early stopping technique was used to select the number of training cycles (epochs) to avoid over-fitting, using the test set to monitor the prediction error. An example of this procedure is reported in Figure 5.5, where the best ANN training was characterized by 18 epochs. Above this point, the error increased further indicating that the ANN tends to overfit. Consequently, the results of ANN are related to these iterations.

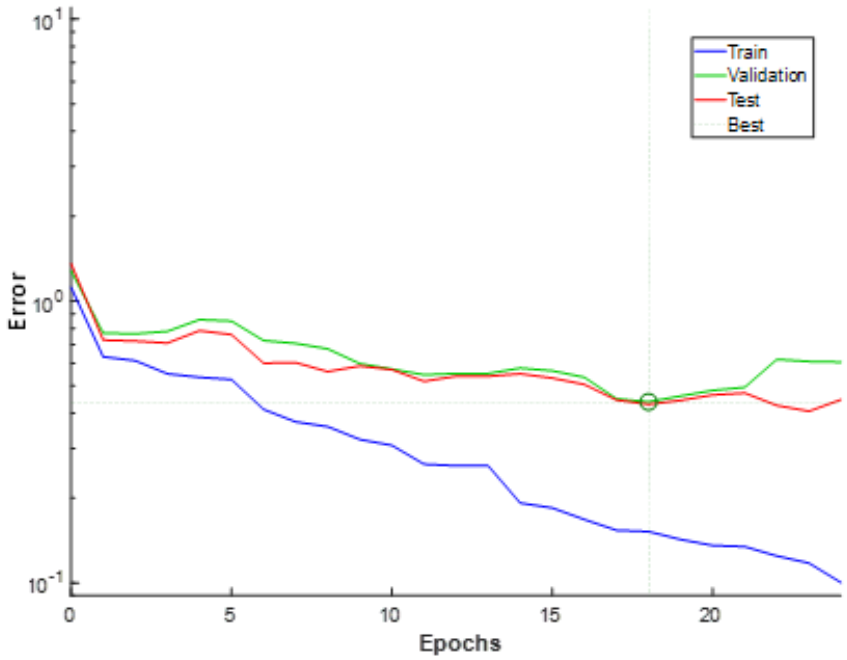

Figure 5.5. Error graph of validation (green line) and test (red line) set.

Training was repeated 5 times and the network's predictions were averaged, since with ANNs convergence is influenced by the initial weight value and the randomized split of data in training, validation, and test sets. The best prediction results were obtained with a three layers network, having 5 nodes; a larger number of nodes did not increase the network performance.

The classification results, in terms of percentage of samples correctly classified, are summarized in Table 5.5. Means and standard deviations (in brackets) were taken into account.

As reported for the PLS-DA model, even in this case the higher percentages (from 93.2% to 98.7%) were achieved for the "EU" category in all the three data sets.

Comparing the results of the external validation (PLS-DA) and testing (ANN), it is possible to note that higher percentages were obtained in the second case for both the "EU" and "Extra-EU" categories. In particular, an increment of 4.7% and 8% of samples correctly classified was obtained. This is probably due to the fact that the ANN model is based on a non-linear approach.

Category	Training (%)	Validation (%)	Testing (%)
EU	98.7 (1.1)	95.4 (3.9)	93.2 (3.2)
Extra-EU	94.4 (7.0)	88.7 (7.8)	88.8 (5.4)

Table 5.5. Percentages (mean of 5 training of the model and standard deviation in brackets) of samples correctly classified for each category using the ANN model. EU: oils from a single state member of European Union and oils obtained mixing EU oils; Extra-EU: oils from a single country outside the European Union and oils obtained mixing EU and Extra-EU oils.

In general, the percentages obtained were slightly lower than those reported by other studies based on volatile compounds and chemometric untargeted data elaboration (Gerhardt et al., 2017; Bajoub et al., 2018; Lukić et al., 2019). This aspect can be explained by the great variability, in terms of geographical origin, olive variety, commercial category, of the samples analyzed, which represents a strong point of this work.

The results described herein confirm the suitability of flash gas chromatography for checking geographical traceability of EVOOs and VOOs, even using untargeted chromatographic signals of the volatile fraction as variables for multivariate analysis (Melucci et al., 2016).

5.3.2 Validation of the analytical method

Intra-day repeatability

Seven replicates of the sample were analyzed by the same operator, with the same equipment and in the same instrument operative conditions within the same day.

For each of point of the chromatogram (after noise exclusion, the data matrix was composed by 8401 points), mean value, SD (Standard Deviation) and RSD were calculated.

More than 97% of the signals presented RSD <10%, and 99.8% of the variables < 20% (Table 5.6).

Class (RSD)	*Frequency*	*%*
10	8185	97.5
15	166	1.5
20	33	0.8
30	17	0.2
40	9	0
50	0	0
60	0	0
70	0	0
80	0	0
90	0	0
100	0	0

Table 5.6. Frequency (number) and percentages of signals of each RSD class measured for the intra-day repeatability.

To analyze the variability as related to the magnitude of the variables, the RSD values were plotted vs. the signal intensity (Figure 5.6). As expected, the repeatability was strongly related with the intensity of the variables, and signals with RSD>10% are those presenting the lowest intensity values. This is in agreement with the trend described by the Horwitz equation for targeted methods (Horwitz, 1982).

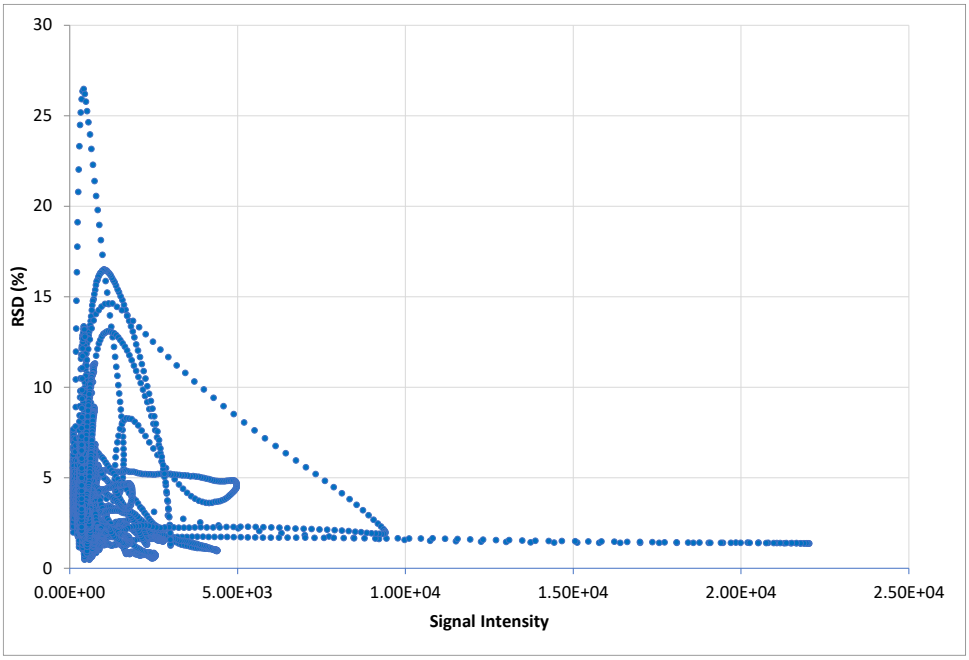

Figure 5.6. Intra-day RSD distribution as related to the signal intensity.

Inter-day repeatability

Seven replicates of the sample were analyzed with the same equipment but in different days, involving different environmental conditions. Also in this case, for each of variable (8401 points), mean value, SD and RSD were calculated.
More than 91% of the signals presented RSD < 10%, and 99.4% of the variables < 20% (Table 5.7).

Class (RSD)	***Frequency***	***%***
10	7690	91.5
15	552	6.6
20	106	1.3
30	16	0.2
40	37	0.4
50	0	0
60	0	0
70	0	0
80	0	0
90	0	0
100	0	0

Table 5.7. Frequency (number) and percentages of signals of each RSD class measured for the inter-day repeatability.

In Figure 5.7 RSD values were plotted vs. the signal intensity. The repeatability was strongly correlated to the intensity of the variables, and variables with RSD>10% are those presenting the lowest intensity values.

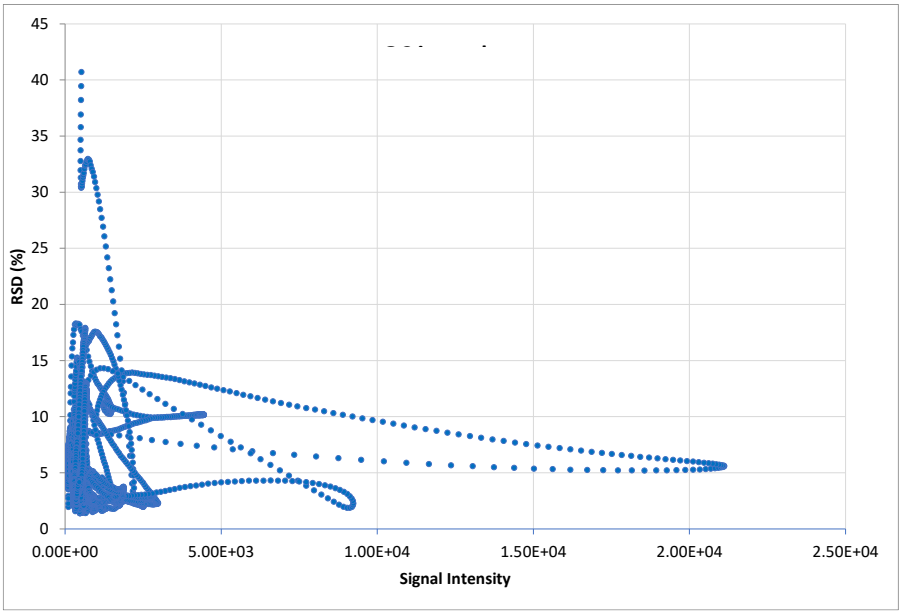

Figure 5.7. Inter-day RSD distribution as related to the signal intensity.

Sensitivity

The sensitivity of the instrument was evaluated analyzing three standard solutions and calculating the ratio S/N. The noise was measured considering the baseline of the chromatogram portion between 43 and 50 sec since no peaks eluted in this zone for all the compounds analyzed.
The results are reported in Table 5.8: the S/N value for the selected analytes in the chromatograms should be > 3.

Compound	*Concentration (mg/kg)*	*S/N*
Ethanol	0.05	3.84 ± 0.99
Hexanal	0.1	5.55 ± 0.96
(*E*)-2-Hexen-1-al	0.75	4.42 ± 1.82

Table 5.8. Concentration (mg/kg) of each compound included in the standard solution and related S/N. S=intensity of the peak of the compound; N=mean intensity of the noise.

5.4 Conclusions

In this work, the application of flash gas chromatography for volatile compounds analysis combined with untargeted chemometric data elaborations (PLS-DA and ANN) to discriminate EVOOs and VOOs with different geographical origin was presented.

For both elaborations, satisfactory results, in terms of percentages of samples correctly classified, were obtained: PLS-DA (external validation) allowed classification of around 89% and 81% of "EU" and "Extra-EU" samples, respectively; for ANN (testing set) the percentages were equal 93.2% and 88.8%, respectively.

It is important to highlight that these promising results were achieved by analyzing a set of samples that are representative of the large variety of parameters (olive cultivar, country of origin, commercial category) that can describe olive oil product and affect its chemical characteristics. The results obtained herein sustained the use of multivariate chemometrics with untargeted detection of volatile compounds as a powerful tool to discriminate EVOOs and VOOs of different origin. Moreover, good performance of the analytical method, in terms of technical repeatability and sensitivity, were obtained.

Other studies have already reported that the analysis of volatile compounds is suitable for tracing the geographical origin of VOOs. However, the methodology proposed herein presents some advantages in comparison with other techniques generally applied for this analysis, as it is very rapid (only 200 sec are needed for each chromatographic run) and easy to use since no sample treatment is required.

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Chapter 6

Application of GC-IMS for assessment of soft deodorized olive oils

6.0 Abstract

Virgin or lampante olive oils with slight sensory defects may be subjected to a soft-deodorization treatment to conceal their negative sensory attributes. The use of temperatures significantly lower (below 140°C) than those applied during conventional deodorization in the refining process limits the formation of those compounds generally used as chemical markers to detect the illegal presence of refined olive oil in extra virgin olive oil. For this reason, the illegal blending of treated oils with extra virgin ones, and possible commercialization of this blend labelled as top-quality grade (EVOO), represents a fraud that is very difficult to detect. Many studies have been conducted and are ongoing to find direct markers of soft deodorization. However, at present there is no method specifically devoted to detecting this treatment. The aim of this work was the application of untargeted GC-IMS (Gas Chromatograph - Ion Mobility Spectrometer) for evaluation of the volatile fraction of soft deodorized olive oils since the soft-deodorization is expected to determine changes in the volatile profile of the oil. A set of extra virgin olive oils, defective olive oils before and after a soft-deodorization treatment, and blends was analyzed and encouraging results confirming that modifications occur in the olive oil volatile profile as consequence of soft deodorization, in terms of amount and composition, were obtained.

This study was realized at Instituto de la Grasa (Seville, Spain) under the supervision of Dr. Wenceslao Moreda during an abroad period (4 months) financially supported by the Marco Polo programme.

Keywords: Virgin olive oil, GC-IMS, Soft-Deodorization, Authenticity.

6.1 Introduction

Extra virgin olive oil (EVOO) is appreciated worldwide for its nutritional, health and pleasant sensory characteristics (Meenu et al., 2019).

According to European regulations, virgin olive oils are *"…obtained from the fruit of the olive tree solely by mechanical or other physical means under conditions that do not lead to alteration in the oil, which have not undergone any treatment other than washing, decantation, centrifugation or filtration…"*, and can be classified as "extra virgin olive oil", "virgin olive oil" or "lampante olive oil" according to specific chemical-physical (free acidity, peroxide value, specific extinctions in UV, fatty acid ethyl esters content) and sensory (positive and negative attributes) parameters.

Lower quality olive oils (virgin or lampante) with slight sensory defects may be subjected to illegal practices, such as neutralization and/or soft deodorization at low temperature, to conceal their negative sensory attributes (Gómez-Coca et al., 2012; Conte et al., 2019). By soft deodorization, oxidation and aroma compounds, responsible for defects, can be removed under vacuum. The use of temperatures significantly lower (below 140°C) than those applied during conventional deodorization in the refining process limits the formation of stigmastadienes, used as chemical markers to detect the illegal presence of refined olive oil in EVOO (Cert et al., 1994; Gallina Toschi et al., 1996). Many studies have been conducted and are ongoing to find direct markers of soft deodorization. However, at present there is no method specifically devoted to detecting this treatment and, therefore, this illegal practice cannot be directly detected at now by any official regulations. In an attempt to solve this problem, the determination of the content of fatty acid ethyl esters (FAEEs) was proposed (Pérez-Camino et al., 2008), since it seems to be the only procedure that can help in some cases (blends with deodorized obtained from fermented defectives virgin or lampante oils) to detect such a fraud (Biedermann et al., 2008; Gómez-Coca et al., 2012). The method was adopted by the European Union in 2011 (EU Reg. 61/2011) and then amended in 2013 for the determination of only ethyl esters of fatty acids in 2013 (EU Reg. 1348/2013). As the matter of fact, ethyl esters can be considered as indirect markers of low quality olives/oils when the fruits have suffered hydrolytic and fermentative processes, and are difficult or impossible to be removed at those low temperature that are supposed to be reached during soft deodorization. Moreover, an additional problem of detection exists for soft deodorized oils obtained from virgin or lampante oils with non-fermentative defects (such as frostbitten olives or rancid), therefore not discoverable by the FAEEs quality parameter. For these reasons, other parameters have been investigated, such as diacylglycerols (DAGs) and pyropheophytin (PPP), but none is used since they are not unequivocal and also change during aging of oils (Conte et al., 2019). The high content of PPP or DAGs could mean the oil was either subjected

to soft deodorization or that the oil is old or was poorly stored. Possible interesting results could be obtained studying in-depth the differences between experimental DAG content and theoretical DAG content (this latter calculated from free acidity) of genuine oils and corresponding samples subjected to the soft deodorization treatment.

A parameter surely affected by soft deodorization, independently of the sensory defect (fermentative or non-fermentative) of the oil, is the content and composition of volatile compounds since the conditions applied during the treatment determine the removal or reduction of volatiles responsible for both the undesirable and desirable odors (Aparicio-Ruiz et al., 2017).

Considering this, the study of the volatile fraction of soft deodorized oils in comparison with EVOOs and strongly defective olive oils could be useful for evaluation of this kind of oil. The aim of this work was the application of untargeted GC-IMS (Gas Chromatograph - Ion Mobility Spectrometer) for evaluation of the volatile fraction of soft deodorized olive oils. This instrumentation is based on a gas chromatograph coupled with an ion mobility spectrometer, able to detect the volatile fingerprints of liquid or solid samples without any relevant sample pretreatment. The volatile compounds present in the sample head space are pre-separated by gas chromatography, inserted in the atmospheric ionization region, and then separated and detected by IMS.

IMS is an emergent technique whose application in food quality and safety has increased in recent years due to its multiple advantages over other conventional analytical techniques (Karpas, 2013). It is based on gas phase ion separation inside a drift tube under the influence of a constant electric field at atmospheric pressure. In an initial step, volatile compounds, extracted from the sample and pre-separated by GC, enter the ionization chamber directed by a carrier gas (N_2) and are ionized through a ionization source (generally a tritium radioactive source). Ionization is based on the emission of primary electrons, which collide with nitrogen causing a combination of reactions that generates reactant ions. The reactant ion peak (RIP) represents the total available ions generated in the source. The analyte molecules react with the reactant ions, if the proton affinity of the original analyte is higher than that of water, yielding product ions such as protonated monomers or proton bound dimers depending on the analyte concentration, their chemical nature or drift tube temperature (Eiceman et al., 2014). Subsequently, generated ions travel to the drift tube through the shutter slit, which opens for a few milliseconds. Inside the drift tube and facing the drift gas current, the sample ion swarm moves at different speeds depending on its size and shape until it reaches the detector. On the detector ions are neutralized and they generate a current in a scale of picoamperes. The results are therefore expressed in terms of voltage units. The generated potential once the ions have reached the detector is the signal that can be correlated with the concentration of sample components. This technique was initially developed for the detection of explosives and chemical warfare agents (Palmer et al., 2001),

but over the years many applications for analysis and characterization of volatile compounds in samples of diverse nature, such as clinic (Chouinard et al., 2016), environmental (Zou et al., 2016; Vautz et al., 2018), and food samples, have been developed. Focusing on the latter category, GC-IMS was amply used for classification of olive oils (Garrido-Delgado et al., 2011; Garrido-Delgado et al., 2012; Garrido-Delgado et al., 2015a, Contreras et al., 2019), identification of the best packaging conditions for EVOOs (Garrido-Delgado et al., 2015b), geographical differentiation of EVOOs (Gerhardt et al., 2017), assessment of authenticity of honey (Gerhardt et al., 2018; Wang et al., 2019; Arroyo-Manzanares et al., 2019), evaluation of egg products freshness (Cavanna et al., 2019), detection of adulteration in sesame oil (Zhang et al., 2016), canola oil (Chen et al., 2018), and peanut oil (Tian et al., 2019), classification of Iberian ham (Arroyo-Manzanares et al., 2018), identification of meat species (Chen et al., 2019). However, at the best of our knowledge, this instrument has never been used for evaluation of soft-deodorized olive oil.

6.2 Materials and methods

6.2.1 Equipment and instrumentations

Usual laboratory glassware; analytical balance for weighing to an accuracy of ± 0.1 mg; GC-IMS (Flavourspec®, Gesellschaft für Analytische Sensorsysteme mbH, Dortmund, Germany) equipped with a capillary column (stationary phase 5% phenyl polysiloxane, length 30 m, internal diameter 0.32 mm, film thickness 0.25 µm).

6.2.2 Samples

The set of samples analyzed was composed by 2 extra virgin olive oils (EVOO, samples 1-2), 10 defective olive oils (DOO, both virgin and lampante olive oils, samples 3-12) before the soft-deodorization, the 10 DOOs after the treatment (SDOO, samples 13-22), and 60 blends of EVOO and SDOOs (samples 23-82) mixed at three different percentages (Table 6.1).

The soft-deodorization treatment, based on a short-path distillation able to discard off-flavours from defective olive oils without generating the usual refining tracers (e.g. stigmastadienes), and the sensory evaluation (Panel Test), to define the category (EVOO, VOO or LOO) of defective oils, were realized in the framework of the OLEUM project.

Samples were stored at 12°C before their analysis.

Sample code	*Sample description*
S1	EVOO with delicate fruitiness
S2	EVOO with medium fruitiness
S3	Defective olive oil (VOO)
S4	Defective olive oil (LOO)
S5	Defective olive oil (VOO)
S6	Defective olive oil (VOO)
S7	Defective olive oil (VOO)
S8	Defective olive oil (LOO)
S9	Defective olive oil (LOO)
S10	Defective olive oil (LOO)
S11	Defective olive oil (LOO)
S12	Defective olive oil (VOO)
S13	S3 after soft deodorization (SDOO)
S14	S4 after soft deodorization (SDOO)
S15	S5 after soft deodorization (SDOO)
S16	S6 after soft deodorization (SDOO)
S17	S7 after soft deodorization (SDOO)
S18	S8 after soft deodorization (SDOO)
S19	S9 after soft deodorization (SDOO)
S20	S10 after soft deodorization (SDOO)
S21	S11 after soft deodorization (SDOO)
S22	S12 after soft deodorization (SDOO)
S23	Blend (30/70 S1/S13)
S24	Blend (50/50 S1/S13)
S25	Blend (70/30 S1/S13)
S26	Blend (30/70 S2/S13)
S27	Blend (50/50 S2/S13)
S28	Blend (70/30 S2/S13)
S29	Blend (30/70 S1/S14)
S30	Blend (50/50 S1/S14)
S31	Blend (70/30 S1/S14)
S32	Blend (30/70 S2/S14)
S33	Blend (50/50 S2/S14)
S34	Blend (70/30 S2/S14)

S35	Blend (30/70 S1/S15)
S36	Blend (50/50 S1/S15)
S37	Blend (70/30 S1/S15)
S38	Blend (30/70 S2/S15)
S39	Blend (50/50 S2/S15)
S40	Blend (30/70 S2/S15)
S41	Blend (30/70 S1/S16)
S42	Blend (50/50 S1/S16)
S43	Blend (70/30 S1/S16)
S44	Blend (30/70 S2/S16)
S45	Blend (50/50 S2/S16)
S46	Blend (70/30 S2/S16)
S47	Blend (30/70 S1/S17)
S48	Blend (50/50 S1/S17)
S49	Blend (70/30 S1/S17)
S50	Blend (30/70 S2/S17)
S51	Blend (50/50 S2/S17)
S52	Blend (70/30 S2/S17)
S53	Blend (30/70 S1/S18)
S54	Blend (50/50 S1/S18)
S55	Blend (70/30 S1/S18)
S56	Blend (30/70 S2/S18)
S57	Blend (50/50 S2/S18)
S58	Blend (70/30 S2/S18)
S59	Blend (30/70 S1/S19)
S60	Blend (50/50 S1/S19)
S61	Blend (70/30 S1/S19)
S62	Blend (30/70 S2/S19)
S63	Blend (50/50 S2/S19)
S64	Blend (70/30 S2/S19)
S65	Blend (30/70 S1/S20)
S66	Blend (50/50 S1/S20)
S67	Blend (70/30 S1/S20)
S68	Blend (30/70 S2/S20)
S69	Blend (50/50 S2/S20)

S70	Blend (70/30 S2/S20)
S71	Blend (30/70 S1/S21)
S72	Blend (50/50 S1/S21)
S73	Blend (70/30 S1/S21)
S74	Blend (30/70 S2/S21)
S75	Blend (50/50 S2/S21)
S76	Blend (70/30 S2/S21)
S77	Blend (30/70 S1/S22)
S78	Blend (50/50 S1/S22)
S79	Blend (70/30 S1/S22)
S80	Blend (30/70 S2/S22)
S81	Blend (50/50 S2/S22)
S82	Blend (70/30 S2/S22)

Table 6.1. Code, description and composition of the samples analyzed. The commercial category (EVOO, VOO or LOO) was defined by Panel Test realized in the framework of the OLEUM project. EVOO: extra virgin olive oil; VOO: virgin olive oil; LOO: lampante olive oil; SDOO: soft deodorized olive oil; Blend: mixture at three different percentages of SDOOs and EVOOs.

6.2.3 Volatile compounds analysis by GC-IMS

The analyses were performed on a GC-IMS (Flavourspec®, Gesellschaft für Analytische Sensorsysteme mbH, Dortmund, Germany) equipped with a capillary column (Figure 6.1).

For analysis, 5 g of oil were placed in a 20 mL vial closed with magnetic caps. After 1 h of incubation at 60°C, 2.5 mL of sample headspace was injected by means of a heated syringe (80°C) into the injector heated at the same temperature. The temperature of the column was set at 40°C maintained during all the analysis. Nitrogen was used as carrier gas with a flow of 1 mL/min from the beginning of the analysis till 2 min, then increased to 10 mL/min within 15 min and maintained constant till the end of the chromatographic run (30 min).

The analytes were driven into the ionization chamber to be ionized prior to spectrometric detection. Molecules were ionized using a Tritium source (6.5 keV) and the resulting ions driven to the drift region via a shutter grid. The drift tube was 9.8 cm long and operated at a constant voltage of 5000 V, a temperature of 45°C and with a drift gas (nitrogen) flow rate of 150 mL/min. Data were acquired in the positive ion mode. Each spectra had an average of 16 scans, obtained using a grid pulse width of 100 μs, a repetition rate of 30 ms and a sampling frequency of 150 kHz.

Data treatment was realized with Laboratory Analytical Viewer (LAV) software version 2.2.1 (G.A.S. Dortmund).

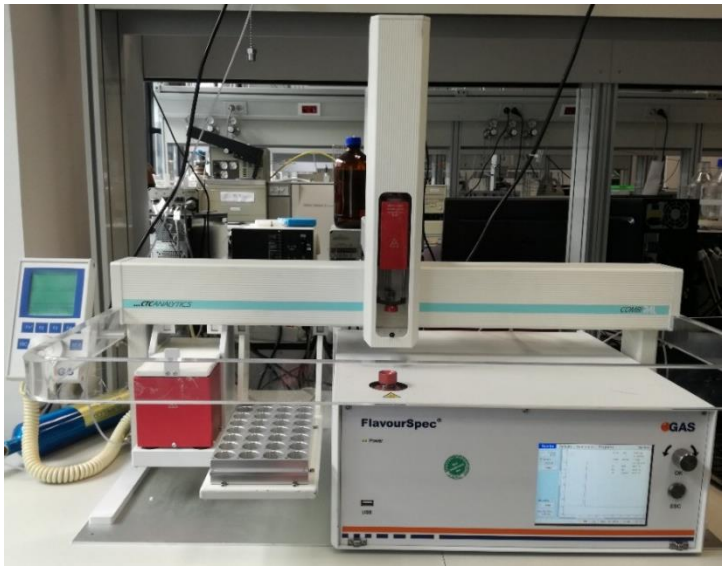

Figure 6.1. GC-IMS Flavourspec® apparatus.

6.3 Results and discussion

A set of EVOOs, DOOs, SDOOs and blends was analyzed for the volatile compounds by GC-IMS. The separation of the analytes by IMS is based on the fact that ions travel at atmospheric pressure versus a flow of inert drift gas (nitrogen) and need to pass a fixed distance (drift tube) in a defined electric field. For these reasons, the data obtained represent a 3D array, in which each point is characterized by the retention time in the chromatographic column, by the drift time and by the intensity of ion current signal. This means that for each sample a tri-dimensional map in which Y axis represents the retention time in the chromatographic column (in seconds), X axis represents the drift time in the drift tube (in milliseconds), and Z axis the intensity value (in V) of each compound is obtained. An example of this topographic plot is presented in Figure 6.2. Each red spot represents a compound and its dimension depends on the molecule concentration.

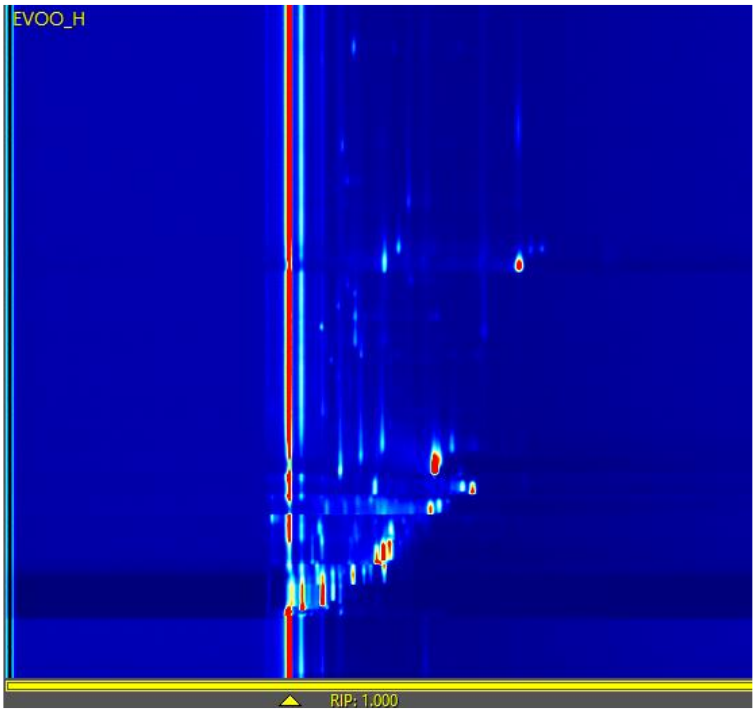

Figure 6.2. Topographic plot of GC–IMS spectra of sample S2 (EVOO with medium fruitiness).

A visual comparison of the spectra obtained for EVOOs, DOOs and SDOOs (blends were not considered in this phase since they were produced mixing different percentages of EVOOs and SDOOs) was carried out in order to identify specific spots (compounds) than can be useful to differentiate the oils according to their quality. A great variety in the spectra profiles of the oil samples was observed due to the different typologies and intensities of sensory defects of the samples.

In general, spectra of SDOOs were poorer in signals than spectra of the respective DOOs samples, confirming that the soft deodorization treatment causes a reduction of volatile compounds (Aparicio-Ruiz et al., 2017). Figure 6.3a shows a comparison of two topographic plots obtained for a SDOO (sample S15) and its corresponding DOO (sample S5), while Figure 6.3b represents the same samples from a 3D perspective. As can be seen, most of the signals appear between 200 and 1000 sec of retention time.

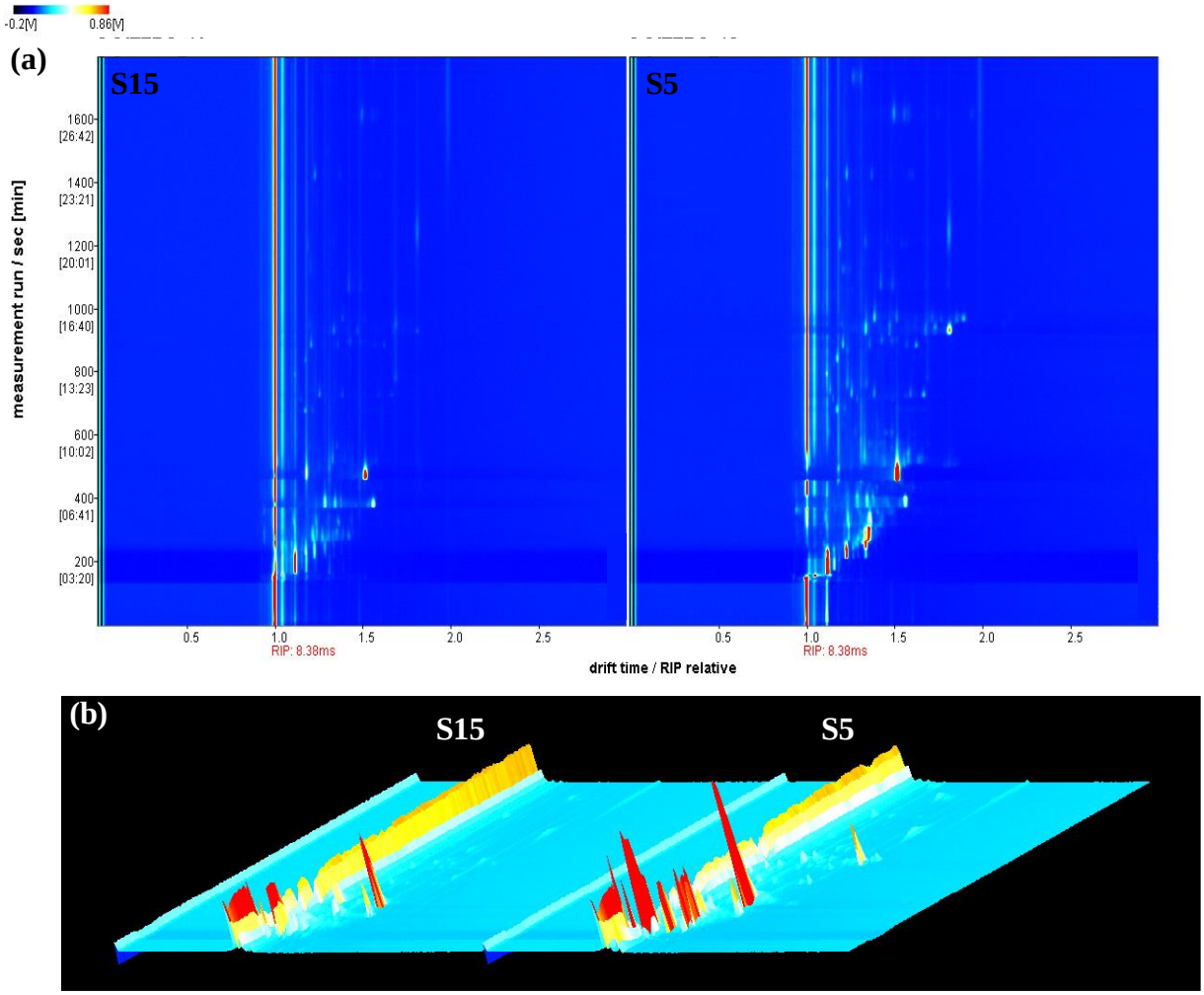

Figure 6.3. Comparison of topographic plots (a) and 3D graphs (b) obtained for a SDOO (sample S15) and its corresponding DOO (sample S5).

A total of 20 spots, considering EVOOs, DOOs and SDOOs, were selected: an overview of the identified spots is presented in Figure 6.4. Each column represents the same spot for all the samples considered, about the color of a specific spot for all the samples, when a spot is red means that the intensity is higher than a blue or white one. Looking at Figure 6.4, it is possible to note that some spots were detected in all the samples (EVOOs, DOOs and SDOOs) even if with a different intensity, and others were detected mainly in DOOs. As already evidenced, SDOOs showed a lower number and amount of volatile compounds.

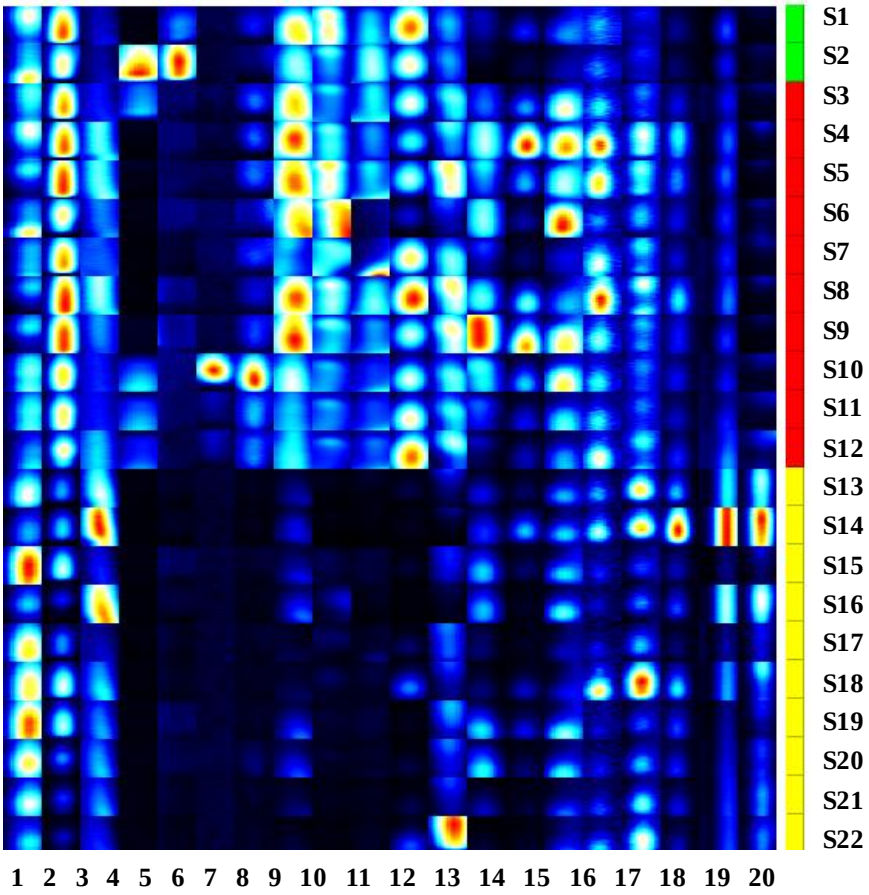

Figure 6.4. Global overview of the 20 spots identified in the samples. S1 and S2: extra virgin olive oil; S3-S12: defective olive oils; S13-S22: soft deodorized olive oils.

Subsequently, considering the area values (calculated by the software) of the 20 spots previously selected a PCA with EVOOs, SDOOs and blends was realized (Figure 6.5, explained variance PC1-PC2 69%). A good separation of the samples according to their categories was evidenced and a satisfactory distribution of blends respects to EVOOs was obtained: the blends placed closest the extra virgin olive oils are the samples that contain a higher percentage of EVOO.

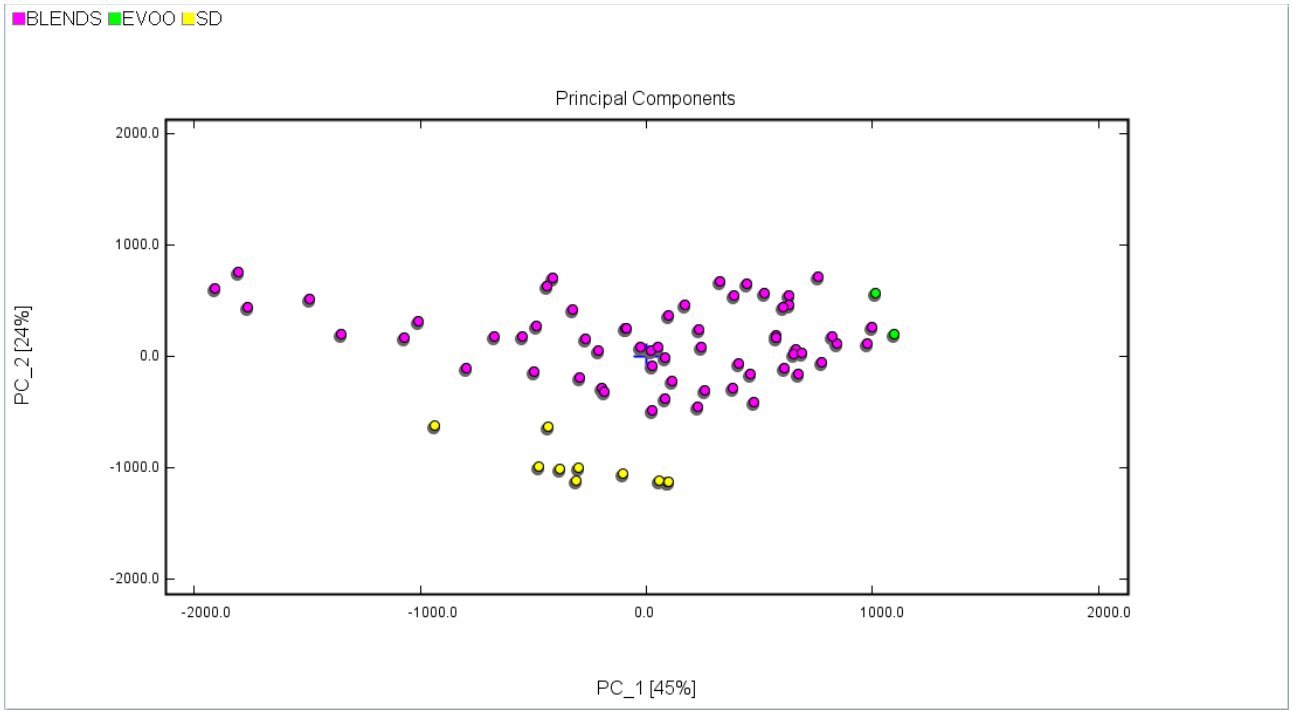

Figure 6.5. PCA (Principal Component Analysis) of the samples considering 20 spots. Green circles: extra virgin olive oils, yellow circles: soft deodorized samples; pink circles: blends.

The identification of volatile compounds analyzed by GC-IMS can be realized by injecting analytical standards or using a software (GCxIMS Library Search) that facilitates the assignment of chemical compounds to signal peaks according to their gas chromatographic retention index (RI) and RIP-relative IMS drift times (Dt). This step could be useful in order to identify those compounds that are expected to be present in certain amounts in EVOOs and whose absence or low concentration can suggest a suspicious blend.

6.4 Conclusions

In this preliminary work, a set of extra virgin olive oils, defective olive oils before and after a soft-deodorization treatment, and blends was analyzed for the first time by GC-IMS, an instrument that comprises advantages of a gas chromatography with regard to selectivity and the sensitivity of an ion mobility spectrometer enabling the analysis of volatiles compounds. The application of this approach showed encouraging results confirming the modifications that occur in the olive oil volatile profile as consequence of soft deodorization.

Analyses of a larger number of samples could further confirm these results and the application of a chemometric approach for the data elaboration could improve the performance of this approach.

In comparison to other methods generally used for determination of volatile compounds, GC–IMS does not require sample treatment, and its relative low cost (compared with other methodologies) and portability, since it works at atmospheric temperature and pressure, make easy its implantation in agri-food laboratories.

6.5 References

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Chapter 7

Conclusions and outlooks

Chapter 7. Conclusions and outlooks

The here presented thesis dealt with the development and improvement of analytical methods for control of quality and authenticity of virgin olive oils. To fulfil this objective different research activities were carried out, some of which related to quality control and others to authentication issues of virgin olive oils.

In particular:

- A revised method for determination of fatty acid ethyl esters was developed and in-house validated with satisfactory results. The method is based on the application of off-line HPLC-GC-FID (with PTV injector), revising both the preparative phase and the GC injector required in the official method. The main advantages of this revised protocol are: *i)* significant reduction of time (more than 50%) and solvents (more than 80%) required for each analytical determination, representing a more environmentally sustainable and rapid alternative to the official method; *ii)* use of HPLC as an alternative to traditional liquid chromatography carried out in manually packed glass columns, allowing for simplification of the technique. In order to confirm and strengthen the reliability and good performances of the approach presented herein, and in view of its proposal to normative bodies for possible adoption, an inter-lab validation of this method, involving several laboratories (also from private industries), is being carried out within the OLEUM project.
- A portable battery-operated electronic system suitable for in-situ measurements of olive oil free acidity was developed and in-house validated. The system is built with low cost electronics and embeds a temperature sensor to compensate for variations in electrical parameters with environmental temperature. Its working principle is based on the estimation of the olive oil free acidity by measuring the conductance of an emulsion between a hydro-alcoholic solution and the sample to be tested. The system has been calibrated and in-house validated: the data obtained showed good performances of the instrument in terms of LOD and LOQ, intra-day and inter-day precision, and accuracy. The procedure is very quick and easy. This makes the system suitable for people without specific training. The application of this analytical device is addressed in particular to estimation of free acidity of newly produced VOOs in oil mills.
- A flash gas chromatography for volatile compounds analysis combined with untargeted chemometric data elaborations (PLS-DA and ANN) to discriminate 210 samples of EVOOs and VOOs with different geographical origin was applied. For both elaborations, satisfactory results, in terms of percentages of samples correctly classified, were obtained.

Considering the PLS-DA, 89% and 81% of EU and Extra-EU samples, respectively, were correctly classified; for ANN the percentages were 93% and 89%, respectively. The results sustained the use of multivariate chemometrics with untargeted detection of volatile compounds as a powerful tool to discriminate EVOOs and VOOs of different origin. The methodology proposed presents some advantages in comparison with other techniques generally applied for the volatile compounds analysis, as it is very rapid (only 200 sec are needed for each chromatographic run) and easy to use since no sample treatment is required.

- GC-IMS was applied for the assessment of soft deodorized olive oils. A set of extra virgin olive oils (2), 10 defective olive oils before and after a soft deodorization treatment, and 60 blends were analyzed. The results obtained confirm that this illegal treatment determines modifications, in terms of amount and composition, of the volatile profile of the oil. Analyses of a larger number of samples could further confirm these results and the application of a chemometric approach for the data elaboration could improve the performance of this approach.

This Ph.D. project results highlighted that, despite the olive oil sector has a very advanced legislation, is still possible to overcome limitations of those analytical methods already officially recognized for control of quality of virgin olive oil, specifically proposing alternative procedures to reduce time and solvent consumption. Moreover, new analytical solutions promising to identify common and emerging frauds (e.g. detection of blends between extra virgin olive oil and soft deodorized olive oil) and to provide all the information required by the international market (e.g. verification of geographical origin of olive oils) could be studied in-depth in order to find procedures suitable to be officially recognized.

Chapter 8

Appendixes

8.1 List of abbreviations and acronyms

ANN: Artificial Neural Network

CART: Classification and Regression Tree

CHL: Chile

CM: Classification Map

COW: Correlation Optimized Warping

DAG: Diacylglycerol

DF: Discriminant Functions

DOO: Defective Olive Oil

EEC: European Economic Community

EIS: Electrical Impedance Spectroscopy

ESP: Spain

EU: European Union

EVOO: Extra Virgin Olive Oil

FA: Free Acidity

FAAE: Fatty Acid Alkyl Esters

FAEE: Fatty Acid Ethyl Esters

FAME: Fatty Acid Methyl Esters

FT-IR: Fourier Transform Infrared Spectroscopy

GC: Gas Chromatograph - Ion Mobility Spectrometer

GC-FID: Gas Chromatograph - Flame Ionization Detector

GRC: Greece

HPLC-UV-Vis: High Pressure Liquid Chromatography - UV/visible detector

HRV: Croatia

IOC: International Olive Council

ITA: Italy

k-NN: k-Nearest Neighbors

LC: Liquid Chromatography

LDA: Linear Discriminant Analysis

LOD: Limit Of Detection

LOO: Lampante Olive Oil

LOQ: Limit Of Quantification

LR: Likelihood Ratio

LR: Logistic Regression

MAR: Morocco

MIR: Mid-infrared spectroscopy

MLP: Multi Layer Perceptron

NIR: Near-Infrared Spectroscopy

OCI: On-column Injector

PCA: Principal Component Analysis

PLS-DA: Partial Least Square - Discriminant Analysis

PPP: Pyropheophytins

PRT: Portugal

PTR-MS: Proton Transfer Reaction-Mass Spectrometry

PTV: Programmed Temperature Vaporizer

RIP: Reactant Ion Peak

RRT: Relative Retention Time

RSD: Relative Standard Deviation

SD: Standard Deviation

SDOO: Soft Deodorized Olive Oil

SIMCA: Soft Independent Modelling by Class Analogy

SPE: Solid Phase Extraction

SVM: Support-Vector Machines

SVN: Slovenia

TAG: Triacylglycerol

TDR: Time Domain Reflectometry

TUN: Tunisia

TUR: Turkey

UNEQ-QDA: Unequal Quadratic Discriminant Analysis

VIP: Variable Importance in Projection

VOO: Virgin Olive Oil

8.2 List of tables

[a] Commercial category defined by Panel Test realized in the framework of the OLEUM project.

[b] Commercial category declared by the company that provided the sample.

8.3 List of figures

8.4 Other publications not related to the topic of the Ph.D. Thesis

8.4.1 Effects of archaic olive and oil storage methods still used in southern Tunisia on olive oil quality

Authors: Sinda El-Gharbi[a,b], Meriem Tekaya[a], Alessandra Bendini[c,d], Enrico Valli[c], Rosa Palagano[c], Tullia Gallina Toschi[c,d], Mohamed Hammami[a], Beligh Mechri[a]

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Published on: Italian Journal of Food Science, 2018, 30, 102-115.

Abstract

The present paper investigated how virgin olive oil quality is influenced by two different storage conditions that residents of Gabes (Southern Tunisia) usually apply to fruits of the Zarazi cultivar: long conservation as oil in glass bottles or traditional storage of olives as sun-dried fruits before processing for oil production. Even if both storage conditions are associated with strong losses in the qualitative characteristics of olive oil, the changes observed were more accentuated for oil stored for two years after its production compared to the oil obtained from olives stored by traditional methods.

Key words

Fatty acid alkyl esters, Olives, Phenols, Traditional storage, Virgin olive oil.

8.4.2 Sensory and instrumental study of Taralli, a typical Italian bakery product

Authors: Sara Barbieri[a], Alessandra Bendini[a, b], Federica Balestra[b], Rosa Palagano[a], Pietro Rocculi[a, b], Tullia Gallina Toschi[a, b].

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Published on: European Food Research and Technology, 2018, 244, 73-82.

Abstract

Taralli is a bakery snack food, typical of the south of Italy, that has currently become very popular worldwide as a savory snack or bread substitute. However, few studies have focused on its physical and sensory characteristics.

The present work aims to select sensory and instrumental information that is able to characterize *Taralli* with similar formulation and size. For sensory characterization purposes, conventional profiling was applied on samples from different producers. All samples were also subjected to physical analysis of appearance and textural proprieties.

Three samples of the set, differing only in storage time, were evaluated to assess changes in sensory characteristics during this period and a discrimination test (triangle test) was also applied for this purpose. The test results confirmed that the sensory analysis allowed a description of the entire range of characteristics resulting from stimulation of senses by physicochemical properties of the food. This methodology was effective in evaluating the quality characteristics and identify differences between *Taralli* samples during different storage times. Instrumental tests were also applied to assess food quality. The results revealed that a combined approach allowed obtaining more information about the product characteristics and definition of quality standards. This study also suggests the use of physical parameters obtained by simple and rapid instrumental tools can support sensory analysis, especially for evaluations that are fatiguing, when decisions made with the sensory data are critical or to provide objective reference standards that are suitable for training purposes.

Keywords

Taralli; Bakery products; Sensory characterization; Image analysis; Texture analysis.

8.4.3 Italian and Spanish commercial tomato sauces for pasta dressing: study of sensory and head-space profiles by Flash Profiling and SPME-GC-MS

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Published on: Journal of the Science of Food and Agriculture, 2017, 97, 3261-3267.

Abstract

BACKGROUND The sensory and head-space profiles of Italian and Spanish commercial tomato sauces have been studied. The Flash Profiling method was used to evaluate the sensory characteristics and samples within each set and were ranked according to selected descriptors. A hundred volatile compounds were identified by SPME-GC-MS. RESULTS For Italian samples, the sensory notes of basil/aromatic herbs, acid and cooked tomato were among the most perceived by assessors, whereas in Spanish ones the sensory attributes of garlic/onion, onion/sweet pepper and, as for the Italian ones, cooked tomato were among the most frequently found. Data were elaborated by multivariate statistical approaches and interesting correlations were seen among different sensory attributes and related volatile compounds. CONCLUSIONS Spanish samples were characterized by highest content of volatiles linked to thermal treatment of tomatoes and to raw and sautéed garlic and onion, whereas the Italian ones by terpenic compounds typical of basil and volatile molecules derived from fresh tomato. These results confirm the influence of formulation and production processes on the aromatic profile (sensory attributes and volatile compounds) of tomato products probably linked to different eating habits and culinary tradition in Italy and Spain.

Key words

Tomato sauce, Sensory profile, Head-space volatiles, Flash Profiling, SPME-GC-MS.

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Rosa