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Metabolomics in action: Towards producing authentic virgin olive oil rich in bioactive compounds and with distinctive organoleptic features

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ABSTRACT

Virgin olive oil (VOO) production is in a constant state of evolution as it adapts to meet the demands of an increasingly competitive global market. However, despite the significant research effort undertaken across multiple scientific disciplines, this vegetable oil still presents challenges and vulnerabilities that require continued research. Within this context, metabolomics has emerged, over the last twenty years, as a promising tool that can resolve several critical issues regarding VOO quality and authenticity. Advanced metabolomics approaches offer unparalleled insights into the molecular mechanisms that influence the sensorial and nutritional features of this vegetable oil in rapid and accurate analyses. This review highlights recent metabolomics applications that unlock the nutritional and sensorial potential of VOO by investigating the effects of various factors on its chemical composition, including environmental conditions, agricultural practices, harvesting time, oil-processing parameters and storage conditions. The principal studies on ensuring product authenticity and traceability are also discussed.

1. Introduction

Virgin olive oil (VOO) is a popular vegetable oil known for its nutritional and health benefits (García-González & Aparicio, 2010). In the current competitive food market and in the face of global climate change, producers are constantly striving to produce the highest quality oils to meet increasing consumer demand. Recent data show that the price of extra virgin olive oil (EVOO) in the main producing countries increased by around 40% in the first quarter of 2023 compared to the same period in 2022 (IOC, 2023a). A similar trend was observed in the harmonized index of consumer prices (HICP) for VOO, which showed an annual rate of change of +26.9% in the EU-27 (IOC, 2023b).

Recent attempts to boost the consumption of VOO have focused on promoting its quality attributes by improving its organoleptic and nutritional properties to meet the growing consumer demand for healthy premium quality VOO that offers a pleasant sensory experience. Many studies have shown that the health-promoting properties of VOO are

mainly due to its balanced fatty acid profile and the antioxidant effect of its phenolic fraction (López-Miranda et al., 2010; Romani et al., 2019; Tsartsou, Proutsos, Castanas, & Kampa, 2019). These compounds not only have an antioxidant and anti-inflammatory effect that protects against chronic diseases, but also contribute to the excellent oxidative stability of VOO.

The taste is also of great importance for marketing strategies, as it strongly influences consumer acceptance and preferences (Aparicio, Morales, & García-González, 2012). The flavor perception of VOO is the result of a complex interplay of taste and aroma compounds, and human olfactory and gustatory receptors (Bendini & Valli, 2012). In addition, although the physicochemical quality criteria such as free acidity, peroxide value and extinction coefficient are mandatory for distinguishing the commercial quality grades of olive oil (extra virgin olive oil, virgin olive oil and lampante) in accordance with European Commission Regulation (EC) 640/2008, the sensory profile also plays a decisive role (European Commission, 2008; IOC, 2013). Indeed, the flavor

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of olive oil is determined by the perception of positive attributes (fruity, bitter and spicy) and negative attributes such as musty, mouldy, vinous, etc. Higher quality VOO is characterized by a well-balanced combination of green and fruity flavors and has no defects. Numerous studies have been carried out to identify and quantify the specific compounds that contribute to the sensory properties of VOO. For instance, phenolic compounds have been largely associated with its bitterness and pungency perceptions (Ajal et al., 2022; Servili et al., 2009). Other authors noted that volatile compounds, especially aldehydes, alcohols, esters and terpenes, are associated with the aroma profile (Genovese, Caporaso, & Sacchi, 2021; Gomes, Freitas, Cabrita, & Garcia, 2012; Procida, Cichelli, Lagazioc, & Conte, 2016; Žanetić, Špika, Ozic, & Bubola, 2021).

Moreover, the issue of authenticity is of pivotal interest for producers, policymakers and regulatory authorities due to the increasing incidence of adulteration in VOOs (Posudin, Peiris, & Kays, 2015). Such fraudulent practices deceive consumers who are prepared to pay a higher price for authentic products of certain varieties or geographical origin (Conte et al., 2020). VOO adulteration may be divided into three principal fraud categories: a) origin (e.g. cultivar, geographical origin, production system), b) substitution with other products of lower commercial value and/or cheaper similar ingredients (e.g. adulteration of olive oil with seed oils, such as sunflower and hazelnut oils), and c) extension of food (e.g. water, colours) (Esslinger, Riedl, & Fauhl-Hassek, 2014). However, due to the complex chemical composition of VOO, an in-depth study of the metabolites involved, especially their qualitative and quantitative contents in response to unexpected variations that might arise from fraudulent practices, is the first step in developing a holistic, promising approach to the production of authentic VOO.

The compounds that give VOO its distinctive characteristics are outcomes of complex interactions between endogenous and external factors that regulate the biosynthesis and the amounts of key compounds in the final product (Ajal et al., 2022; Clodoveo, Hbaieb, Kotti, Mugnozsa, & Gargouri, 2014). They can indeed vary significantly with factors such as harvesting time, climate, cultivar, environment, farming methods and oil-production conditions. Although various conventional methods are available and have been used widely for the analysis of changes in the chemical composition of biological matrices, their accurate and in-depth analysis in VOO poses a great analytical challenge (Tena, Wang, Aparicio-Ruiz, García-González, & Aparicio, 2015). The chemical compounds of VOO present a complex set of analytes that

entail a wide range of chemical diversity and variable concentration levels (Olmo-garcía & Carrasco-pancorbo, 2021). The presence of various structural isomers and the overlapping spectra of these compounds in different matrices further complicate their characterization and quantification (Alvarez-Rivera, Ballesteros-Vivas, Parada-Alfonso, Ibáñez, & Cifuentes, 2019; Olmo-garcía & Carrasco-pancorbo, 2021). Sophisticated analytical methods are therefore needed to overcome these challenges and obtain accurate and reliable analyses of the chemical changes in VOO.

The scientific community has been supporting producers by providing appealing and distinctive added value to their high-quality VOO and protecting consumers from fraudsters over the past two decades (Fig. 1) (Tena et al., 2015). To achieve this goal, it is important to conduct a thorough and comprehensive investigation of all the potential factors that may impact upon the quality, bioactive compounds and organoleptic characteristics of VOO, while developing innovative methods to prevent adulteration and fraudulent practices.

Within this context, metabolomics, which can be defined as the screening and/or quantitative determination of all or particular chemical substances in the biological matrix under study, has emerged in the last two decades to overcome all the above-mentioned challenges (Fiehn, 2001). Unlike genomics and proteomics, which reflect changes in the expression of genes and proteins, respectively, metabolomics provides a picture of the whole set of primary and secondary metabolites present in a biological matrix using advanced technologies such as mass spectrometry (MS)-based techniques and nuclear magnetic resonance (NMR) spectroscopy (Cevallos-Cevallos, Reyes-De-Corcuera, Etxeberria, Danyluk, & Rodrick, 2009; Hu & Xu, 2013; Ibáñez, García-Cañas, Valdés, & Simó, 2013; Klassen et al., 2017; Oms-Oliu, Odriozola-Serrano, & Martín-Belloso, 2013; Valdés, Cifuentes, & León, 2017). Typically, metabolomics can be classified as targeted and non-targeted approaches. A targeted analysis, often named synonymously as metabolic profiling, aims to identify and/or quantify one or more pre-defined metabolites that either belong to the same chemical class, or are involved in a specific pathway (Klassen et al., 2017), whereas non-targeted, or untargeted analysis covers the detection of as many groups of undefined metabolites as possible in a particular biological matrix (Klassen et al., 2017). Targeted approaches are influenced by *a priori* knowledge of chemical composition and possibly by expected concentrations. Careful selection of the appropriate metabolomics

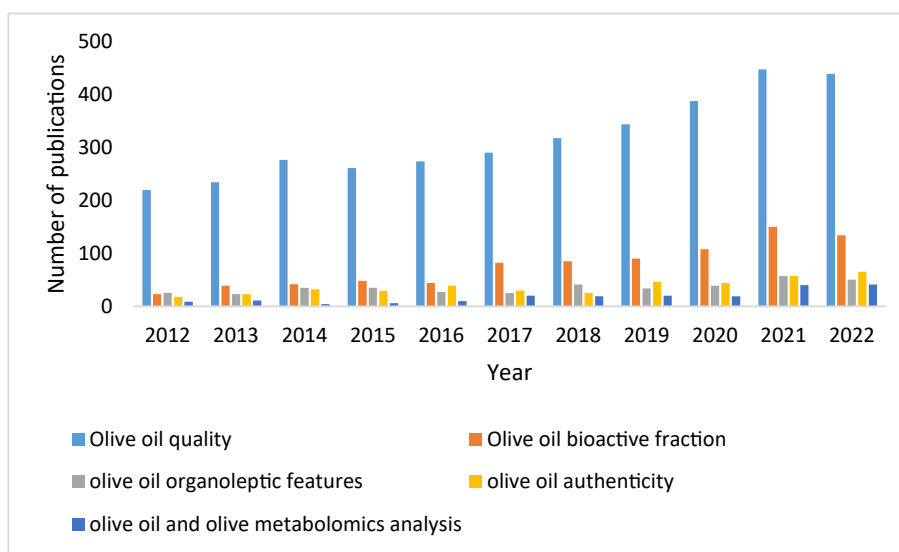


Fig. 1. Number of articles published on VOO quality, organoleptic features, the bioactive fraction, authenticity and metabolomics analysis over the last decade according to the Scopus database. The word search in the article titles, keywords and abstract for each area are as follows: for olive oil quality – olive oil quality; for olive oil bioactive fraction – olive oil bioactive; for olive oil organoleptic features – olive oil organoleptic and olive oil sensorial; for olive oil authenticity – olive oil authenticity and olive oil traceability; for olive oil and olive metabolomics analysis; olive oil metabolomics and olive metabolomics.

approach, based primarily on the goal of the study, is paramount to the success of an experiment.

Metabolomics has recently proven itself to be a powerful tool with which to tackle a broad range of issues related to the analysis of VOO, including its quality, bioactive fraction, sensory features and authenticity (Lioupi, Nenadis, & Theodoridis, 2020; Olmo-garcía & Carrasco-pancorbo, 2021). Through studying all potential changes in the chemical composition of VOO by using sophisticated analytical platforms, metabolomics approaches could help in tracking the impact of various factors. As part of this strategy, it is possible to monitor the entire process of oil production from the planting system and accurate selection of the suitable fruits to the technical parameters of the processing system and storage conditions.

The aim of the present review is to highlight the main findings from metabolomics-based studies that reveal the health-promoting and sensory properties of VOO by investigating the effects of various factors on its chemical composition. The paper also outlines a metabolomics workflow developed for analysing VOOs and emphasizes the major contribution of these approaches to the production of healthy, authentic oils that provide a pleasant sensory experience. The main applications for ensuring authenticity and traceability are also discussed, with a special focus on relevant developments in VOO analysis.

2. Methodology of the study

The current review has been conducted in accordance with the PRISMA guidelines. Web of Science and SCOPUS databases were included for the bibliographic research using the following search terms in titles, abstracts and keywords: Olive AND oil OR VOO; AND bioactive

OR nutritional OR sensorial OR organoleptic OR authenticity OR adulteration. All the found research papers were checked and the studies that do not include a metabolomics-based approach were not considered. Reference lists from all selected articles were also examined for additional relevant studies. The search was limited to studies in English language and the literature was searched from inception to January 01, 2023.

However, this study is not intended to be exhaustive and comprehensive, but rather as a summary of the developments and applications that are of particular interest to the production of authentic and high-quality VOO rich in health-related compounds and with particular sensory properties. Hence, only relevant papers were considered and included in this contribution.

3. Metabolomics workflow in VOO analysis

Metabolomics consists of specific sequential steps including, among others, sample preparation, metabolite extraction, data acquisition and chemometrics (Cevallos-Cevallos et al., 2009). A clear comprehension of each step will be discussed herein. The most common metabolomics steps involved in olive oil analysis are shown in Fig. 2.

3.1. Sample preparation

The first concern in a metabolomics analysis is the establishment of suitable experimental design to provide the right answers to a biological inquiry. Likewise, sample collection is of pivotal importance and may lead to the erroneous interpretation of obtained results if performed poorly. There are various factors to take into consideration during the

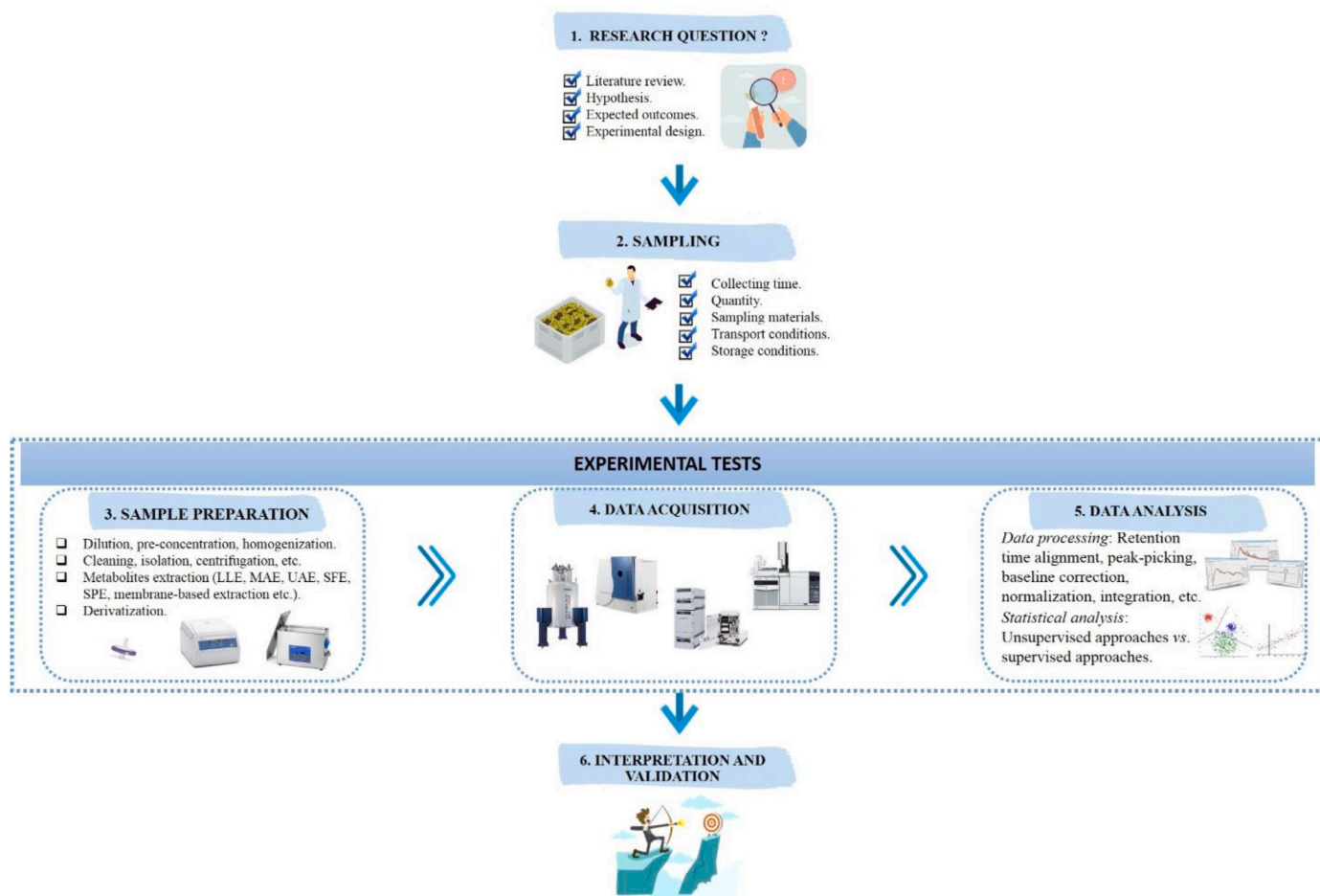


Fig. 2. Most common metabolomics steps involved in VOO analysis.

sampling step, and these include quantity, cultivar, geographical origin, harvest period, environmental conditions, VOO production process, olives' maturity degree, agricultural practices and irrigation modes (Anastasopoulos, Kalogeropoulos, Kaliora, Kountouri, & Andrikopoulos, 2011; Dabbou et al., 2009; Di Giovacchino, Sestili, & Di Vincenzo, 2002; José Motilva, Jesús Tovar, Paz Romero, Alegre, & Girona, 2000). The harvesting time and the collected part of the tree are also critical during sample collection (Kim & Verpoorte, 2010). Fingerprinting and profiling studies conducted on different part of the fruit including the epicarp, mesocarp and endocarp have showed that different tissues have distinctive metabolites (Alagna et al., 2012; Goulas & Manganaris, 2012). Different preparation steps might be considered for fruits analysis depending mainly on the purpose of the metabolomics study. A prior separation of some parts of the olives might be considered when focusing on specific compounds which are known to be present in particular components of the fruit or when studying the repartition of investigated compounds in all tissues. Considering metabolites' extraction, various methods are used ranging from conventional such as Soxhlet and heat reflux extractions to advanced techniques such as ultrasound and microwave assisted extractions (Goulas & Manganaris, 2012). Collected olive fruits must be packed properly and stored in stable conditions to preserve their composition. The analysis of olive fruits may be of utmost interest when looking for new applications or new sources of bioactive compounds in the processed VOO or for understanding the distribution of secondary metabolites in *Olea europaea* L. (Luque De Castro, 2014; Olmo-García et al., 2018). Finally, once VOOs are produced, they are usually stored in glass bottles in the dark, while adding gaseous N₂ to the headspace is recommended to prevent oxidation (Ros et al., 2019).

Even though metabolomics analyses are generally straightforward and well identified, sample preparation is often based on personal experience rather than well-validated scientific protocols. Sample-preparation methods mainly depend on sample type, the metabolomics approach (targeted vs. untargeted), and the selected analytical technique (Klassen et al., 2017). In contrast to targeted approaches, sample preparation in untargeted metabolomics should be kept to a minimum so that a wide range of metabolites can be covered. Targeted metabolomics usually requires preparation methods that are highly selective for the metabolites under study.

Although a simple delipidation step is most commonly used for VOO phenolic profiling, metabolites extraction includes various methods depending on the purpose of the study. For an efficient recovery, the main parameters to be considered are the type of solvent, solvent/sample ratio, extraction period and temperature (Kim & Verpoorte, 2010). Several innovative methods have emerged to overcome the drawbacks of conventional extraction techniques, such as Soxhlet and maceration, and improve extraction efficiency. Among them, ultrasonic-assisted extraction (UAE), microwave-assisted extraction (MAE), pressurized liquid extraction (PLE) and supercritical fluid extraction (SFE), have gained considerable attention in recent years.

Focusing our attention on green solvents, deep eutectic solvents (DES) have been described as recyclable, inexpensive and safe alternatives to conventional solvents such as methanol, ethanol, ethyl acetate and hydroalcoholic mixtures, for the extraction of molecules with high added value. DES have been developed to overcome the main drawbacks of ionic liquids, primarily their toxicity and low biodegradability. DES exhibit good biodegradability, low toxicity and high biocompatibility, and have been shown to have great potential as green solvents when combined with advanced extraction technologies such as UAE, MAE and PLS, among others (Benvenuti, Zielinski, & Ferreira, 2019). For instance, a phenolic profiling-based study conducted by Francioso et al. (2020) has demonstrated the feasibility of using DES in combination with preparative high-performance liquid chromatography for the isolation of strong antioxidants, including hydroxytyrosol, tyrosol, oleacein and oleocanthal, from VOO.

A truly in-depth discussion of sample-preparation techniques is

beyond the scope of this review. However, other techniques deserve to be mentioned such as: Solid-liquid extraction; dispersive-solid-phase extraction; solid-phase microextraction; headspace extraction; subcritical water extraction and membrane extraction (nanofiltration, microfiltration and ultrafiltration). Moreover, extensive information about the abovementioned techniques can be found in previous reports (Brehm-Stecher, Young, Jaykus, & Tortorello, 2009; Jalili, Barkhordari, & Ghiasvand, 2020; Kim & Verpoorte, 2010; Knoll, Rösch, & Huhn, 2020; Martinović, Šrajter Gajdošik, & Josić, 2018; Osorio-Tobón, 2020; Urbanowicz, Zabiegała, & Namieśnik, 2011).

3.2. Data acquisition

The tremendous diversity in the composition of biological matrices makes the development of a single analytical platform that can precisely and accurately detect and quantify the full range of metabolites an unrealistic prospect. Selecting the most suitable technology is thus of utmost priority. The choice must be made on the basis of the type of sample to be analyzed (solid, liquid or gas), sample's quantity, analyst's technical expertise, availability of materials and reagents and expected concentrations (Martins, Sentanin, & De Souza, 2019). There are two conceptually different analytical systems that are the most widely used in contemporary research in VOO metabolomics; Mass Spectrometry (MS)-based and Nuclear Magnetic Resonance (NMR)-based technologies (Araújo, Pimentel, Alves, & Oliveira, 2015; Ibáñez et al., 2013; Lioupi et al., 2020).

The rapidly growing use of NMR spectroscopy can mainly be attributed to its non-destructive character, high accuracy and high reproducibility (Cox, Oh, Keasling, Colson, & Hamann, 2014; Hatzakis, 2019; Maestrello, Solovyev, Bontempo, Mannina, & Camin, 2022; Mannina & Segre, 2002). NMR analysis is equally suitable for both structural elucidation and quantification. Sample preparation in NMR-based metabolomics applied for VOO analysis is minimal (the sample is always dissolved in a deuterated solvent) (Hatzakis, 2019). After analysis, the NMR spectra provides a screening of the chemical compounds present in the studied sample. Interestingly, NMR analysis can also be used for structural elucidation of unknown compounds, fact which helps in the discovery of novel biomarkers in VOO. In this regard, several studies exploring the potential use of NMR-based metabolomics have been published in the last decades (Dais & Hatzakis, 2013; Lioupi et al., 2020; Mannina & Segre, 2002; Tang, Polari, Green, Wang, & Hatzakis, 2022). However, when compared to MS-based methods, NMR is still an underused technique in the field of VOO metabolomics due to its low sensitivity and high cost as well as the need for *a priori* experience in NMR analysis (Hatzakis, 2019; Medina, Pereira, Silva, Perestrelo, & Câmara, 2019; Medina, Perestrelo, Silva, Pereira, & Câmara, 2019).

In this context, the MS-based method has established itself as the top choice for food scientists due to the fact that it enables the analyst to detect and quantify a large number of metabolites in a simple manner (Hu & Xu, 2013; Medina, Perestrelo, et al., 2019; Quirantes-Piné et al., 2013). Innovation in novel soft-ionization MS-based technologies, such as electrospray ionization (ESI), matrix-assisted laser desorption/ionization (MALDI) and atmospheric pressure chemical ionization (APCI), among others, has played a key role in covering a vast number of polar and thermally unstable molecules (Hu & Xu, 2013). MS is usually combined with a prior separation step to guarantee extensive coverage of the full metabolome of a biological matrix (Cifuentes, 2012). Commonly employed separation methods in VOO analysis include: Gas chromatography (GC), liquid chromatography (LC), and capillary electrophoresis (CE) (Araújo et al., 2015; Lioupi et al., 2020; Olmo-garcía & Carrasco-pancorbo, 2021).

MS-based GC analysis is one of the most robust and cost-effective technologies used in food metabolomics (Hernández, Portolés, Pitarch, & López, 2011; Lioupi et al., 2020; Waktola, Zeng, Chin, & Marriott, 2020). It is mostly related to fatty-acid and volatile-organic-compound profiling (Cox et al., 2014). The main advantages of the GC approach

are the availability of extensive mass spectra databases for metabolite identification and the great resolution ability of GC columns (Griffiths et al., 2010). Nonetheless, GC-MS usually requires a derivatization step for the analysis of non-volatile compounds. This fact has enhanced the use of alternative separation methods, such as LC and CE technologies.

Indeed, LC has tremendous potential in the global screening of VOO chemical components as it can separate and identify a wide range of metabolites with unparalleled sensitivity (Hu & Xu, 2013; Ibáñez et al., 2013; Medina, Perestrelo, et al., 2019; Pyrzyńska & Sentkowska, 2015). Although high-performance liquid chromatography (HPLC) is the most popular LC method, ultra-high performance liquid chromatography (UHPLC) has become the top priority for chemical analysts in the last decade. UHPLC typically requires smaller columns and minimum solvent volumes, making it less time consuming than the standard HPLC method (Medina, Perestrelo, et al., 2019; Pyrzyńska & Sentkowska, 2015). However, despite the significant progress in LC technologies, there is a lack of global LC-MS libraries with extensive information for rapid and accurate metabolite identification unlike GC databases, while inter-laboratory reproducibility is also a concern.

Capillary electrophoresis (CE) is an electrophoretic technique that can be coupled to MS for the separation of polar and charged molecules (García-Cañas, Simó, Castro-Puyana, & Cifuentes, 2014; Knoll et al., 2020; Ramautar, Somsen, & de Jong, 2015). The rapid development of CE is mainly attributed to its high-resolution ability, fast analysis speed, low sample size and low solvent volume requirements (Cifuentes, 2006; García-Cañas et al., 2014; Ibáñez et al., 2013). Unlike other separation techniques, reduced sensitivity and low reproducibility in quantitative analyses are the main drawbacks of CE, precluding its use for the analysis of trace compounds (Medina, Perestrelo, et al., 2019; Ramautar et al., 2015; Valdés et al., 2017).

Besides the abovementioned techniques, other analytical technologies have been applied in the study of VOO, including vibrational spectroscopy (Fourier-transform infrared spectroscopy (FTIR), mid-IR (MIR), near-IR (NIR) and Raman spectroscopy), inductively coupled plasma MS (ICP-MS), Fourier transform ion cyclotron resonance MS (FT-ICR-MS) and proton transfer reaction-MS (PTR-MS) (Araújo et al., 2015; Brenner, 2017; Lioupi et al., 2020; Wang, Sun, Zhang, & Liu, 2016).

3.3. Chemometrics

The complexity of raw metabolomics datasets entails various processing and treatment steps prior to data analysis and interpretation. Processing can reduce and even remove noise and unimportant variations. There is a variety of software that can enable users to handle raw data and perform retention-time alignment, peak-picking, baseline correction, filtering, normalization, centering and scaling, integration, etc. (Hu & Xu, 2013; Waktola et al., 2020). These steps are of utmost interest because, if inadequately performed, data interpretation can be disordered and thus lead to fallacious results (Hu & Xu, 2013).

The data, once processed, is subject to statistical analysis. This can be achieved using several statistical approaches. Although some studies may involve simple statistical analyses, such as one-way analysis of variance (ANOVA), they may be classified into two basic categories; unsupervised and supervised analysis (Kumar, Bansal, Sarma, & Rawal, 2014; Mendlein, Szkudlarek, & Goodpaster, 2013). Unsupervised approaches include principal component analysis (PCA), correspondence analysis (CA), hierarchical cluster analysis (HCA) and k-means are performed without any preliminary information about the studied data. Within this category, PCA is considered the most commonly used multivariate method in metabolomics as it can be used for a wide range of multivariate data sets including spectra and chromatograms (Cevallos-Cevallos et al., 2009; Mendlein et al., 2013; Messai, Farman, Sarraj-Laabidi, Hammami-Semmar, & Semmar, 2016; Rathore, Bhushan, & Hadpe, 2011; Richards & Holmes, 2015). The principal idea of PCA is to identify trends of individuals through the condensation of large-dimension data sets into reduced and significant matrices

(principal components), while preserving the variation present in the initial data set (Kumar et al., 2014; Messai et al., 2016).

Likewise, supervised methods are guided by *a priori* knowledge, which will direct the final outcomes of the study (Messai et al., 2016). This class includes, among others, linear discriminant analysis (LDA), k-nearest neighbor (k-NN), partial least squares (PLS), support vector machines (SVMs), and soft independent modeling by class analogy (SIMCA), while artificial neural networks (ANNs) include various supervised and unsupervised methods (Messai et al., 2016).

Another classification can be made if we consider intended outcomes. In this way, multivariate data analysis can either be informative, discriminative or predictive (Cevallos-Cevallos et al., 2009; Trygg, Gullberg, Johansson, Jonsson, & Moritz, 2006). Informative analyses aim to establish links between samples and variables, as well as then identifying the most influential variables (Callao & Ruisánchez, 2018). Discriminative methods focus on the identification of distinguishing characteristics prior to the classification of samples into homogeneous groups (Cevallos-Cevallos et al., 2009). The most commonly used methods in this category include PCA, HCA, LDA, k-NN, PLS-discriminant analysis and SIMCA. Finally, predictive approaches allow the elaboration of mathematical models with the intent of predicting a target variable (number, property, behavior, etc.) (Cevallos-Cevallos et al., 2009; Singh, Juneja, Kaur, & Kumar, 2013). Within this category, PLS, multi-linear regression (MLR) and principal component regression (PCR) are the most widely used methods.

More in-depth information on chemometric methods in the field of metabolomics can be found in previous papers (Cook & Rutan, 2014; Kanginejad & Mani-Varnosfaderani, 2018; Kumar et al., 2014; Messai et al., 2016; Pinto, 2017; Roberts & Cozzolino, 2016; Savorani, Rasmussen, Mikkelsen, & Engelsen, 2013; Singh et al., 2013; Valdés et al., 2017).

Obviously, once the data is analyzed, the obtained results must be interpreted and discussed in a clear and complete manner that considers all influencing factors.

4. Metabolomics for the investigation of the nutritional, health-promoting and sensory properties of VOO

4.1. Nutritional and health-promoting properties

Metabolomics strategies have proven to be effective in revealing the nutritional value and health-related properties of VOO through the in-depth study of its bioactive matrix. For instance, the phenolic fraction of the latter olive tree-derived product has displayed remarkable inhibitory effects on enzymes involved in chronic diseases, including diabetes, obesity and hypertension in a MS-based metabolomics study conducted by Loizzo, Lecce, Boselli, Menichini, and Frega (2011).

On a related note, an MS-based profiling approach has been developed for the identification of phenolic compounds in plasma and urine after the intake of a phenols-rich VOO (Rubió et al., 2014). The results identified hydroxytyrosol sulfate and hydroxytyrosol acetate sulfate as compliance biomarkers that are expected to have promising health benefits (Rubió et al., 2014). The intake of phenolic compounds-rich VOO was also found to be linked to a significant decrease in LDL oxidation leading to a better protection against cardiovascular disorders, as reported by Castañer et al. (2012). Likewise, replacing saturated fatty acids with phenols-rich VOO can induce beneficial cardiometabolic and hepatic effects as it reduces oxidative stress and modulates the circulation of amino-acid levels (Ruocco et al., 2022).

De la Torre et al., (2020) have demonstrated the positive effects of maslinic and oleanolic acids, which are naturally occurring in VOO, on endothelial function in humans. VOO supplementation was also found to ameliorate diet-induced metabolic syndrome, mainly *via* the modulation of the biosynthesis pathway of branched-chain amino acids, while oleic acid has been identified as one of the main biomarkers in a recent untargeted metabolomics study based on UPLC-MS coupled to

chemometrics (Zhi-Hao et al., 2022).

VOO is characterized by a high content of monounsaturated fatty acids, being oleic acid the major component, and lower concentrations of both polyunsaturated fatty acids (mainly linoleic ($\omega 6$) and linolenic ($\omega 3$) fatty acids) and saturated fatty acids (mainly palmitic and stearic fatty acids). This well balanced fatty acid profile together with the quite high $\omega 6:\omega 3$ ratio (ranging between 1 and 21) link VOO with protective effects against various health issues such as cardiovascular diseases, inflammatory disorders and several types of cancers (Caravita et al., 2007; Lombardo, Grasso, Lanciano, Loria, & Monetti, 2018; Mariotti & Peri, 2014). In this regard, an MS-based approach that combines the LC-MS method and chemometric tools, principally PCA and OPLS-DA, has demonstrated VOO's protective activity against oxidative stress in parenteral nutrition mixtures containing ω -3 polyunsaturated fatty acids (Kosek et al., 2020). Specifically, its main compound, oleic acid, has been highlighted as useful in improving immune response and preventing cardiovascular diseases, metabolic disturbances and cancers (Bermudez et al., 2011; Lopez et al., 2014; Sales-Campos, Souza, Peghini, Silva, & Cardoso, 2013; Servili et al., 2014).

Vitamin E (α -tocopherol), another important component of VOO, has also been widely targeted using metabolomics for quantification purposes, which may boost its commercialization from a nutritional point of view (Aresta & Zambonin, 2017). A metabolomics study based on reversed phased LC was also developed for a rapid determination of tocopherols (α -, sum of (β + γ), and δ), pigments (chlorophylls and carotenoids) and squalene in VOO (Martakos, Kostakis, Dasenaki, Pentogennis, & Thomaidis, 2020). Seçmeler and Güçlü Üstündağ (2017) have developed a rapid and accurate GC-based method for the identification of various lipophilic bioactive molecules in VOO, including α -tocopherol, squalene and β -sitosterol. Another relevant study quantified tocopherols together with other bioactive compounds (lipophilic and hydrophilic phenols) in VOO using an LC-MS approach combined with chemometrics (Róžańska et al., 2020). The work suggested that high-quality VOOs were associated with a higher concentration of secoiridoids and flavonoids, while lower quality samples were mostly characterized by a higher content of gallic acid and β -tocopherol. However, it seems difficult to attribute low-quality VOOs to the latter compounds in order to assess its nutritional value, as both compounds are known to be present in low amounts in VOO.

Considering the above-mentioned metabolomics-based evidence for the health-promoting properties of VOOs and the great progress in the development of robust targeting approaches for the quantification methods of its bioactive compounds, the use of nutrition and health claims on VOO bottles can help producers and stakeholders in positioning their products on the market and promote their consumption. Although the regulated quality of VOO has long been well-defined by national and international standards and regulations based on specific physico-chemical and organoleptic criteria (Aparicio, Morales, Aparicio-Ruiz, Tena, & García-González, 2013), it is only recently that VOO has been allowed to carry labels that emphasise its potential as a functional food with beneficial effects on consumer health (Lockyer & Rowland, 2014). Legislation such as that of the European Union (EU) and the United States of America (Boskou, 2015) as well as in some Mediterranean countries such as Morocco (Gouvernement du Maroc, 2013) strictly regulate the conditions for the use of these claims. Interestingly, in the absence of a standardised classification system for vegetable oils based on their nutritional quality, this approach can play a crucial role in maintaining the loyalty of traditional consumers and capturing new markets by promoting the product as a distinctive and healthy functional food with a unique taste and a variety of health benefits. This will further drive the switch from vegetable oils to VOO, as VOO consumption accounts for around 1% of total vegetable oil consumption worldwide (FranceAgriMer, 2022).

4.2. Sensory quality

Although the nutritional and health-promoting properties of VOO are of utmost interest in marketing strategies, consumers' preferences depend also on its sensory perceptions. The intensity and activity of the enzymes involved in the formation of the volatile compounds responsible for the flavour of VOO influence both their qualitative and quantitative composition (Campestre, Angelini, Gasbarri, & Angerosa, 2017). Indeed, it is a major challenge to associate each volatile compound with a distinctive sensory attribute. However, chemometric tools have proven useful to establish links between sensory notes provided by different panels and instrumental data. For example, Angerosa and colleagues applied a linear regression analysis to the sensory notes perceived by a panel of tasters and to C5 and C6 compounds (Angerosa, Mostallino, Basti, & Vito, 2000). Their results suggest that hexanal plays a key role in the formation of most green attributes. Likewise, in a volatile profiling-based study, C6 and C5 volatile compounds were strongly related to the green fruity sensory attributes of Tunisian and Italian VOO samples (Kotti, Cerretani, Gargouri, Chiavaro, & Bendini, 2011). Procida and co-workers have also identified the correlation between C5 and C6 aldehydes and alcohols and the aroma profile of Italian samples (Procida et al., 2016).

A combination of mid-infrared (MIR) spectroscopy data and the PLS-DA method allowed the identification of musty, winey, fusty and rancid organoleptic defects in VOOs based on specific spectral regions, and thus may be considered a complement to the official Panel Test (Borràs et al., 2015). Such fingerprinting methods are highly recommended mainly on an industrial scale due to their rapidity and nondestructive nature. The predictive models used within this study were able to discriminate between the defective and non-defective oil categories with a prediction accuracy ranging between 70 % and 90 % (Borràs et al., 2015). One year later, the same research group developed a low-level data fusion strategy of three analytical techniques, head space-MS, MIR spectroscopy and UV-visible spectrophotometry, which, together with PLS-DA statistical analysis, was able to effectively identify musty, winey and fusty defects in olive oil samples (Borràs et al., 2016). However, data fusion strategies require the combination of several analysis methods, which limits their use in routine analysis at the industrial level due to the lack of cost-effectiveness and time efficiency.

In another relevant study conducted by Tomé-Rodríguez and colleagues, the highest contents of 1-penten-3-one, hexanol, 2-hexenal and 2-hexenol were found in EVOOs with 'medium' fruitiness intensity, while hexanal, 3-hexenol, 1-penten-3-ol, 2-pentenal and 3-hexenal were more abundant in 'highly intense' oils (Tomé-Rodríguez, Ledesma-Escobar, Penco-Valenzuela, Calderón-Santiago, & Priego-Capote, 2022). The same study attributed the 'ripen' fruitiness with high concentrations of 3-hexenal, 2-hexenal and 2-hexenol, while the highest concentrations of 2-pentenal and 1-penten-3-ol were related to 'green' notes and high levels of 1-penten-3-one, hexanal and hexanol to EVOOs with 'green' notes. The phenolic profile of VOO can also induce an effect on the intensity and timing of the release of some aroma compounds (ethyl butyrate, *cis*-3-hexenyl acetate, ethyl acetate, 1-penten-3-one, *trans*-2-hexenal, hexanal, 1-hexanol, and linalool) as revealed in a study conducted by Genovese, Yang, Linforth, Sacchi, and Fisk (2018).

Furthermore, phenolic compounds were widely related to the pungent and bitter taste characteristics of VOO (Servili et al., 2009). For instance, metabolomics has been able to link phenolic compounds, mainly tyrosol and oleuropein aglycone, with the bitterness and pungency of VOO (Cerretani, Salvador, Bendini, & Fregapane, 2008). A few years later, a rapid screening of VOO samples using near-infrared spectroscopy confirmed the positive correlation between its bitter taste and its phenolic composition (Inarejos-García, Gómez-Alonso, Fregapane, & Salvador, 2013). Other researchers have correlated the bitter sensory notes of VOO to oleuropein and ligstroside aglycons such as *p*-HPEA-EDA, 3,4-DHPEA-EDA and 3,4-DHPEA-EA (Mateos, Cert,

Pérez-Camino, & García, 2004; Tovar, Motilva, Luna, Girona, & Romero, 2001) while the pungency was mainly related to the deacetoxylogistroside aglycon *p*-HPEA-EDA (Beauchamp et al., 2005).

An in-depth discussion on the link between VOO chemical composition and its sensory characteristics has been perfectly presented in a book chapter by Taticchi, Esposto, and Servili (2014).

5. Metabolomics applied for studying the influence of various factors on health-related compounds and sensory properties

5.1. Environmental conditions and agricultural practices

Since olive tree water status has been shown to induce a non-significant effect on the physicochemical criteria of VOO (Caruso et al., 2014; Sánchez-Rodríguez et al., 2019), it is of paramount importance to investigate the in-depth changes in its chemical composition. In view of the efficiency, robustness and high-selectivity of advanced high-throughput analytical technologies, in combination with the power of chemometric methods, metabolomics has therefore great potential to study the changes that occur in the chemical fingerprints of VOOs in response to different environmental conditions and agricultural practices. For instance, daily irrigation has been linked to lower contents of the pigments, α -tocopherols, oleic acid and most phenolic compounds, compared with oil samples produced from rain-fed trees in a study conducted by using LC for tocopherols' analysis, GC-MS for fatty acids and LC-MS/MS system for phenolic compounds (Faghim et al., 2021). The impact of three irrigation strategies, namely sustained deficit irrigation (SDI), low-frequency deficit irrigation (LFDI) and full irrigation (FI), on the quality of VOO from 'Arbequina' cv. olive trees grown in southwest Spain has also been assessed by García et al. (2013). The results show that the two deficit irrigation systems resulted in better quality in terms of carotenoid, chlorophyll, phenolic and mono-unsaturated fatty acid contents, whereas the LFDI strategy led to high contents of oleacein (dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol, or 3,4-DHPEA-EDA) and oleocanthal (dialdehydic derivative of decarboxymethyl elenolic acid linked to tyrosol, or *p*-HPEA-EDA) in a targeted metabolomics study based on both LC and GC (García et al., 2013). Likewise, Sánchez-Rodríguez and co-workers concur that using regulated deficit irrigation (RDI), instead of full irrigation, during pit hardening improved the nutritional and sensorial quality of VOO (Sánchez-Rodríguez et al., 2019, 2020). The reported studies, conducted on Spanish 'Arbequina' cv. samples, highlighted significant increases in the levels of phenolic compounds and mono-unsaturated compounds as well as a well-balanced volatile profile and a fruitiest flavor, and thus confirming the sustainability and positive effect of deficit irrigation strategies on high-quality VOOs.

In a volatile profiling study, Fernandes-Silva and coworkers revealed a decreasing tendency for the content of volatile compounds with increasing amounts of water, as well as higher intensities of bitterness and pungency in processed oils linked to deficit irrigation of 30% of crop evapotranspiration and rain-fed regimes (Fernandes-Silva, Gouveia, Vasconcelos, Ferreira, & Villalobos, 2013). Through a phenolic and volatile profiling-based metabolomics study, Caruso et al. (2014) have also proved that changes occur in VOOs in response to soil water availability in a three-year experiment in a high-density orchard located at Venturina, Italy. VOOs linked to trees with high water status had lower concentrations of phenols and *O*-diphenols than those obtained from trees with only complementary irrigation (Caruso et al., 2014). Furthermore, trees with lower water stress produced olive oil with higher contents of (*E*)-2-hexen-1-ol, which is associated to the 'fruity' and 'cut grass' flavors. The same trend was observed in a phenolic and volatile profiling of Chilean VOOs (Romero, Saavedra, Tapia, Sepúlveda, & Aparicio, 2016).

Considering that the main volatile compounds are formed during oil accumulation via the lipoxygenase pathway from linoleic and linolenic acids (García et al., 2017; Servili et al., 2007), the general increase in

their concentrations in VOOs associated with deficit irrigation systems suggests an enhanced effect of this pathway in response to water stress. An increase in water supply to olive trees was also associated with a decreasing trend in phenolic compounds, which can be attributed to the plant's limited antioxidant defence system. However, a sharp increase in phenolic content can lead to a VOO with excessive bitterness, which limits consumer acceptance. Therefore, regulated deficit irrigation systems seem promising for a balanced presence of phenolic and volatile compounds in VOO. However, the use of reclaimed water for irrigation systems would be more favourable from a sustainability point of view. A research paper on FI (100% of daily evapotranspiration) and RDI (50% of daily evapotranspiration) with two water sources - desalinated water (DW) and reclaimed water (RW)- reported that the levels of total phenolic content and ω 6/ ω 3 ratio were higher in oils from Italian 'Arbosana' cv. olive trees treated with RW and RDI, while reduced oleic acid content was also observed (Romero-Trigueros et al., 2019). Further research in this regard would fill the current gap in understanding the effects of various types of treated water on the chemical composition of processed VOOs, taking into account the characteristics of each type. Minerals are crucial for plant growth and knowledge of their impact on the chemical composition of VOO can lead to specific fertilization practices. Nitrogen foliar fertilization during the oil-accumulation phase failed to induce any significant changes in VOO chemical composition in a four-year study conducted by Regni and Proietti (2019) on Italian 'Frantoio' cv. olive trees. Tekaya and co-workers have revealed the negative influence of two nutrient-based fertilizers - one rich in nitrogen and the other in boron, magnesium, sulfur and manganese - in a two-year study on VOO quality (Tekaya et al., 2013). This study linked foliar fertilization with a considerable decline in phenolic and *O*-diphenol contents, whereas no significant changes were observed in the fatty-acid profile of samples from 'Picholine' cv. olive trees grown in Tunisia.

In a related area of expertise, soil type was found to be a significant factor for VOO quality in a metabolomics study performed, using a HS-SPME and GC-MS approach, on samples from olives of the variety 'Chemlali' grown in Sousse, Tunisia (Ben Rached et al., 2017). Even though all samples displayed similar volatile profiles, significant differences were found in the individual concentrations of volatile compounds in VOOs from various soil types (sandy, clay, stony, brown and limestone). 1-Hexanol was the principle volatile constituent in VOOs from sandy and brown soils, while 2-hexenal was the main volatile component in clay, stony and limestone soils (Ben Rached et al., 2017). In addition, β -sitosterol was the most abundant sterol, while stigmasterol was found in minor quantities in all analyzed samples. The highest concentration of β -sitosterol was found in sandy soil, while clay soil had the lowest. Regarding tocopherols, the highest content of α -tocopherol was obtained in VOOs samples from stony soil, and the lowest in sandy soil.

Furthermore, temperature can be a critical factor among environmental parameters. High temperatures have been linked to considerable reductions in phenolic- and oleic-acid contents in VOO (Nissim et al., 2020). However, five varieties ('Barnea', 'Koroneiki', 'Coratina', 'Souri' and 'Picholine') were used in the experiment, and each cultivar responded differently to the high temperature environment. 'Souri' was reported to be the most tolerant to high temperatures in the cultivars studied. The same declining trend in oleic-acid content in VOO has also been reported in trees grown in hot climates in Tunisia, in a GC-based study conducted by Ben Rouina et al., (2020). This trend suggests that olive cultivars are usually able to develop various distinctive mechanisms and defense systems in response to environmental stress.

5.2. Optimal harvesting time

From an agronomic point of view, harvesting time is one of the key factors in achieving satisfactory production yields, balanced chemical composition and distinctive organoleptic properties. To determine the

optimal harvest time, VOO producers generally rely on the change in fruit color, which is directly related to the stage of ripeness. However, fruit color alone is not sufficient to make an accurate assessment, considering the significant variations that can be caused by environmental, genetic and agronomic factors. A comprehensive analysis of the chemical composition of olives at different stages of ripeness is therefore crucial for determining the optimal harvest time.

Late harvesting should be avoided to produce high-quality VOO, as most studies have reported. For instance, Amanpour, Kelebek, and Selli (2019) have performed an MS-based phenolic profiling of Turkish cv. 'Nizip Yaglik' VOOs of different ripening periods to appraise the influence of olive-ripening stage on the phenolic composition of the produced oils (Amanpour et al., 2019). In this study, both the total phenolic content and the concentration of individual phenols decreased significantly with advancing olive-fruit ripening stage, and all samples presented quite similar phenolic profiles. A similar trend was observed in a study, over two consecutive years, of 'Frantoio' and 'Manzanilla' VOOs samples processed from olives grown in Australia (Alovaesh, Singh, Fang, & Kailis, 2018). An early harvesting date also appeared to be most suitable for samples from the 'Coratina' cultivar grown in Chongqing, southwest China (Huang et al., 2020). Indeed, the chemical profiles revealed that advanced harvesting periods led to a serious decline in the contents of hydroxytyrosol, rutin, oleic acid, total phenols and total flavonoids.

A maturity index of 2.4 has been suggested as the most suitable period for collecting olives in north-central Algeria to produce 'Chemlal' VOO of superior quality (Bengana et al., 2013). The results obtained in this study revealed that a decrease in phenol and chlorophyll content occurred with later ripening stages. This significant loss led to a lessening of the VOO throat irritating sensation and made the oil's color more yellow (Bengana et al., 2013). Similar outcomes have been observed in samples processed from three olive cultivars grown in southern Tunisia ('Jemri', 'Fakhari' and 'Touffehi') (Ben Brahim & Bouaziz, 2019).

Another research paper reported by Nsir et al. (2017) showed a significant decline in the contents of tocopherols, squalene, carotenoids and polar phenols, at later maturity stages, while high intensity was observed in the desirable organoleptic characteristics (fruity, bitterness and pungency) in Tunisian 'Sayali' cv. VOOs processed with less ripe olive fruits.

In a study conducted on 'Picual' and 'Hojiblanca' samples, Jimenez, Sánchez-Ortiz, Lorenzo, and Rivas (2014) have established that maturity stage has a higher effect on tocopherol contents, fatty-acid composition and phenolic-compound contents than farming system (organic vs. conventional) (Jimenez, Sánchez-Ortiz, Lorenzo, & Rivas, 2014). The contents of palmitic acid, α -tocopherol and most phenolic compounds showed significant declines during the fruit-ripening process. One year later, the same group revealed the effect of ripening stage on the nutritional properties of 'Picual' and 'Hojiblanca' VOOs (Jimenez, Sánchez-Ortiz, Lorenzo, & Rivas, 2015). The results confirmed the considerable influence of olive maturation stage on the bioactive micro-constituents and suggested that the lignoceric and stearic acids are linked to unripe and ripe olive fruits, respectively.

In another work, the effect of ripening stage on the C6 and C5 compounds in various Turkish VOOs was investigated using a MS-based profiling approach. The results showed an increase in the total content of esters when olive-fruit ripeness progressed (Karagoz et al., 2017). Ouni, Flamini, and Zarrouk (2016) have demonstrated the feasibility of the volatile-profiling approach in establishing the link between maturity stage and oil quality. Their results suggested that an optimum ripening index between 2.0 and 3.0 was the best period for harvesting 'Oueslati' olive fruits in the center of Tunisia.

Reboredo-rodríguez et al. (2020) have revealed that an increase in the concentrations of oleic and linoleic acids, total tocopherols, oleocanthal, tyrosol, luteolin and apigenin in cv. 'Brava Gallega' VOOs from Spain occurred as ripening progressed, whereas total phenolic content

remained relatively unchanged. Meanwhile, El Sohaimy, El-Sheikh, Refaay, and Zaytoun (2016) have found that 'Manzanilla' and 'Kalamata' cv. VOOs gave the highest quality, in terms of their phenolic and fatty-acid compositions, during their reddish ripening stage.

However, Piscopo and co-authors (2018) have stated that the effect of harvest period on the quality of 'Carolea' and 'Sinopolese' VOOs produced in south of Italy is not worth considering, whereas 'Otto-bratica' and 'Grossa di Gerace' samples displayed lower quality as maturation progressed (Piscopo, Zappia, Bruno, & Poiana, 2018).

It is generally recognised that late harvest times lead to higher oil yields, while the quality concept is of particular interest to producers who want to market their products at top prices. In general, an early ripening stage has been associated with a higher content of nutrients and bioactive compounds and a better organoleptic perception. An optimal ripening index between 2.0 and 3.5 seems to be highly recommended to produce a VOO rich in health-relevant ingredients and with pleasant sensory properties. Late ripening can expose the olive flesh to mechanical and parasitic attacks that lead to fermentation and esterification of fatty acids (Piscopo et al., 2018). Nevertheless, the differences observed in the response of the varieties must be taken into account and therefore the genetic factor should be considered when determining an optimal ripening time.

5.3. Oil processing parameters

The mechanical extraction of VOO leads to substantial variations in nutritional and aroma attributes. Hence, investigating the impact of various extraction parameters is of pivotal interest, and has therefore been widely reported over the last decade. Some fascinating studies on metabolomics applications for assessing the changes occurring in VOO composition in response to various processing parameters are discussed herein.

Great differences in the aroma profiles of VOO samples processed using two extraction systems (discontinuous vs. continuous) have been reported in a GC-MS-based analysis conducted by Issaoui et al. (2015). The oils processed in a discontinuous (pressing) system had the highest contents of alcoholic constituents and fusty/muddy sensory notes, whereas the levels of hexanal and (*E*)-2-hexenal were greater in samples produced by a continuous (centrifugation) processing system. The same trends have been reported by Volpe et al. (2014). In this latter volatile profiling study, the authors reported the presence of a significant amount of isoamyl alcohol, which is related to some negative aroma attributes, in samples from the traditional discontinuous system.

Furthermore, as centrifugation, by means of a decanter, is currently the most recommended system, Antonini, Farina, Scarpa, Frati, and Ninfali (2015) have investigated the differences in the phenolic profiles of VOOs processed using two-phase and three-phase decanters. Interestingly, samples from the two-phase system had the highest contents of oleacein, oleocanthal, oleuropein aglycone, lignans, (+)-pinosresinol and (+)-1-acetoxypinosresinol. Besides enhancing the nutritional and bioactive contents of the produced oil, a key advantage of the two-phase decanter relies on its capability to operate without the generation of wastewater. This stands in contrast to press and three-phase systems, thus underscoring its environmental sustainability and efficiency in the processing of VOO.

On a different note, an untargeted metabolomics study has shown that a decrease in inner-fruit temperature before processing can positively affect VOO sensory quality (Dourou et al., 2020). A significant decrease in the content of several volatile components that are related to some particular defects in VOO organoleptic properties, namely 1-penten-3-ol, 1-penten-3-one, acetic acid and ethyl alcohol, was reported as fruit temperature decreased (from 19 to 10 °C). In fact, although 1-penten-3-one has been associated with green notes and, to a lesser extent, with bitterness and pungency, it may have a notable negative correlation with the fruity attribute, while 1-penten-3-ol has been negatively correlated to almond note, and both ethanol and acetic acid

show a direct association with the winey/vinegary defect (Campestre et al., 2017). Furthermore, the concentrations of hexanal and 2-hexenal, which have been reported to be responsible for the green leafy sensory notes, were higher when the fruit inner temperature was reduced to 15 and 10 °C (Dourou et al., 2020).

Likewise, in a phenolic-profiling study, Veneziani et al. (2017) have demonstrated the positive effect that olive-cooling treatment can have on VOO quality characteristics. The results show a tremendous increase in phenolic concentration levels in processed ‘Coratina’, ‘Peranzana’ and ‘Ottobratica’ cv. VOO as olive-paste temperature declined from approximately 27 to 15 °C. The phenolic profile of VOO is largely determined by the actions of polyphenol oxidase, peroxidase and β -glucosidase (García-Rodríguez, Romero-Segura, Sanz, Sánchez-Ortiz, & Pérez, 2011). The increasing trend of phenolic compounds levels during olive-cooling treatment may therefore be attributed to a reduced action of these enzymatic activities at lower temperatures (<20 °C) (Taticchi et al., 2013; Veneziani et al., 2017).

Although easy to manage, crushing speed can also have a considerable effect on the health and sensorial properties of VOO. Indeed, Guerrini, Migliorini, Giusti, and Parenti (2017) have demonstrated, using a volatile and phenolic-profiling approach, that the contents of chlorophylls and the main phenolic compounds responsible for oxidative stability, oleacein, and oleocanthal, increased linearly with crushing speed, while volatile constituents were less impacted. The concentration of *E*-2-hexenal, which is associated to the ‘green’ taste, was negatively correlated to crushing speed (from 17.80 mg/kg at 2200 rpm, to 16.50 mg/kg at 3200 rpm). Another four volatile compounds showed some very limited changes; hexyl acetate, *Z*-3-hexenal, *Z*-3-hexenyl acetate and *Z*-3-hexenol (Guerrini et al., 2017). A similar tendency has been reported by Polari, Garcá-Aguirre, Olmo-garcía, Carrasco-Pancorbo, and Wang (2018), who investigated the influence of industrial hammer-mill rotor speed on VOO quality, and revealed that the contents of total phenols, oleacein, oleocanthal, oleanolic acid and maslinic acid increased when the speed was intensified from 2400 to 3600 rpm. The volatile profiles in studied samples were quite similar regardless of rotor speed, whereas the pungency taste was more pronounced at higher rotor speeds. The increased pungency in VOO may be explained by the higher levels of phenolic compounds in samples generated under higher rotor speeds, thus affirming the correlation between the phenolic content and the perceived intensity of pungency in the processed oil.

Selecting the most suitable time and temperature conditions for oil-processing during the paste malaxation step is also crucial to ensure high contents of desired aroma and bioactive constituents. Phenolic compounds are more strongly influenced by the malaxation temperature than by time. In general, higher amounts of phenolic compounds can be obtained at a temperature between 27 and 30 °C, which then steadily decrease, as shown by a study on the phenolic profile carried out by Parenti, Spugnoli, Masella, and Calamai (2008). This could be explained by the thermal degradation of these compounds or the increased enzymatic activities, such as polyphenol oxidase and peroxidase, at higher temperatures, while the lower amount at lower temperatures is probably due to the limited release of certain phenolic compounds bound to other molecules in the fruit. An opposite trend was observed in the phenolic profile when the temperature was increased from 27 to 47 °C, as most phenolic compounds, including hydroxytyrosol, tyrosol, pinosresinol and *p*-coumaric acid, increased linearly with malaxation temperature in a study conducted on ‘Ayvalik’ and ‘Memecik’ cv. VOOs (Jolayemi, Tokatli, & Ozen, 2016).

Another study conducted by Cevik, Ozkan, and Mustafa (2016) using (GC-MS)-based metabolomics profiling showed that low temperatures and short times are associated with a VOO of good organoleptic attributes, and, using the response surface methodology, the authors identified 28 °C/38 min and 29 °C/40 min as the most appropriate for samples processed from ‘Memecik’ cv. purple and black olives, respectively. Although all samples studied were classified as EVOO, Olmo-Cunillera et al. (2022) have revealed a positive effect of cold malaxation

at 20 °C on the aroma and flavor of VOO when compared with 25 and 30 °C. Temperatures above 25 °C can decrease the activity of two key enzymes involved in the lipoxygenase (LOX) pathway, namely LOX and hydroperoxide lyase, resulting in a decrease in the formation of C6 saturated and unsaturated aldehydes, alcohols, and esters, which are responsible for the cut grass and floral sensory notes of VOO (Angerosa, Mostallino, Basti, & Vito, 2001; Salas & Sanchez, 1999). The opposing findings found in the literature indicate that the cultivar used could potentially impact the release of key compounds during oil extraction. It is therefore necessary to conduct a thorough investigation into the mechanisms involved in the distribution of key compounds within both fruits and their respective oils during the malaxation, taking genetic factors into account.

Focusing our attention on malaxation time, Jimenez and co-workers (2014) have indicated that shorter times (45 min vs. 90 min) gave ‘Hojiblanca’ and ‘Picual’ VOOs higher contents of secoiridoids and major phenyl alcohols (hydroxytyrosol and tyrosol) (Jimenez, Sánchez-Ortiz, et al., 2014). Another targeted metabolomics study has shown that the contents of total phenols and oleic acid were higher in ‘Chetoui’ and ‘Chemlali’ cv. VOOs at a malaxation time of 30 min, and that these contents decreased continuously with longer time periods, regardless of the cultivar (Ouni et al., 2013). Regarding volatile compounds, the same study noted a significant increase in the levels of C6 and C5 alcohols and carbonyl compounds, mainly hexanal and (*E*)-2-hexenal, as the malaxation time increased from 15 to 60 min, whereas a significant decline was reported for C6 esters, particularly (*Z*)-3-hexenyl acetate, in both cultivars. However, another team revealed that the effect of malaxation time and temperature conditions on the composition of ‘Oblica’ cv. VOOs is weak compared to that of the harvest time (Lukić et al., 2017).

In a quite similar context, Catania and co-workers have demonstrated the effect of oxygen (O₂) content in the headspace of the malaxer on the phenolic and volatile compound profiles of VOO (Catania, Val-lone, Farid, & Pasquale, 2016, 2017). Optimizing phenolic concentrations in VOO could be facilitated by adjusting O₂ level during processing. The phenolic composition of olive fruits is highly variable and dependent on various agronomic factors. Hence, regulating oxygen level during the malaxation by treatment with inert gases may result in optimal phenolic concentrations in VOO. A reduced level during the malaxation can inhibit the activity of some oxidative enzymes leading to less oxidation and higher amounts of antioxidant compounds in VOO (Clodoveo, 2012). Regulating O₂ content during the olive-fruit crushing process may also help to control the volatile profile of VOOs and thus modify the sensorial characteristics of non-balanced oils (Sánchez-Ortiz et al., 2016).

The installation of advanced technologies, such as non-thermal ultrasound and pulsed electric field treatment, in industrial VOOs processing units may also help to enhance the content of bioactive compounds, mainly phenolic compounds and tocopherols, in the produced oil (Grillo et al., 2022). These emerging technologies can improve the oil recovery process and help break down the cell walls, ultimately releasing trapped minor compounds from the uncrushed olive tissue. In addition, these technologies can increase the labour capacity of the olive oil mill and significantly reduce the overall time required for the process (Clodoveo et al., 2014). The use of advanced technologies in the extraction process can also provide the opportunity to influence the sensory and health-promoting properties of VOO resulting from endogenous enzymatic activities.

5.4. Optimal storage conditions

Suitable storage conditions (exposure to light, packaging materials, contact with O₂ and temperature) are crucial to lengthening the storage life, and preserving the nutrition status and sensory properties of VOOs. Bozdogan, Eker, Konuskan, Tulin Oz, and Kafkas (2019) have demonstrated that storing olive fruit under different conditions prior to VOOs

processing is linked to considerable variability in some of the phenolic constituents of 'Gemlik' cv. samples. Although storage under an N₂ and CO₂ atmosphere did not significantly affect the oils' phenolic and fatty-acid profiles, storing olives under an N₂ atmosphere at 5 °C led to the fast degradation of a bitter-tasting compound; oleuropein.

Once processed, VOO is usually stored under dark conditions at room temperature. Generally, its conservation under these conditions preserves its nutritional and sensory status for more than a year (Kotsiou & Tasioula-Margari, 2015; Stefano & Melilli, 2020). Díaz, Pega, Primrose, Sancho, and Nanni (2019) have reported the considerable loss of α -tocopherol and chlorophyll content in VOO samples during light-exposure treatment in a fluorescence spectra-based study combined with statistical analysis. In addition, Torre-Robles et al. (2019) have demonstrated in a phenolic profiling study that a significant decrease in phenolic content occurs in samples stored in clear bottles and polyethylene containers under light. A similar tendency has been observed in a 1H NMR-based study, as a decrease in the 1H signal intensities of phenolic compounds and other minor compounds, such as fatty acids, squalene and (*E*)-2-hexenal, present in fresh VOO, has been observed together with the appearance of new low intensity signals after storage over one year, in the light, and two years, in the dark, revealing that both light and increased temperature enhance hydrolytic and oxidative degradation (Alonso-Salces et al., 2021). However, the highest levels of phenolic acids, flavonoids and lignans were found in samples stored in dark glass bottles regardless of the light-exposure level (Torre-Robles et al., 2019). Similarly, a GC-based study by Gargouri, Zribi, and Bouaziz (2015) found that tin and dark glass packaging materials preserve the contents of fatty acids and sterols better than polyethylene and clear glass containers. Multilayer (plastic-coated paperboard aluminum foil) packaging may also play a crucial role in protecting VOO bioactive composition than green and ultraviolet grade absorbing glass (Esposito et al., 2021).

In another targeted metabolomics study, Guillaume, Gertz, and Ravetti (2014) have confirmed the fact that dark bottles (glass, plastic, etc.) and a temperature of 20 °C are better at preserving the quality characteristics of VOO. Indeed, samples exposed to light treatment or/and a higher temperature could no longer be considered as Extra Virgin after a period of 12 months. This study also revealed that pyropheophytins, chlorophyll pigment breakdown compounds from the thermal degradation of VOO, and 1,2-diacyl-glycerols (determined as a percentage of the total amount of 1,2-diacylglycerols and 1,3-diacylglycerols) can be used to track the overall quality of VOO, and thus predict its shelf-life (Guillaume et al., 2014). Pyropheophytins increased linearly with temperature and period of light exposure and are directly related to UV coefficients and rancid defects. 1,2-Diacyl-glycerols content declined at advanced storage times and is associated to free fatty acids and UV coefficients. Interestingly, another targeted metabolomics study pointed out the total diacylglycerols/1,3-diacylglycerols ratio as an indicator of the freshness of VOO suggesting that the ratio increases linearly with storage period (Caponio et al., 2013).

Another team investigated the effect of cold temperatures (4.5 and -27 °C) and made a comparison to the optimal temperature (25 °C) in a phenolic and volatile-profiling-based study (Li, Zhu, Shoemaker, & Wang, 2014). The obtained results revealed the great potential that cold storage conditions have on preserving hydroxytyrosol, tyrosol, oleuropein and α -tocopherol contents for up to 18 weeks. A similar tendency was reported by Bubola, Koprivnjak, Sladonja, and Belobrajčić (2014). Importantly, the temperature of 4 °C was found to maintain the VOO volatile profile better than -20 °C and room temperatures, which ranged from 10 to 27 °C.

The shelf-life of VOO has been principally related to its initial phenolic content as reported by Esposito et al. (2017, 2020). Indeed, samples with lower phenolic content displayed a significant reduction in phenol and tocopherol concentrations together with a remarkable increase in some undesirable volatile compounds during storage under dark conditions. Similar trend was observed in a phenolic profiling study

conducted by Castillo-Luna, Criado-Navarro, Ledesma-Escobar, López-Bascón, and Priego-Capote (2021) using LC-MS/MS. However, in an (LC-MS)-based metabolomics analysis, Montesano et al. (2019) demonstrated that enriching VOO with a carotenoid-rich extract from *Lycium barbarum* L. may preserve its phenolic, tocopherol and carotenoid contents over 28 weeks of storage at room temperature, leading to extended shelf-life.

To conclude this section, it should be emphasised that the choice of optimal conditions for effective monitoring of VOO quality must take account of seasonal and environmental variations and the specific characteristics of each olive variety. Genetic factors can indeed play an important role in monitoring the release of key compounds, and several mechanisms have been shown to be cultivation-dependent. In addition, the unpredictability of climatic conditions, especially in the context of global warming, poses a major challenge that requires careful consideration.

6. Metabolomics for assessing VOO authenticity and traceability

The adulteration of VOO, the emblematic ingredient of the Mediterranean diet, is a common issue due to its premium price, unique nutritional value and pleasant taste. The main authenticity issue related to VOO is its adulteration with a lower quality product or other cheaper vegetable oils. The deliberate misdescription of this premium-foodstuff (including, among others, selling low quality olive oil as EVOO, mislabeling it as an 'organic' product, and attributing it with desirable varietal and/or geographical origins) is also widespread in the global food market. However, remarkable progress has been made in VOO authenticity assessment over the last decade thanks to the revolution in 'omics' technologies. Indeed, although no single method has been applied as a universal standard for all authenticity matters, metabolomics offers great potential in the deep screening of unexpected variations in the chemical fingerprint, which is a clear advantage that can severely hamper dishonest traders (Aparicio et al., 2013; Chaji et al., 2021; Esslinger et al., 2014; Medina, Pereira, et al., 2019). Table 1 shows some interesting applications of various metabolomics approaches for assessing VOO authenticity, mainly involving adulteration, misclassification of quality grades, varietal authentication and geographical traceability.

Ozcan-sinir (2020) have demonstrated a clear distinction between EVOO, adulterated olive oil and other vegetable oils using MS-based volatile profiling and chemometrics, mainly SIMCA and PLS regression. 1-octanol, 1-penten-3-one, 2-phenylethanol, dodecane, anisole, ethyl nonanoate, isobutanoic acid, ocimene, phenol and toluene were presented as the most prominent discriminant markers in the study.

Furthermore, the potential of volatile profiling using headspace GC coupled with MS and IMS for the differentiation of olive oil according to its quality grades has been investigated (García-Nicolás, Arroyo-Manzanares, Arce, Hernández-Córdoba, & Viñass, 2020). A headspace solid-phase microextraction/gas chromatography-mass spectrometry (HS-SPME-GC-MS) method has also been developed for the geographical discrimination of Cretan EVOO samples, with 13 volatile compounds being highlighted as potential markers, including four terpenic hydrocarbons (6-methyl-5-hepten-2-one, copaene, (*E*)-4,8-dimethyl-1,3,7-nonatriene and (*Z*)-beta-ocimene), two esters ((3*Z*)-hex-3-en-1-yl acetate and hexyl acetate), two aldehydes ((*E*)-2-pentenal and pentanal), three hydrocarbons (dodecene, (*E*)-2-dodecene, undecene and 1-ethyl-2-methylbenzene), one alcohol ((*Z*)-11-hexadecen-1-ol) and one ether (1-methoxy-2-propanol) (Lioupi et al., 2022).

The fatty-acid profile can also play a key role in olive oil authentication, and this can be done using the metabolomic-profiling approach based on GC with a flame ionization detector (GC-FID), which can differentiate pure VOO from samples blended with other vegetable oils, such as corn, peanut, sunflower, soybean and palm (at various levels up to 50% w/w) (Siano & Vasca, 2020).

Table 1
Some relevant applications of various metabolomics approaches for assessing virgin olive oil authenticity.

Authentication issue	Sampling	Metabolomics analysis technique				Model's accuracy/Marker (s)	Reference
		Metabolomics approach	Extraction technique	Analytical platform	Statistical analysis		
Olive oil adulteration	<ul style="list-style-type: none"> 43 pure EVOO from different suppliers, production years, geographical origin and storage conditions. 3 "soft deodorized", 2 "soft deacidified", and 2 "soft deacidified and then deodorized" samples prepared from virgin and lampante olive oils on the laboratory scale. Preparing 6 mixtures at different percentages of adulteration (40, 45, 50, 60 and 75%). 	Fingerprinting	LLE using methanol (MeOH): water (80:20, v/v).	HPLC-HRMS	PCA, PLS-DA and one-way ANOVA	Markers: Propylene glycol 1 stearate, (2R,3E)-5-(3-chloro-5-formyl-2,6-dihydroxy-4-methylphenyl)-3-methyl-1-[(1S,2R,6R)-1,2,6-trimethyl-3-oxocyclohexyl]-3-penten-2-yl acetate, 2,3,4-trihydroxy-6-methyl-5-[(2E,6E)-3,7,11-trimethyl-2,6,10-dodecatrien-1-yl]benzaldehyde, and 4 unidentified compounds.	Cavanna et al. (2020)
	<ul style="list-style-type: none"> 13 'Arbequina', 6 'Arbosana', and 6 'Koroneiki' cv. EVOOs. 11 grapeseed oils, 3 soybean oils, 7 canola oils, 4 high-oleic safflower oils, and 5 high-oleic sunflower oils from different suppliers. Preparing 7 blends of 'Arbequina' EVOO and adulterants at various percentages: From 95% EVOO with 5% of the adulterant to 10% EVOO and 90% of the adulterant. 	Profiling	<ul style="list-style-type: none"> Simple dilution with MeOH/chloroform (50:50, v/v) for triacylglycerol analysis. Dissolution in toluene then in MeOH and MeOH/hydrochloric acid (80:20, v/v) for fatty acid profiling. 	UHPLC-CAD	PCA	Markers: Triacylglycerol profile.	Green et al. (2020)
	<ul style="list-style-type: none"> 4 pure EVOOs from different Italian cultivars, and 4 commercial pure corn oils (from different brands). Preparing corn oil/EVOO mixtures at different 	Profiling	Treating samples with CHCl ₃ /TBA-CHCA (1:2) then chloroform and water.	MALDI-TOF MS	HC, PCA	Classification accuracy: 100%/Markers: Lipid spectra profile.	Girolamo et al. (2015)

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Table 1 (continued)

Authentication issue	Sampling	Metabolomics analysis technique				Model's accuracy/ Marker (s)	Reference
		Metabolomics approach	Extraction technique	Analytical platform	Statistical analysis		
	percentages: 0.5/99.5, 1/99, 5/95, 10/90 and 20/80.						
	<ul style="list-style-type: none"> EV, ordinary olive oil and adulterated olive oil with other vegetable oils (corn, sunflower, soybean and canola) in different proportions (between 0.5 and 20%, w/w). 	Fingerprinting	LLE with MeOH/deionized water (1:1, v/v) with 1.0% formic acid.	MS	PLS-DA	Classification accuracy: 100%/ Markers: tyrosol, hydroxytyrosol, sinapic acid, pinoresinol, trioleylglycerol, acetoxypinoresinol, coumaric acid, diacylglycerols, monoacylglycerols and other unidentified compounds.	Alves, Botelho, Sena, and Augusti (2013)
	<ul style="list-style-type: none"> 40 EVOO of various geographical origins, different brands and different batch numbers purchased from local markets. Adulterants: 5 camellia oils, 5 soybean oils, 5 sunflower oils and 5 corn oils obtained from local markets. Preparation of olive oil samples adulterated with camellia oils at various percentages: 1, 2, 3, 4, 5, 10, 15, 20, 35, 50, 70, 90% (W/W). 	Fingerprinting	Direct injection	FTIR spectroscopy	SLLE, locally linear embedding, PCA, nearest centroid classification and PLS	Classification accuracy ranging between 92.23% and 96.58%.	Sun, Lin, Li, Shen, and Luo (2015)
	<ul style="list-style-type: none"> EVOO samples from different brands. Adulteration with corn oil, sunflower oil, high oleic sunflower oil and olive oil purchased from local market. 	Profiling	Direct injection	SIFT-MS	One way-ANOVA, SIMCA and PLSR	Markers: 1-octanol, 1-penten-3-one, 2-phenylethanol, dodecane, anisole, ethyl nonanoate, isobutanoic acid, ocimene, phenol and toluene.	Ozcan-sinir (2020)
	<ul style="list-style-type: none"> Adulteration levels: 1, 2.5, 5, 10, and 20%. 						
	<ul style="list-style-type: none"> 8 EVOO, 6 seed oils, 4 sunflower oils, 1 corn oil. EVOO adulteration in different proportions (10, 20, 30, 40 and 50%). 	Fingerprinting	Direct injection	UV-IMS	PCA, LDA, K-NN and PLS	Classification accuracy: Sensitivity ranging between 76.5 and 93.7% and specificity ranging between 95.8 and 100%.	Garrido-Delgado, Muñoz-Pérez, and Arce (2018)
	<ul style="list-style-type: none"> Commercial EVOO, soybean (SB) and sunflower (SF) 	Fingerprinting	Direct injection	FTIR spectroscopy	PLS and PLS-DA	Classification accuracy: 100%.	Oussama, Elabadi, Platikanov, Kzaiber, and Tauler (2012)

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Table 1 (continued)

Authentication issue	Sampling	Metabolomics analysis technique				Model's accuracy/ Marker (s)	Reference
		Metabolomics approach	Extraction technique	Analytical platform	Statistical analysis		
	oils of one single origin.						
	<ul style="list-style-type: none"> 58 EVOO-soybean samples and 46 EVOO-sunflower samples prepared at different percentages (from 1 to 24% weight ratios). Refined rapeseed-oil and olive oil samples. Preparing blends at: 1%, 2.5%, 5%, and 10% of refined rapeseed oil to olive oil and olive oil to refined rapeseed oil. 	Profiling	Dissolution in dichloromethane	GC-FID	none	1,2-palmitoyl-3-linolein- <i>sn</i> -glycerol, 1-palmitoyl-2-stearoyl-3-oleoyl- <i>sn</i> -glycerol, 1,2-palmitoyl-3-oleoyl- <i>sn</i> -glycerol and 1,2-oleoyl-3-linolenoyl- <i>sn</i> -glycerol.	Qian, Rudzińska, Grygier, and Przybylski (2020)
	<ul style="list-style-type: none"> 64 olive oil from different categories and 55 samples from other vegetable oils (7 canola, 5 corn, 5 peanut, 13 sunflower, 5 no specified seed, 4 grapeseed, 7 palm, 3 sesame and 6 soybean oils). 	Fingerprinting	<ul style="list-style-type: none"> Methyl-transesterification using [MeOH (10% MeONa)–<i>tert</i>-butyl methyl ether, (4 + 6, v/v)]. 	HPLC	PCA, k-NN, PLS-DA, SIMCA, OCPLS, SVC and SVM-C	Efficiency ranging between 44 and 98%, and classification accuracy ranging between 41 and 97%.	Jiménez-Carvelo et al. (2017)
	<ul style="list-style-type: none"> 2 'Chemlali' EVOO from 2011/2012 and 2 from 2012/2013. • Similar maturity indices (4.5). Different refined oil samples (corn, sunflower, olive, pomace olive, soybean and palm oils). Preparation of 66 EVOO mixtures with refined oils at different percentages: 0.1, 0.2, 0.3, 0.4, 0.5, 1, 2, 3, 4, 5, and 10 %, w/w). 	Profiling	<ul style="list-style-type: none"> For <i>trans</i>-fatty acids analysis: Extraction with <i>n</i>-heptane and methanolic potassium hydroxide. For stigmasta-3,5-diene analysis: Saponification with alcoholic potassium, extraction with <i>n</i>-hexane and washing with ethanol/water (1/1) until neutral pH, then extraction through a silica gel column with <i>n</i>-hexane. 	GC-FID	LDA	Classification accuracy: 100%/ Markers: <i>trans</i> -fatty acids and stigmasta-3,5-diene.	Jabeur, Zribi, and Bouaziz (2016)
	<ul style="list-style-type: none"> 104 samples of different categories (EVOO, VOO and olive oil) and 47 olive oils adulterated with vegetable oils (corn, 	Fingerprinting	Direct injection	FTIR and Raman spectroscopies	SIMCA and PLSR	Classification accuracy: excellent sensitivity (100%) and specificity (100%).	Aykas et al. (2020)

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Table 1 (continued)

Authentication issue	Sampling	Metabolomics analysis technique				Model's accuracy/Marker (s)	Reference
		Metabolomics approach	Extraction technique	Analytical platform	Statistical analysis		
Varietal authenticity	sunflower, soybean, and canola oils) from various geographical origins (Turkey, Italy, Spain, Greece, Turkey, Tunisia, Portugal, and Peru).	Fingerprinting	Direct injection	FTIR spectroscopy	PLS, PCR and DA	Excellent classification accuracy with only one misclassified sample.	Rohman, Man, and Yusof (2014)
	• EVOO and other vegetable oils (canola, grape seed, rice bran, and walnut oils) purchased from local market. • Preparation of 20 EVOO samples adulterated with canola oil at different levels (from 1 to 50 %). • Preparation of EVOO samples adulterated with other vegetable oils (canola, grape seed, rice bran and walnut oils).						
	• 55 monovarietal and varietal blends olive oil samples. • Preparation of adulterated samples using EVOO and 9 vegetable oils (10 and 20% (w/w) of pure EVOO and each adulterant). • 93 commercial samples.	Fingerprinting	Direct injection	FT-NIR spectroscopy	CI, PCA and SIMCA	Classification accuracy: 100%.	Karunathilaka, Kia, Srigley, Chung, & Mossoba, (2016)
	• 25 monovarietal EVOO from different trademarks and from five cultivars: 'Arbequina', 'Cornicabra', 'Hojiblanca', 'Picual' and 'Frantoio'.	Profiling	<ul style="list-style-type: none"> • SPE with Diol-cartridges. • Non-polar fraction removal by hexane. • Dilution (1:10, v/v) with MeOH. • Derivatization for GC-MS analysis. 	HPLC-MS and GC-MS	PCA, PLS-DA and ANOVA	PLS-DA: Good discriminant ability and excellent predictability (0.541 < R2X > 0.620, 0.983 < R2Y > 0.995 and predictability 0.940 < Q2 > 0.995 for the LC-based approach and 0.439 < R2X > 0.460, 0.896 < R2Y > 0.983 and predictability 0.819 < Q2 > 0.925 for the GC-based approach)/Markers: 19 phenolic compounds.	Bajoub, Pacchiarotta, et al. (2016)
• 66 olive oil samples from four cultivars:	Profiling	Direct injection	NMR	PCA, OPLS-DA, RF	Accuracy: R2/Q2 values obtained by OPLS-DA were 0.97/	Tang et al. (2022)	

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Authentication issue	Sampling	Metabolomics analysis technique				Model's accuracy/Marker (s)	Reference
		Metabolomics approach	Extraction technique	Analytical platform	Statistical analysis		
	<ul style="list-style-type: none"> 'Arbequina' (n = 18), 'Arbosana' (n = 18), 'Koroneiki' (n = 18), and 'Sikitita' (n = 12). Each cultivar was harvested six times per year during 2016, 2017, and 2018, except that 'Sikitita' was harvested only during 2017 and 2018. •3 kg of olives were randomly collected from a block consisting of three lines of 20 trees each. 					0.94, 0.98/0.93, 0.97/0.91 and 0.98/0.94 for 'Arbosana'/'Koroneiki', 'Koroneiki'/'Sikitita', 'Arbequina'/'Koroneiki', and 'Arbequina'/'Sikitita', respectively while a relatively low error of 6% for classification was obtained by RF/Markers: esters of phytol and geranylgeraniol, and linolenic acid for 'Arbequina'; β -sitosterol for 'Arbosana'; linolenic acid, linoleic acid, squalene and 1,2-diglycerides for 'Sikitita'; unsaturated FA, squalene, linoleic acid, 1,2-diglycerides for 'Koroneiki'.	
	<ul style="list-style-type: none"> • 202 micro-milled monovarietal olive oil samples from different cultivars ('Frantoio', 'Leccino', 'Moraiole', 'Pendo-lino', 'Maurino', 'Leccio del Corno', 'Rossellino', 'Morchiaio', 'Lazzero', 'Maremmano', 'Mignolo cerretano', 'Oliustra seggianese' and 'Razzaio') including 10 Tuscan PGI cultivars. • Molecular analysis for varietal confirmation. 	Fingerprinting	Dissolution in chloroform-d (13.5: 86.5, %w/w).	¹ H NMR	PCA, PLS-DA and OPLS-DA	Classification accuracy: 100% for the most representative cultivars ('Moraiole', 'Frantoio' and 'Leccino') as well as good discriminative and predictive abilities (R ² X = 0.83, R ² Y = 0.74, Q ² = 0.57).	Girelli et al. (2018)
	<ul style="list-style-type: none"> • 32 VOO samples from eight cultivars (cv. 'Carolea', 'Casaliva', 'Cayon', 'Frantoio', 'Kalamon', 'Maurino', 'Moraiole' and 'Taggiasca'). • Olive oil processed from hand-picked olives with ripening indices ranging 	Profiling	<ul style="list-style-type: none"> • LLE with three portions of ethanol/water (80:20, v/v) and one portion of ethanol/water (60:40, v/v). • Derivatization using N,O-bis(trimethylsilyl) trifluoroacetamide plus 1% of trimethylchlorosilane. 	GC-MS	ANOVA, PCA and PLS-DA	Markers: tocopherols, luteolin, β -sitosterol and tyrosol.	Olmo-García, Polari, et al. (2019)

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Authentication issue	Sampling	Metabolomics analysis technique				Model's accuracy/Marker (s)	Reference
		Metabolomics approach	Extraction technique	Analytical platform	Statistical analysis		
	between 2.3 and 2.9. <ul style="list-style-type: none"> 51 Greek monovarietal EVOOs from the varieties: 'Manaki', 'Ladoelia', 'Koroneiki', 'Amfissis', 'Chalkidikis' and 'Kolovi'. 2015–2016 harvest year. Conventional agriculture and 3-phase extraction system. 	Fingerprinting	LLME using MeOH:H ₂ O (80:20, v/v)	UHPLC-MS	ACO-RF and PCA	Classification accuracy: 100%/ Markers: Apigenin, vanillic acid, luteolin 7-methyl ether and oleocanthal.	Kalogiouri et al. (2018)
	<ul style="list-style-type: none"> 112 VOO samples: 69 'Koroneiki' cv. samples from the region of Messinia and 43 'Mastoides' cv. From the southeast area of Lakonia. Both regions have similar climatic conditions. 2014–2015 harvest year. Fruits harvested at optimal ripening stage. 	Profiling	For sterol and triterpene dialcohol analysis: <ul style="list-style-type: none"> Saponification with potassium hydroxide in ethanolic solution. Separation of the sterol and triterpene di-alcohol fractions by thin-layer chromatography. Transformation of the recovered fractions into trimethylsilyl ethers by adding pyridine-hexamethyldisilazane-trimethylchlorosilane (9:3:1, v/v/v). For fatty acid methyl ester analysis: Cold alkaline transesterification with methanolic potassium hydroxide solution and extraction with <i>n</i>-heptane. 	GC-FID	ANOVA and PCA	Markers: 13 fatty acids and 13 sterols.	Skiada et al. (2020)
Classification of olive oil grades	<ul style="list-style-type: none"> 40 EVOO, 40 VOO and 40 LOO samples. 	Profiling	<ul style="list-style-type: none"> LLE with hexane and MeOH/water (1:1, v/v). SFE with O₂ and methanol (5%) Direct injection for GC-IMS analysis 	<ul style="list-style-type: none"> Polar fraction: CE-UV, HPLC-UV/FLD. Volatile fraction: GC-IMS 	PCA, LDA and KNN	Classification accuracy: Ranging between 65.5% (HPLC-UV/FLD data) and 86.7% (GC-IMS data)	Jurado-campos, Arroyo-manzanares, Viñas, and Arce (2020)
	<ul style="list-style-type: none"> Binary blends of EVOO and vegetable oils (soybean, corn, sunflower, and canola) at different proportions (0; 1; 3; 5; 10; 15; 20; 25; 30; 35; 40; 45; 50; 55; 60; 70, 80, 90 and 100% w/w). 40 binary blends of 7 EVOO brands (at 25:75 and 50:50, w/w%) 	Fingerprinting	Direct injection	NIR spectroscopy	PLS, PCA and SIMCA	Classification accuracy: 100%.	Borghi et al. (2020)
	<ul style="list-style-type: none"> 98 EVOO, 159 VOO, and 35 LOO samples collected during the harvest year: 2014–2015. 92 EVOO, 196 VOO and 121 	Fingerprinting	Direct injection	GC-IMS	PCA, LDA, KNN, OPLS-DA	Classification accuracy: Between 79.40 and 93.9%.	Contreras, Jurado-Campos et al. (2019)

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Authentication issue	Sampling	Metabolomics analysis technique				Model's accuracy/Marker (s)	Reference
		Metabolomics approach	Extraction technique	Analytical platform	Statistical analysis		
	<ul style="list-style-type: none"> • LOO collected during harvest year: 2015–2016. • Heterogeneous samples (different cultivars, geographical origins, ripeness stages, processing practices and storage conditions). • 104 EVOO, 84 VOO, and 80 LOO samples. 	Fingerprinting	Direct injection	HS-GC-IMS	PCA, PLS-DA, OPLS-DA and ANOVA	Classification accuracy: between 82 and 100%/ Markers: 128 aroma compounds.	Contreras, Arroyo-Manzanares et al. (2019)
	<ul style="list-style-type: none"> • 52 EVOO, 56 VOO, and 52 LOO samples. 7 EVOO, 7 VOO, and 7 LOO samples for external validation. • All samples are from various geographical origins. • 331 samples (EVOO, VOO, and LOO) from the most common cultivars and from two harvest years (2016/2017 and 2017/2018). • 54 EVOO, 78 VOO, and 48 LOO collected during harvest year 2016/2017. • 69 EVOO, 51 VOO and 33 LOO collected during harvest year 2017/2018. 	Profiling	Direct injection	HS-GC-IMS/MS	PCA, LDA and KNN	Classification accuracy: 85.71%.	García-Nicolás et al. (2020)
	<ul style="list-style-type: none"> • 331 samples (EVOO, VOO, and LOO) from the most common cultivars and from two harvest years (2016/2017 and 2017/2018). • 54 EVOO, 78 VOO, and 48 LOO collected during harvest year 2016/2017. • 69 EVOO, 51 VOO and 33 LOO collected during harvest year 2017/2018. 	Fingerprinting	Direct injection	FGC	PLS-DA	Classification accuracy: between 72 and 85%.	Barbieri et al. (2020)
	<ul style="list-style-type: none"> • 120 EVOO, 120 VOO, 60 LOO and 125 blind samples (Unknown quality). 	Fingerprinting	SPE with cartridges and hexane-diethyl ether (50:50, v/v).	GC-MS	PCA, PLS-DA and OPLS-DA	Classification accuracy 70%/ Markers: Methyl 2-methyl butyrate, diethyl carbonate, ethyl 2-methyl butyrate, guaiacol and 11 unidentified compounds.	Sales et al. (2017)
	<ul style="list-style-type: none"> • 70 monovarietal olive oil samples of four commercial grades: extra virgin, virgin, ordinary virgin and lampante. 	Fingerprinting	Direct injection	FTIR spectroscopy	PCA, PLS-DA and PLS2-DA	Precision accuracy: 100%.	Hirri, Bassbasi, Platikanov, Tauler, & Oussama, (2016)

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Table 1 (continued)

Authentication issue	Sampling	Metabolomics analysis technique				Model's accuracy/Marker (s)	Reference
		Metabolomics approach	Extraction technique	Analytical platform	Statistical analysis		
	<ul style="list-style-type: none"> • 30 Spanish olive oils from different quality categories: EVOO, VOO and LOO. 	Fingerprinting	Dilution in dichloromethane (CH ₂ Cl ₂)/MeOH/water (10:9:1) containing 0.025% hydrochloric acid and LLE using MeOH:H ₂ O (50:50, v/v).	DMA-MS	PCA and OPLS-DA	Classification accuracy: 89% for the LLE samples, 67% for the diluted samples and 100% when combining both methods/ Markers: Unidentified spectral fingerprints.	Piñero et al. (2020)
Geographical traceability	<ul style="list-style-type: none"> • 90 EVOO samples from different geographical origins. • 15 samples for model testing. • 1020 olive oil samples collected over four harvest years (2014–2017) and 105 over two recent years (2018 and 2019). • Sampling of various geographical origins. 	Fingerprinting	<ul style="list-style-type: none"> • Polar compounds: LLE with methanol then MeOH:water (1:1, v/v). • Less polar compounds: Dilution with butanol. 	UHPLC-MS	PCA, PLS-DA and OPLS-DA	Classification accuracy: 87%/ Markers: 12 compounds.	Gil-Solsona et al. (2016)
	<ul style="list-style-type: none"> • 1020 olive oil samples collected over four harvest years (2014–2017) and 105 over two recent years (2018 and 2019). • Sampling of various geographical origins. 	Fingerprinting	LLE using a mixture of acetonitrile and acetonitrile-d ₃ (95:5, V/V) and sodiumtrimethylsilylpropionate-d ₄ .	1H NMR	PCA, CA and KNN	Classification accuracy: 96%	Winkelmann and Küchler (2019)
	<ul style="list-style-type: none"> • 33 VOO samples of different geographical origins. 	Profiling	Dissolution in chloroform-d.	1H NMR	PCA and ANOVA	Markers: linolenoyl, linoleoyl, oleoyl and saturated fatty acyls.	Ün and Ok (2018)
	<ul style="list-style-type: none"> • Olive fruits from 'Picholine Marocaine' cv. • Samples from different regions. * Harvesting over two crop seasons (2011/2012 and 2012/2013). • Maturity index between 3.5 and 4. • Oil extraction on the laboratory scale. 	Profiling	1. Dilution in n-hexane. 2. Solid-phase extraction using silica-gel cartridges and n-hexane-diethyl ether (87:13, v/v). 3. Dissolution in acetone.	HPLC	PCA, S-LDA, PLS-DA and SIMCA	PLS-DA model: Prediction accuracy ranging between 77.46 and 99.43%. S-LDA: classification accuracy ranging between 81.25 and 100%/Markers: 21 triacylglycerols.	Bajoub, Medina-Rodríguez, et al. (2016)
	<ul style="list-style-type: none"> • 125 Spanish EVOO from various geographical origins. 	Profiling	MAAD	ICP-MS/OES	PCA, LDA, PLS-DA, SVM and RF	Classification accuracy: Sensitivity ranging between 50 and 100%, and specificity ranging between 93.4 and 100%/Markers: 55 elements.	Sayago, González-Domínguez, Beltrán, and Fernández-recamales (2018)
	<ul style="list-style-type: none"> • 63 bottled and branded VOO samples from both organic (n = 19) and conventional (n = 44) olive groves. • Samples of different local olive cultivars 	Profiling	HS-SPME	GC-MS	SIMCA, PCA, OPLS-DA, ANOVA	Markers: four terpenic hydrocarbons (6-methyl-5-hepten-2-one, copaene, (E)-4,8-dimethyl-1,3,7-nonatriene, and (Z)-beta-ocimene), two esters ((3Z)-hex-3-en-1-yl acetate and hexyl acetate), two	Lioupi et al. (2022)

(continued on next page)

Table 1 (continued)

Authentication issue	Sampling	Metabolomics analysis technique				Model's accuracy/Marker (s)	Reference
		Metabolomics approach	Extraction technique	Analytical platform	Statistical analysis		
	('Koroneiki' and 'Tsounati') harvested in 2018–2019 in different regions of Crete, Greece.					aldehydes ((E)-2-pentenal and pentanal), three hydrocarbons (dodecene, (E)-2-dodecene, undecene, and 1-ethyl-2-methylbenzene), one alcohol ((Z)-11-hexadecen-1-ol), and one ether (1-methoxy-2-propanol)	

Abbreviations: ACO-RF: ant colony optimization-random forest; ANOVA: analysis of variance; CE-UV: capillary electrophoresis-ultraviolet; CI: conformity index; DA: discriminant analysis; DMA-MS: differential mobility analysis-mass spectrometry; EVOO: extra virgin olive oil; FGC: flash gas chromatography; FTIR: Fourier transform infrared; FT-NIR: Fourier transform near-infrared; GC-FID: gas chromatography-Flame ionization detection; GC-IMS: gas chromatography-ion mobility spectrometry; GC-MS: Gas chromatography-mass spectrometry; HC: hierarchical clustering; HPLC: high-performance liquid chromatography; HPLC-HRMS: high performance liquid chromatography-high resolution mass spectrometry; HPLC-MS: high performance liquid chromatography-mass spectrometry; HPLC-UV/FLD: high performance liquid chromatography-ultraviolet-fluorescence detector; HS-GC-IMS/MS: headspace gas chromatography-ion mobility spectrometry/mass spectrometry; HS-SPME: headspace-solid phase micro extraction; ICP-MS/OES: inductively coupled plasma-mass spectrometry/optical emission spectroscopy; K-NN: k-nearest neighbors; LDA: linear discriminant analysis; LLE: liquid-liquid extraction; LLME: liquid-liquid micro extraction; LOO: lampante olive oil; MAAD: microwave-assisted acid digestion.; MALDI-TOF MS: matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry; MS: mass spectrometry; NIR: near-infrared; NMR: nuclear magnetic resonance; OCPLS: one-class partial least squares; OPLS-DA: orthogonal projections to latent structures discriminant analysis; PCA: principal component analysis; PCR: principal component regression; PGI: protected geographical indication; PLS: partial least square; PLS-DA: partial least squares discriminant analysis; PLSR: partial least squares regression; RF: random forest; SFE: supercritical fluid extraction; SIFT-MS: selected ion flow tube - mass spectrometry; SIMCA: soft independent modeling of class analogies; S-LDA: stepwise-linear discriminant analysis; SLLE: supervised locally linear embedding; SPE: solid phase extraction; SVC: support vector classification; SVM-C: support vector machines-classification; UHPLC-CAD: ultra-high-performance liquid chromatography-charged aerosol detection; UHPLC-MS: ultra-high-performance liquid chromatography-mass spectrometry; UV-IMS: ultraviolet photoionization-ion mobility spectrometry; VOO: virgin olive oil.

Untargeted approaches have also been widely explored for the discrimination of VOO from cheaper oils. For instance, a fast LC fingerprinting approach has been proposed by Jiménez-Carvelo, González-Casado, Pérez-Castaño, and Cuadros-Rodríguez (2017) for the differentiation of VOO from various vegetable oils (canola, corn, peanut, sunflower, non-specified seed, grapeseed, palm, sesame soybean oils). Chemometrics models displayed accuracies ranging from 41 to 97%. Similarly, Aykas, Karaman, Keser, and Rodríguez-Saona (2020) have compared the ability of two non-destructive methods - FT-IR and Raman spectroscopies - to discriminate high-quality VOO and detect its adulteration with lower grades or cheaper oils. The data obtained were then used for SIMCA multi-class analyses, which showed high sensitivity (100%) and specificity (100%) for both methods, whereas the SIMCA single-class method exposed reduced specificity, leading to a misclassification of lower quality olive oil as VOO (89 and 66% for FT-IR and Raman, respectively) (Aykas et al., 2020).

Interestingly, Contreras et al., (2019) have compared an untargeted fingerprinting methodology, based on overall (GC-IMS) data, and a targeted approach, based on specific markers, and their abilities to classify olive oil from different quality grades (extra virgin, virgin and lampante). The results obtained revealed that the two approaches had similar classification accuracies (ranging between 74.29 and 91.46% for the targeted approach, and between 79.40 and 93.90% for the untargeted). However, the fingerprinting approach generates a large amount of data, whereas the second approach is considered less-time consuming, which makes it appropriate for routine analyses.

Ensuring the varietal origin of VOO is another concern that can guarantee legal and honest globalized trade. In a metabolomics study performed using GC-FID and a PCA tool, the sterol and fatty-acid profiles were presented as powerful markers for the varietal authentication of 112 VOO from two widely spread cultivars in Greece, 'Koroneiki' and 'Mastoides' cv. (Skiada, Tsarouhas, & Varzakas, 2020). In another stimulating example, fatty-acid profiling, using the same metabolomics analysis, was successfully applied for the varietal discrimination of Iranian VOO samples of three cultivars: 'Beleydi', 'Mission' and

'Koroneiki' cv. (Noorali, Barzegar, & Sahari, 2014).

Likewise, two different phenolic profiling approaches have been developed to identify potential markers for the varietal discrimination of VOOs obtained from 'Arbequina', 'Cornicabra', 'Hojiblanca', 'Picual' and 'Frantoio' cv. (Bajoub, Pacchiarotta, et al., 2016). The authors used an MS-based metabolomics approach coupled with two separation methods - LC and GC - together with chemometrics (PCA and PLS-DA). The outcomes highlighted 19 phenolic compounds as being the most varietal discriminating, while PLS-DA models showed good discriminating ability and excellent predictability ($0.541 < R^2_X > 0.620$, $0.983 < R^2_Y > 0.995$ and predictability $0.940 < Q^2 > 0.995$ for the LC-based approach and $0.439 < R^2_X > 0.460$, $0.896 < R^2_Y > 0.983$ and predictability $0.819 < Q^2 > 0.925$ for the GC-based approach) (Bajoub, Pacchiarotta, et al., 2016).

In another relevant study, a fingerprinting approach that is based on reversed-phase UHPLC coupled to electrospray ionization-quadrupole-time-of-flight MS and chemometrics was found to be very promising for the varietal traceability of 51 monovarietal VOOs processed from 'Manaki', 'Ladoelia', 'Koroneiki', 'Amfissis', 'Chalkidikis' and 'Kolovi' cv. olive fruits (Kalogiouri, Aalizadeh, & Thomaidis, 2018). The authors found that apigenin, vanillic acid, luteolin 7-methyl ether and oleocanthal are the key markers that lead to a discrimination accuracy of 100%.

Great attention must be paid to geographical origin, which is not surprising since many studies have demonstrated that the nutritional and sensorial features of VOO are significantly affected by its geographical provenance. A fingerprinting approach that combines (UHPLC-QTOF-MS)-based analysis and PCA, PLS-DA and OPLS-DA methods has been applied to trace the geographical origin of VOOs from 6 Spanish regions (Gil-Solsona et al., 2016). The study identified 12 markers as being responsible for the correct classification of 87% of samples to their origins.

In the same context, Olmo-García, L., Wendt, et al. (2019) have explored the possible differentiation of VOOs from 6 geographical indications using two different analytical techniques (LS-MS and GC-MS)

in combination with chemometrics. The data obtained from the platforms were used to build a two-class PLS-DA model, which showed a clear differentiation between samples according to their geographical origins.

NMR spectroscopy has also been applied to the geographical tracing of VOOs. Girelli et al. (2018) have indeed demonstrated that the accurate discrimination of Tuscan PGI monovarietal samples can be achieved using 1H NMR spectroscopy combined with multivariate statistical analyses (Girelli et al., 2018). They found that NMR-based metabolomics could not only discriminate samples based on their olive cultivar (OPLS-DA model discriminative and predictive parameters: $R2X = 0.83$, $R2Y = 0.74$, $Q2 = 0.57$) but also according to their geographical origin (OPLS-DA model showed good discriminative and predictive abilities: $R2X = 0.74$, $R2Y = 0.75$, $Q2 = 0.65$).

In other publications, 1H NMR-based metabolomics has been used to target the VOOs polar minor fraction to assess its geographical provenance (Winkelmann & Kuchler, 2019). The statistical model gave a classification accuracy of 96%, which proved the method's efficiency in the rapid and accurate verification of VOOs geographical labeling.

In the same context, Peršurić, Saftić, Mašek, and Pavelić (2018) have compared the triacylglycerol and fatty-acid profiling approaches using MALDI-TOF/MS and GC-MS, respectively, for the geographical classification of VOOs. The PCA method was found to be more powerful than triacylglycerol profiling, which was later confirmed using a PLS-DA model.

The recent review paper by Kalogiouri, Aalizadeh, Dasenaki, and Thomaidis (2020), in which the authors present an exhaustive overview of the latest advances in, and applications of, (HR-MS)-based metabolomics coupled with chemometrics for VOO authenticity determination, is highly recommended reading. However, as authenticity and traceability are not main focus of this review, more extensive information on this topic can be found in the following contributions (Aparicio et al., 2013; Esslinger et al., 2014; Lioupi et al., 2020; Mannina & Segre, 2002).

7. Conclusion and future perspectives

Metabolomics is experiencing faster growth compared to other 'omics' fields and is establishing itself as a robust bioanalytical approach to the analysis of VOO. The knowledge gained through metabolomics over the past decade has significantly improved our comprehension of possible changes in the chemical composition of VOO, providing a deeper understanding of how various agrotechnological factors influence its nutritional and sensory properties. Furthermore, metabolomics consolidates its role as an essential tool in addressing all challenges related to its authenticity, including adulteration by cheap vegetable oils, misclassification of quality grades and mislabeling of certain varieties and geographical origins.

Future interest will need to focus on the development of advanced technologies that ensure the simultaneous determination of a broader range of metabolites, as no current metabolomic approach allows the detection of all metabolites in a single run. Future metabolomics studies must also focus on examining interannual variability to accurately identify all potential changes; otherwise, questionable results and misinterpretation may occur.

Regardless of the metabolomics approach used, regularly updated databases of VOO chemical compounds are essential for the rapid identification of unknown metabolites. They must include not only reference compounds or spectra, but also complete information on how the data were provided, including the analytical platform used, the conditions for sample preparation and the type of VOOs analyzed (cultivar, geographical origin, processing system, storage conditions, etc.).

Furthermore, the integration of artificial intelligence (AI) tools into metabolomics databases promises to improve the nutritional and sensory properties of VOO. Interestingly, the development of AI models that

suggest optimal conditions to produce premium quality oil, taking into account variables such as country of origin, cultivar selection, production parameters, and expected environmental conditions, has the potential to make production processes more efficient.

CRedit authorship contribution statement

Salah Chaji: Conceptualization, Investigation, Writing – original draft. **Aadil Bajoub:** Conceptualization, Funding acquisition, Supervision, Writing – original draft. **Christian Cravotto:** Investigation, Writing – original draft, Writing – review & editing. **Monica Voss:** Investigation, Writing – original draft. **Silvia Tabasso:** Conceptualization, Data curation, Writing – original draft. **Hafida Hanine:** Supervision, Writing – original draft. **Giancarlo Cravotto:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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