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CHEMICAL AND SENSORY CHARACTERIZATION OF DOCG WINES FROM MARCHE REGION (ITALY)

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List of abbreviation

ANOVA: Analysis of Variance

CO: Cònero

DL: Decreto Legislativo

DO: Denominazione di Origine

DOC: Denominazione di Origine Controllata

DOCG: Denominazione di Origine Controllata e Garantita

DoE: Design of Experiment

DOP: Denominazione di Origine Protetta

EU: European Union

FIA: Flow Injection Analysis

GC : Gas Chromatography

HPLC: High Performance Liquid Chromatography

ICP-MS: Inductively Coupled Plasma- Mass Spectrometry

IG: Indicazione Geografica

IGP: Indicazione Geografica Protetta

IGT: Indicazione Geografica Tipica

IMT: Istituto Marchigiano di Tutela Vini

LLE: Liquid-liquid Extraction

MCFAs: Medium Chain Fatty Acids

MRM: Multiple Reaction Monitoring

MS: Mass Spectrometry

OIV: International Organization of Vine and wine

PA: Offida Passerina

PE: Offida Pecorino

PCA: Principal Component Analysis

PFM: Polyfunctional Mercaptan

RO: Offida Rosso

SBSE: Stir Bar Sorptive Extraction

SCFAs: Short Chain Fatty Acids

SPE: Solid Phase Extraction

SPME: Solid Phase Micro-Extraction

TD: Thermal Desorption system

UV: Ultraviolet

VD: Vernaccia di Serrapetrona sweet

VM: Verdicchio di Matelica

VJ: Castelli di Jesi Verdicchio Riserva

VS: Vernaccia di Serrapetrona dry

VOCs: Volatile Organic Compounds

Summary

The present thesis work has been divided into two main chapters. The objective of the first and principal project was to perform the chemical and sensorial characterization of the five DOCG wines produced in Marche region, with the aim to valorise a product of excellence that is worldwide exported and appreciated.

The chemical characterization has been performed by taking into account the fundamental classes of compounds able to contribute to the organoleptic characteristics of wines, being volatile and phenolic substances, minerals and organic acids. This has been carried out to try to identify those molecules which could be used as markers to assess Marche region DOCG wines authenticity.

The sensorial analysis of wine samples has been performed with the aim to describe their organoleptic features and to find the possible correlation with the chemical fingerprint discriminating one DOCG over the other and thus, further valorising a product of excellence of Marche region. This was carried out by taking into consideration also the DOCGs subgroups. Indeed, for "Offida" DOCG, "Pecorino", "Passerina" and "Rosso" have been considered and, in the same way, for "Vernaccia di Serrapetrona" DOCG the two typologies "sweet" and "dry" were included.

Furthermore, in order to obtain a sampling that was more representative of the wine market in the region, 18 wine samples (2-3 for every DOCG) were collected considering the wineries with higher sales.

The analysis of the volatile substances was performed due to their importance in defining wine aroma. This was carried out by using a new developed solid-phase microextraction method coupled to gas chromatography-mass spectrometry (SPME-GC-MS) analysis. Extraction by SPME includes two steps: a direct immersion followed by head space with the use of an overcoated-fiber. By the analysis of the 18 wine samples, it was possible to detect and identify 50 volatile compounds and, in order to better investigate the results, they were divided into six different groups, according to the chemical class (ethyl esters, other esters, alcohols, acids, terpenes and others). The predominant classes of compounds were ethyl esters, followed by alcohols, even if the composition was quite variable among DOCGs. Moreover, the analyses underlined the presence of compounds which were associated to characteristics scents of the samples investigated, or which could be possibly used as authenticity markers for DOCG wines investigated. For example, the high presence of ethyl esters in "Verdicchio di Matelica" was able to explain the yellow pulp fruity aroma perceived by the panel during the sensorial analysis.

The analysis of short and medium chain free fatty acids was performed by using a new developed and validated liquid-liquid extraction procedure followed by gas chromatography coupled to flame ionization detection (LLE-GC-FID) analysis. This class of compounds is of huge importance when considering the wine quality and the method permitted the quantification of acetic, propionic, isobutyric, isovaleric, hexanoic and octanoic acids. The main differences raised between the composition of white and red wines, the first being more enriched in hexanoic and octanoic acid, while the latter in acetic, propionic, isobutyric and isovaleric acids. For every analyte, the AOV (active olfactory value) was also calculated to assess the compounds that may play an active role in the aroma of the samples investigated. Interestingly, for every analyte (with except for propionic acid) AOV values >1 were obtained, meaning that these analytes may actually take part to the overall aroma of DOCG wines.

Concerning the phenolic profile, it was assessed by performing high performance liquid chromatography coupled to tandem mass-spectrometry (HPLC-MS/MS) analyses and a total of 37 compounds were detected and quantified. As expected, red wines were much more enriched in polyphenols when compared to white DOCG wines. The different statistical approaches evidenced the presence of some peculiarities able to discriminate wines made with same grape variety, or wines produced in close geographical areas. As an example, “Offida Rosso” and “Cònero” which are produced with a minimal amount of 85 % of Montepulciano grape variety showed a similar polyphenolic profile. At the same time, “Offida Rosso” showed the highest concentration of vanillic acid, which makes this compound a possible authenticity marker of this wine typology.

When considering minerals, among the 40 elements quantified by inductively coupled plasma-mass spectrometry (ICP-MS) analysis, five were selected, being considered the ones not consistently altered by oenological practises (and thus, more linked to the soil composition of origin). These elements were Sr, B, Ba, Li and Mn, but, when performing different statistical approaches, a clear discrimination did not raise between wines produced in different part of the region, which can be due to the relatively small production geographical area.

Regarding organic acids, six compounds were identified by HPLC coupled with diode array detector (HPLC-DAD) analysis with an analytical method which is currently under optimization. In particular, tartaric, isocitric, shikimic, lactic, succinic, citric and malic acids were identified and quantified. The statistical analyses of samples underlined a difference in the concentrations of acids when comparing white and red DOCGs. White wines were generally enriched in tartaric and isocitric acid, with the first being the main responsible of wine acid taste. As expected, red wines showed higher concentrations of lactic and malic acids, but also of succinic acid. Shikimic acid, which is considered to be able to discriminate grape varieties, showed

characteristic concentrations. At the same time, every DOCG was characterized by a peculiar organic acid profile, which should be better investigated to confirm its usefulness to assess their authenticity.

Finally, through the sensorial analysis it was possible to point out features able to characterize the 5 DOCGs with distinctive organoleptic profiles. Indeed, for example, "Verdicchio di Matelica Riserva" and "Castelli di Jesi Verdicchio" were described by the typical anise aroma and were the samples more enriched in methyl salycilate, which is a compound able to give the particular note. Also, some differences raised between the two "Offida" white wines ("Pecorino" and "Passerina"), which were described by different scents. Also differences between red wines raised, being the "Còmero" and "Offida Rosso" described by diverse aspect and scents. Finally, "Vernaccia di Serrapetrona" wines were the ones more described by tertiary aromas, such as cinnamon scents.

In this project, both the chemical fingerprint and the sensorial analysis were able to underline substantial differences between the five DOCG wines produced in the region and, by further confirmations, their chemical fingerprints could be used in the future to assess their authenticity. In order to do that, further developments should be performed, by considering both more wineries and different vintages. Also, the sensorial analysis should be performed by following quantitative methods. Indeed, by selecting a panel of judges and by training the panel to describe the sensory characteristics of the DOCG wines investigated, a more reliable correlation could be performed able to fully characterize the sensorial and chemical profile of these wines of excellence.

The second part of the thesis regards the project that was carried out during the period January-June 2022, in the Laboratorio de Análisis Aroma y Enología (LAAE) at the University of Saragozza (Spain).

The study was focused on the optimization of a solid-phase extraction (SPE) procedure in order to concentrate and isolate five polyfunctional mercaptans from wine. These compounds are known to play essential roles in the organoleptic characteristics of wines. In particular, in this study, the five mercaptans considered were 4-mercapton-4-methyl-2-pentanone, furfurylthiol, benzyl mercaptan, 3-mercaptohexanol and 3-mercapto hexyl acetate, which are known to be amongst the most abundant in wine. The SPE procedure has been optimized by taking into consideration all the steps involved (loading of the sample, cleaning, washing and final elution) and by also exploiting the CuCl salt to form strong Cu(I)-S complexes that were found to be essential for the retention of mercaptans in the sorbent phase. Finally, an SPE procedure was

optimized which enabled to obtain very pure extract. The conditions for the final extract analysis are still under optimization through stir bar sorptive extraction (SBSE) coupled to a thermal desorption system (TC-GC-GC-MS) aimed to lower the sensitivity of the method and to proceed with the subsequent validation.

Chapter I: Characterization and valorisation of DOCG wines from Marche region, through the study of their chemical fingerprints and sensorial attributes

1. Introduction

1.1 Products of excellence: geographical indication and designations of origin

The European Union (EU) geographical indications systems are applied to protect products names, in particular food and food-related ones, which are made in a specific geographical area. They establish intellectual property rights for products, whose quality has a specific connection with the place in which they are produced. These recognitions enable consumers to distinguish and to trust a quality product, while also helping producers to valorise and elevate their products (European Commission Official Webpage, Geographical and rural development section. Available online at: https://agriculture.ec.europa.eu/farming/geographical-indications-and-quality-schemes/geographical-indications-s-quality-schemes-explained_en). The designations are used to indicate that the characteristics of a product are essentially linked to the *terroir*, a French term that is used to describe all the environmental factors (soil, climate, etc.) which can influence the quality of a product by imparting unique peculiarities.

Geographical indications comprise:
Geographical Indication ("Indicazione Geografica: IG")
Protected Geographical Indication ("Indicazione Geografica Protetta: IGP")
Protected Designation of Origin ("Denominazione di Origine Protetta: DOP")

The most general and less restrictive designation is IG, which is applied only to spirit drinks or aromatised wines. In this case at least one of the distillation or preparation stage must take place in the region, but for example raw materials don't need to come from the region.

The IGP designation is an acknowledgment used to emphasise the relationship that exists between the name of the product and the geographical region where it is produced and that is applied for food, agricultural products and wines.

By EU definition:

"«geographical indication» means the name of a region, a specific place or, in exceptional cases, a country, used to describe an agricultural product or a foodstuff: originating in that region, specific place or country and which possess a specific quality, reputation or other characteristics attributable to that geographical origin and the production and/or processing and /or preparation of which take place in the defined geographical area"

(EU Regulation n.510/2006, Art. 2)

For the IGP products at least one of the production stages, preparation or processing must take place in the region. The labelling for foods granted as IGP is mandatory, while it is optional for wines (**Figure 1, A**).

Finally, the Protected Designation of Origin (DOP), introduced in the European Union in 1992, is an even more prestigious designation compared to IGP and follows more restrictive rules.

By EU definition:

"«designation of origin» means the name of a region, a specific place or, in exceptional cases, a country, used to describe an agricultural product or a foodstuff: originating in that region, specific place or country; the quality or characteristics of which are essentially or exclusively due to a particular geographical environment with its inherent and human factors, and the production, processing and preparation of which take place in the defined geographical area"

(EU Regulation n.510/2006, Art. 2)

As IGP designation, even in this case it is applied to food, agricultural products and wines but for DOP every part of the production processes must take place in the region. Concerning labelling, it is mandatory for food and agricultural products, but not for wines (**Figure 1, B**).

A

B

Figure 1. A) EU IGP logo. B) EU DOP logo.

Italy can boast a huge variety of products that are protected by designations of origin and that are greatly appreciated and exported worldwide; one of particular prestige is surely wine. A

system regulating, labelling and legally protecting Italian wines was already introduced in 1963, which was subsequently overhauled to match the more general previously cited European Union DOP. The updated classification of wines was introduced by the Legislation 164/1992 which stated that wines can be classified as the ones having geographical indications, IGP or DOP, and the ones that are not protected by the geographical indication.

Wines in Italy can follow 4 different classifications:

- **DO:** Designation of Origin, rarely used ("Denominazione di Origine")
- **IGT:** Indication of Geographical Typicity ("Indicazione Geografica Tipica")
- **DOC:** Controlled Designation of Origin ("Denominazione di Origine Controllata")
- **DOCG:** Controlled and Garanteed Designation of Origin ("Denominazione di Origine Controllata e Garantita")

The IGT designation is used to indicate wines with a quality higher than the so-called table wines, but that at the same time, do not meet the extreme rigorous requirements of DOC and DOCG. This designation is the equivalent of the European Union "Protected Geographical Indication" (PGI).

Subsequently, with DOC are designed wines that satisfy defined quality standards and that are produced in a restricted geographical area. Furthermore, with respect to the IGT wines, they are regulated with more stringent rules which regard grape variety, harvest yield, minimum alcohol content, minimum ageing and other factors.

Finally, DOCG is a superior classification of DOC wines, representing the highest level of classification that a wine can reach. In order to be classified as DOCG, a wine needs to follow a restrictive disciplinary protocol and before being bottled, it is analysed by a competent government-licensed judgment panel. Moreover, these wines are guaranteed by the presence of a numbered governmental seal across the cork or cap. (**Figure 2**). The procedure to recognise a wine as DOCG follows European legislation (Reg. (CE) n.479/2008). Both DOC and DOCG fall into the European category of DOP. The legislation which regulates the designations and labelling for IGT and DOP wines follows the Reg. (CE) n. 607/2009.

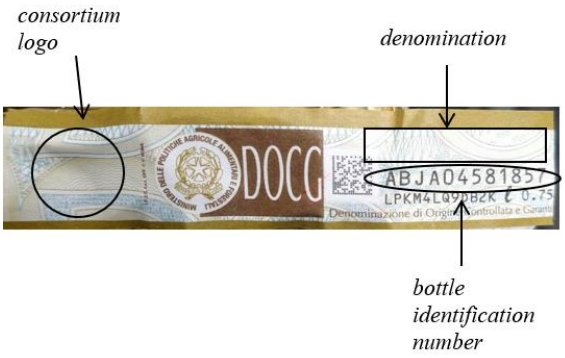

Figure 2. Mandatory labelling introduced for DOCG wines.

Then, by the introduction of DL (CE) n.61/2010 also wines which were previously referred as "table wines" can be further classified in "table wines" or "varietal wines", where the production year of grape variety can be optionally specified (**Figure 3**).

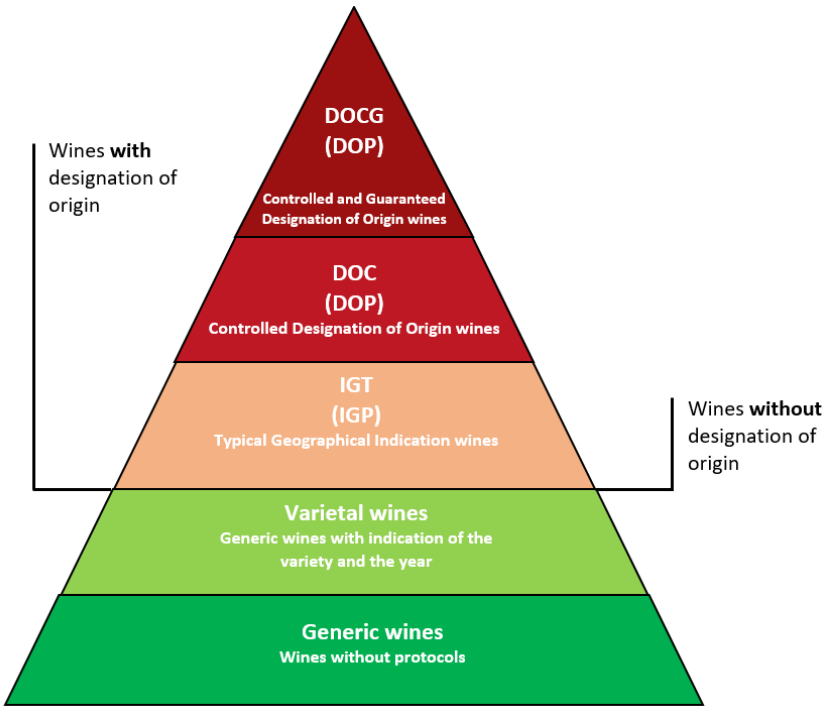

Figure 3. Italian wine classification according to DL (CE) n.61/2010.

1.2 DOCG wines from Marche region

According to Federdoc ("Confederazione italiana dei consorzi volontari per la tutela delle denominazioni dei vini italiani") and to Masaf ("Ministero dall' Agricoltura, della Sovranità Alimentare e delle Foreste") Italy can count 76 DOCG wines, most of them produced in Piemonte (19), Toscana (11) and Veneto (14) regions (last updated 2022. Data available online at:

https://www.federdoc.com/i-vini-italiani-a-denominazione-dorigine-2022). Marche Region can count 5 DOCGs and 15 DOCs (**Figure 4**).

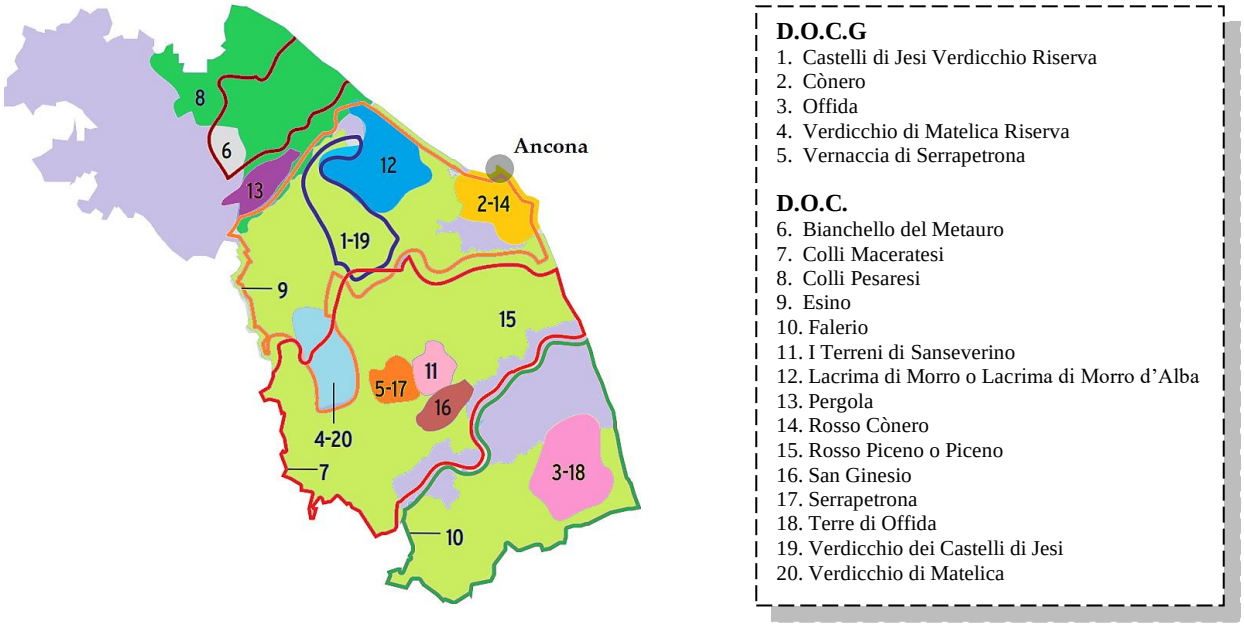

Figure 4. Marche region DOC and DOCG wines (Federdoc, 2022).

Marche region was historically and geographically divided by rivers l and from the borders of the various Marche areas and it is characterized by a great *terroir* diversification due to the different influence that Apennine Mountains to the west, and Adriatic Sea to the east, have (Mignani *et al.*, 2019).

In particular, two consortia, territorially divided in north and south provinces, protect and promote the 20 wines with protected designation of origin. "Istituto Marchigiano di Tutela Vini" (IMT) covers the provinces of Pesaro, Ancona and Macerata, while the provinces in the south of Marche Region, namely Fermo and Ascoli Piceno, are covered by the consortium "Vini Piceni". The first is the biggest one, territorially covering over 8000 ha and representing 16 over the 20 designations of origin. IMT presents 12 DOCs designation and 4 DOCGs designation, and Vini Piceni, the remaining 3 DOCs and 1 DOCG designation.

The 5 DOCGs are:

1. Castelli di Jesi Verdicchio Riserva
2. Verdicchio di Matelica Riserva
3. Offida
    a. Pecorino
    b. Passerina
    c. Rosso
4. Còner
5. Vernaccia di Serrapetrona
    a. Sweet
    b. Dry

For Offida wines all the three different typologies Pecorino, Passerina and Rosso are acknowledged with DOCG designation. Vernaccia di Serrapetrona can also count two DOCGs since it is produced as sweet or dry wine. Wineries and producers are obliged to follow the related production disciplinary in order to obtain wines that can be granted as DOCG.

Some of the main characteristics of the 5 DOCGs, according to their production specifications, are briefly reported below.

Verdicchio di Matelica Riserva

"Verdicchio di Matelica Riserva" is produced with grapes cultivated in 5 municipalities in Macerata province and in delimitated part of 2 municipalities in the Ancona province. The wines must be obtained from Verdicchio grape variety with a minimal amount of 85%. The requirements that must be respected in order to be designed as DOCG, are the following:

Colour: straw yellow
Odour: delicate, characteristic
Taste: dry, harmonic with a light bitter aftertaste
Minimal alcoholic degree: 12.5%
Total acidity: 5.00 g/L
Minimal non-reductive extract: 18.0 g/L

Castelli di Jesi Verdicchio Riserva

"Castelli di Jesi Verdicchio Riserva" (and Riserva Classico) is produced with grapes cultivated in the provinces of Ancona and Macerata. The wines must be obtained from Verdicchio grape variety with a minimal amount of 85 %. The requirements that must be respected in order to be designed as DOCG, are the following:

Colour: straw yellow, more or less intense
Odour: delicate, characteristic
Taste: dry, harmonic, with a pleasant bitter aftertaste
Minimal alcoholic degree: 12.5 %
Total acidity: 4.5 g/L
Minimal non-reductive extract: 17.0 g/L

Offida

"Offida" wines are produced with grapes cultivated in the provinces of Ascoli Piceno and Fermo, including near delimitated territories. The wines must be produced with Pecorino,

Passerina or Montepulciano grapes variety with a minimal amount of 85 %. The requirements that must be respected in order to be designed as DOCG, are the following:

Offida Pecorino

Colour: straw yellow with gold reflections
Odour: characteristic, pleasant
Taste: dry, typical, characteristic
Minimal alcoholic degree: 11.5 %
Total acidity: 4.5 g/L
Minimal non-reductive extract: 16.0 g/L

Offida Passerina

Colour: straw yellow with greenish reflections
Odour: characteristic, pleasant
Taste: dry, typical, characteristic
Minimal alcoholic degree: 12.0 %
Total acidity: 4.5 g/L
Minimal non-reductive extract: 18.0 g/L

Offida Rosso

Colour: ruby red with tendency to garnet with aging
Odour: pleasant, complex, slightly ethereal
Taste: dry, harmonic, typical, characteristic
Minimal alcoholic degree: 13.0 %
Total acidity: 4.5 g/L
Minimal non-reductive extract: 24.0 g/L

Cònero

"Cònero" is produced with grapes cultivated in Ancona province. The wines must be obtained from Montepulciano grape variety with a minimal amount of 85 %. The requirements that must be respected in order to be designed as DOCG, are the following:

Colour: ruby red
Odour: pleasant, winery
Taste: harmonic, dry, body rich
Minimal alcoholic degree: 12.5 %
Total acidity: 4.5 g/L
Minimal non-reductive extract: 24.0 g/L

Vernaccia di Serrapetrona

"Vernaccia di Serrapetrona" is produced with grapes cultivated in Serrapetrona municipality and in part of Belforte del Chienti and San Severino Marche municipalities. The wines must be obtained from Vernaccia Nera with a minimal amount of 85 %. The requirements that must be respected to be designed as DOCG, are the following:

Sweet and dry
Foam: persistent, fine-grained
Colour: from garnet to ruby red
Odor: characteristic, winey
Taste: characteristic, from dry to sweet, with pleasant bitter aftertaste
Minimal alcoholic degree: 11.5 %
Total acidity: 4.5 g/L
Minimal non-reductive extract: 22.0 g/L

2. The quality of a wine explained by its chemical composition

Wine quality is a very difficult parameter to define, since a unique and general definition cannot be accepted. It is strictly correlated to subjective perceptions, but it can also depend on many other factors, for example the differences between populations and thus, social factors (Charters & Pettigrew, 2007). A review was recently published by Yang & Lee (2020) concerning sensory studies on wine to understand consumer perception performed in the last decade.

Only few characteristics are generally known to lower wine quality, which are the most common defects, such as vinegar-like odour or the well-known cork taint, but that are usually associated with occasional process and/or storage problems.

The International Organization of Vine and Wine (OIV) considers the quality as a set of parameters that can discriminate one wine from another, one of these being the consumer taste and preferences. However, this definition makes wine quality relative and variable and also dependent on factors such as the country or population habits. On the other hand, the Regulation (EEC) No. 823/87 established a more permanent concept of wine quality, by defining parameters such as: delimitation of production area, varieties distribution, cultivation systems, vinification methods, yield per hectare, minimum natural volumetric alcoholic strength and analysis and evaluation of sensory characteristics. Consequently, wine quality will depend on several and different factors which will be also important in the development of aromatic compounds.

When searching for a high-quality wine, consumers look for suggestions coming from wine experts and/or other trusted sources, followed by price, geographical area and age. Furthermore, for most of consumers, quality reflects satisfaction and since quality is a cerebral construct, is not surprising that it has been detected on a neuronal level and that different consumers can experience different emotions in wine consumption (Plassmann *et al.*, 2008; Calvo-Porral *et al.*, 2020).

Hence, wine quality perception is the sum of different factors, and it is a parameter which is not easy to assess, even if some critical aspects are surely able to determine it. One of the most crucial factors in the development of a wine's quality is certainly represented by the winemaker (**Figure 5**).

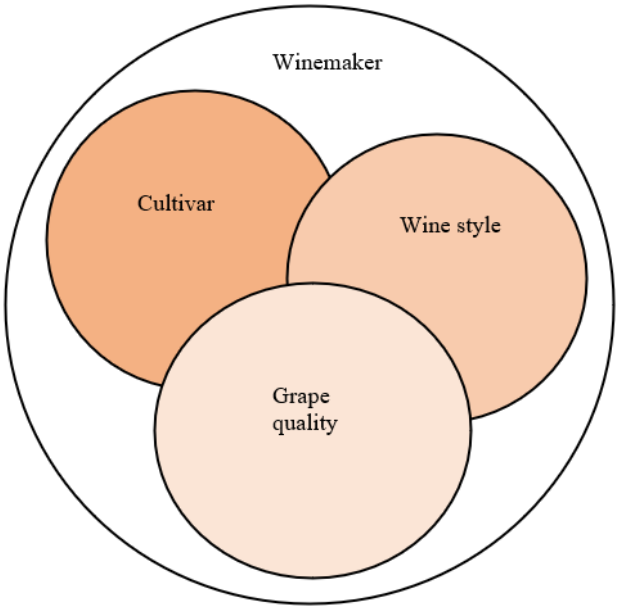

Figure 5. Diagram with the major wine quality sources (Jackson, 2009).

The attributes and the evolution of wine are strictly related to the vinification process planned by the important figure of the winemaker. Grape cultivar and wine production style, for instance, are the factors which more easily donate detectable differences between wines, with respect, for example, to regional characteristics. The choice of grape cultivar (or variety) is essential to produce a precise wine typology but, at the same time, even if some grape cultivars are known to lead to wine characterized by a distinct aroma, the production style is one of the predominant factors shaping a defined flavour wine profile. Indeed, most of aromatic compounds evolve during fermentation processes and wine aging, hence the choice of the proper winemaking and refinement conditions is of paramount importance. The grape quality, that comprises maturity, flavour content and health, is also important since it fixes the limits on the wine quality potential

and in this context the role of winemaking is also essential. The wrong choice of the harvest time, for example, would compromise the success to obtain a wine with targeted characteristics.

Quality can be viewed also in two different factor types: intrinsic (sensory, physicochemical) and extrinsic (context, prestige, price) where ideally only the intrinsic factors would have legitimacy. Actually, it is widely known that extrinsic factors are able to play the preeminent role in the peoples' concept of wine quality. For this reason, one goal of paramount importance for sensory analysis would be to understand the physiological, psychological and chemical bases of quality. In fact, even if wine quality is framed in terms of varietal origin, prestige, provenance, style, age, vintage and other attributes, its legitimate quality is the results of the sensory characteristic which arise from its chemical composition.

By now more than 800 compounds have been found to be potentially present in wine. The chemical composition also changes during time due to chemical reactions such as oxidation, reduction, polymerization and other transformations. Moreover, most of the chemicals are present in quantities below their detection threshold levels, hence, only few compounds are really able to play a role in the sensorial perception in a particular wine and these substances are mainly alcohols, terpenes, carboxylic acids, esters, phenolics and sugars. In the past some works were published aimed to explain the relation between the sensorial properties of some wines and their chemical composition. As an example, Gawel *et al.* (2007) performed the statistical correlation between the in-mouth textural characteristics and the chemical composition (polyphenols, acidity and red pigments) of Shiraz wines. Bindon *et al.* (2014) reported a study based on a multidisciplinary approach which combined a detailed chemical analysis to sensory analysis and consumer testing to define the relationship between grape maturity wine composition and quality (Bindon *et al.*, 2014).

3. State of Art

Autochthonous grape varieties and wines of excellence produced with them have gone through a fast development in the recent years. Even if sensory analysis is widely used in the food industry, in Italy, Marche region was the first one that exploited the results from the sensory analysis in order to promote regional wines (Magnani *et al.*, 2019a). Moreover, the IMT consortium wanted definitions of the objective quality characteristics of DOC and DOCG wines to promote Marche region wines in the international market. In fact, consumers benefit from reading the sensory profiles of wines since it details the distinctive notes of the product. Furthermore, an oenologist who curated the IMT promotional campaign affirmed that "there was

a need for a scientific tool that could objectively define the sensory characteristics of denominations of origin, in order to have specific information for expressing the Marche region "*terroir*". The sensory analysis uses quantitative descriptive analysis, which is a reproducible method for describing the peculiarities of a wine, objectively and trough a language which is easily accessible to consumers. To this, the chemical characterization should be put beside as an essential tool to explain wine quality, with the aim to correlate the chemical fingerprint and composition to precise sensorial characteristics.

In this context, scientific methods to assess authenticity are gaining more and more attention and the chemical profile of wines plays an essential role. In 2017, a multivariate approach was proposed in order to relate the typicality of a red wine with DOP designation to its chemical composition and the chemical composition of its grape variety (Canuti *et al.*, 2017). The chemical characterization of grapes and wine demonstrated that their quality was geographically controlled since they frequently varied with the geographical origin of grapes or with the place of wine manufacture (Vaudour, 2002). Then, Canuti *et al.* (2019) demonstrated that it was possible to determine the different provenance of wines made with Sangiovese grape variety. It was performed over 46 Sangiovese wines of 2016 vintage produced in Italy and California by analysing volatile fraction, elemental content, colour and phenolic composition, confirming how the study of the chemical fingerprint could play an essential role in wines characterization and in particular on those protected by designation of origin.

Wines produced in Marche region have been subject of few studies by now, even if most of the investigated wines were not DOCG. Concerning "Castelli di Jesi Verdicchio" for example, a sample of Classico was characterized by Carlin *et al.* (2019) who studied its volatile fraction. The phenolic content of Verdicchio DOC and Passerina was assessed in by Boselli *et al.* (2006). In 2019 three different Marche region wines "Verdicchio di Matelica" DOC, "Verdicchio dei Castelli di Jesi" DOC, "Offida Pecorino" DOC, together with a Veneto Region wine were investigated in a very interesting study with the aim to understand unconscious reactions in wine tasting by consumers (Magnani *et al.*, 2019b). The volatile profile and phenolic fraction of "Vernaccia di Serrapetrona" sweet wines, designed as DOCG, was investigated by Fiorini *et al.* (2013) by considering different vintages.

Hence, among the few works present in literature, some of which were above mentioned, there is still a lack in a more specific chemical characterization of DOCG wines produced in Marche region, which are products of excellence widely known and appreciated. The great variability of terroir, climatic conditions together with the different winemaking techniques

performed by different wineries favour the obtaining of products which are characteristic and unique and whose quality can be better explained by investigating their chemical fingerprint.

4. Aim of the work

The EU and the national politics and legislation are oriented toward the defence and the development of local production by Legislation EEC n. 2081/1992, and update with the new EEC n.510/2006 which rules the protection of geographical indications and designation of origin. The scope is to preserve biodiversity in order to make it easier to determine those parameters useful to assess authenticity and typicality of regional food products, such as wine (Boselli *et al.*, 2008). Also, the enhancement of the methods to verify the geographical origin of authentic wine is of huge importance.

Starting from these promises, the objective of this work was to characterize different samples of DOCG wines produced in Marche region investigating in detail their chemical composition and their sensory properties in order to identify also peculiarities and compounds which could be eventually used as authenticity markers. Moreover, the study has been carried out with the aim to find the possible correlation existing between the chemical fingerprint which characterizes the samples under investigation with the deriving sensorial attributes that acknowledge these wines as products of excellence. To this purpose, different analyses were performed, with the important collaboration of different research groups.

The volatile fraction was analysed after optimizing a new method which involves the solid-phase microextraction (SPME) followed by gas-chromatography analysis coupled to mass spectrometry (GC-MS). The application of the method for analysing the wine samples was performed at the Università Politecnica delle Marche (UNIVPM). Concerning the volatile profile, the subclass of short and medium chain free fatty acids (SCFAs and MCFAs), known to have an important role from the sensorial point of view, was also taken into consideration. A new method for their analysis was developed and validated, that consists of the liquid-liquid extraction (LLE) of the analytes followed by gas chromatography analysis coupled to flame ionization detector (GC-FID).

Also, polyphenols, minerals and organic acids were analysed with the help of other research groups from UNICAM. These classes of compounds are known to play essential roles in the definition of wine quality and in their traceability. Finally, a sensorial analysis was performed by a panel, to describe characteristic attributes for all wine samples in terms of visual, olfactive and gustative perceptions. This part of the work was performed at Valoritalia (Jesi, Italy).

This study can be considered as the base for the identification of authenticity markers of DOCG wines produced in Marche region. As a future perspective, in fact, these analyses should be performed by taking into consideration more wineries and also different vintages of the same DOCG wines. The comparison with other wines, could unequivocally allow the identification of those compounds that characterize the wines under investigation.

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1: Analysis of Volatile Organic Compounds (VOCs): Optimization and application of a solid—phase microextraction (SPME) procedure followed by GC-MS analysis

1.1 Introduction

1.1.1 The importance of volatile substances in wine

The aromatic profile of wine, which results from grapes, fermentation and aging processes, is one of the most important parameters to define its quality. Furthermore, the complexity of wine aroma can vary greatly depending on a huge number of different factors, such as *terroir*, grape variety, yeast, fermentation process, aging and bottling. Even if the volatile fraction composes only a small percentage (estimated around the 0.5%) of the total wine volume, it has a fundamental role and it greatly influences the consumer preferences and its acceptance or rejection (**Figure 1**).

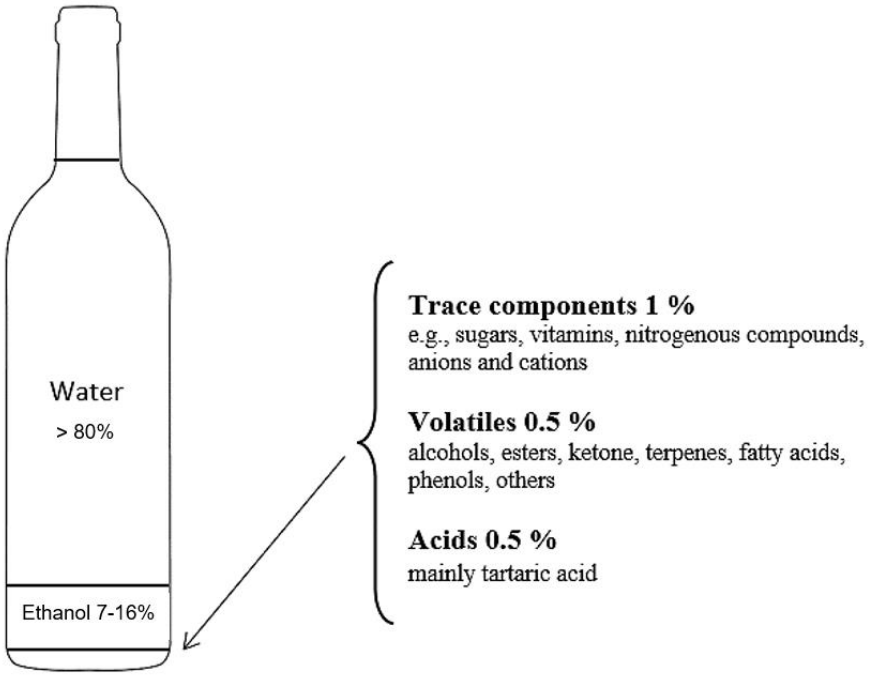


Figure 1. General chemical composition of wine (Jackson, 2014).

Hence, it results clear why the study of wine (and grape) volatile fraction, is very important and it's gaining more and more attention.

Wine is a fermented alcoholic beverage, and it is a very complex matrix which comprises several hundreds of compounds (more than 800) of different nature, even it only few of them can really contribute to the so-called *bouquet* (Guth, 1997; Cullere *et al.* 2004; Zhang *et al.* 2007; Li &

Tao, 2008). Some of the most relevant volatiles, with their ranges of concentrations, chemical and physical characteristics, and sensorial attributes and arising from different wines (white, young and red aged) are reported in **Table 1**.

Table 1. Chemical and sensorial properties of relevant aroma compounds in wine (Remedios & Ross, 2013).

Compound	Molecular weight (g/mol)	Boiling point (°C)	Log *P* Value[d]	Concentration (μg L^{-1})	Threshold (μg/L)	Aroma descriptor[k]
Ketones						
β-damascenone [a,b,c]	190	265	4.21	2[a], 29,[b] 3.5[c]	0.05 [e,h]	apple, honey
β-ionone[c]	192	263	3.84	0.23[c]	0.09[g]	seaweed, violet
3-octanone[b]	128	168	2.22	17[b]	21-50[j]	herb, butter
Esters						
ethyl 2-methylbutyrate[c]	130	135	2.26	32[c]	18[g], 1[e]	apple
ethyl 3-methylbutyrate[c]	130	135	2.26	20[c]	3[g]	fruit
ethyl butyrate[a,b]	116	122	1.85	680,[a] 69[c]	20[g,h]	apple
ethyl hexanoate[a,b,c]	144	167	2.83	650,[a] 140,[b] 29[c]	14[g,h],5[e]	apple peel, fruit
ethyl cinnamate[c]	176	271	2.99	1.22[c]	1.1[g]	honey, cinnamon
isoamyl acetate[a,b,c]	130	142	2.25	60,[a] 142,[b] 120[c]	30[e,g,h]	banana
Alcohols						
isoamyl alcohol[a,b,c]	88	131	1.16	150,000,[a] 1,412,[b] 112,800[c]	30,000[e,h]	whiskey, malt, burnt
hexanol[a,b,c]	102	158	2.03	8,000,[a] 617,[b] 780[c]	8,000[e],[g],[h]	resin, green
phenyl ethyl alcohol[a,b,c]	122	218	1.36	34,000,[a] 6,089[b] 60,300[c]	14,000,[g,h] 10,000[e]	honey, rose
methionol[c]	106	178	0.44	3,750[c]	1,000[g]	sweet, potato
1-heptanol[b]	116	177	2.62	15[b]	3[j]	chemical, green
Phenols						
guaiacol[c]	124	205	1.32	47.3[c]	9.5,[g] 10[e]	smoke, medicine
eugenol[c]	164	253	2.27	60[c]	6,[c] 5[e]	clove, honey
4-vinylguaiacol[c]	150	247	2.24	30[c]	40,[e] 10[j]	clove, curry
Terpenes						
β-citronellol[b,c]	156	224	3.91	21,[b] 1.2[c]	100[f]	rose
linalool oxide[b]	170	233	2.08	3.0[b]	4–10[k]	flower, wood
geraniol[b,c]	154	230	3.56	19,[b] 3.2[c]	20,[c] 30[e]	rose, geranium
Lactone						
cis-whiskey lactone[c]	156	261	2.00	151[c]	67[i]	coconut
Acids						
isopentanoic acid[c]	102	176	1.16	1,670[c]	33[g]	unpleasant, acid
hexanoic acid[a,b,c]	116	205	1.92	5,300[a] 120[b], 2,730[c]	420[g,h]	goat, cheese
octanoic acid[a,b,c]	144	239	3.05	26,000[a] 555[b] 1,910[c]	500[g,h]	fat, cheese

[a]Li *et al.*, 2008; [b]Tao *et al.*, 2008; [c]Escudero *et al.*, 2007; [d]Hydrophobic constant from EPI Suite™ software; [e]Guth *et al.*, 1997 (in 10% ethanol/water); [f]Etievant, 1991 (in wine); [g]Ferreira *et al.*, 2000 (in 11% ethanol/water with 7g/L glycerine, 5g/L tartaric acid, pH 3.4); [h]Cullere *et al.*, 2004 (in 10% ethanol/water with 5 g/L tartaric acid at pH 3.2); [i]Lopez *et al.*, 2002 (in 10% ethanol water at pH 3.2; [j]Burdock, 2009 (unspecified matrix); [k] Flavornet by Terry Acree & Heinrich Arn.

Volatile compounds are also characterized by the so-called olfactory threshold (OTH), which is the lowest concentration of an odour compound that can be perceived by the human sense of smell. The presence of a compound in wine in concentration above its OTH does not automatically leads to its perception. In fact, the final aromatic profile is the result of the synergic or depressive effects of the volatiles substance (mainly ester, alcohols, aldehydes, terpenes, thiols, etc.) with the non-volatile matrix (rich in acids, polysaccharides, glucose, fructose, proteins and polyphenols) and the sensory olfactory receptors (Styger *et al.*, 2011). Therefore, the overall sensations perceived during wine consumption is not strictly correlated to the individual aromatic compounds in wine. Indeed, it has been observed specific volatile-non volatile component interactions able to influence the sensory characteristics of wines (Muñoz-González *et al.*, 2014).

Also, the perception of an aromatic compound is affected by the other volatile substances. For example, it has been confirmed that some molecules, such as dimethyl sulfide (truffle notes), diaceyl (butter notes), acetic acid (vinegar notes) are able to modulate the aroma perceptions by masking the fruity scents of ethyl esters and higher alcohols in red wines (Cameleyre *et al.*, 2015).

Beyond the complexity of wine aroma and its perception, during its consumption, physiological factors can also play a very important role in the aroma release, leading to different concentrations of the aromatic molecules that reach sensorial receptors and that can change its retro-nasal perception (Lyu *et al,* 2021).

When considering wine aroma, a very important distinction must be highlighted, regarding their origin. The flavouring substances can be divided into 3 main categories, depending mainly on the vinification step from which they can derive. They are, namely, primary, secondary and tertiary aromas, described below more in detail and with some examples.

Primary aromas are also known as varietal aromas. They are aromatic molecules already present in the grape where they can be found in the form of their non-odorant precursors and stored as sugars or amino acid conjugates. They are strictly related to the grape variety and are released through the mechanical crushing and pressing steps of vinification and during fermentation.

Primary aromas that belong to the same grape variety can present different features depending, for example, to the terroir, and thus on the natural factors that can differently impact primary aromas (Pereira *et al.*, 2020). Compounds associated to the class of varietal aromas are usually terpenes, norisoprenoids and pyrazines.

Some examples of the most important terpenes with their sensorial attributes are represented in figure below (**Figure 2**).

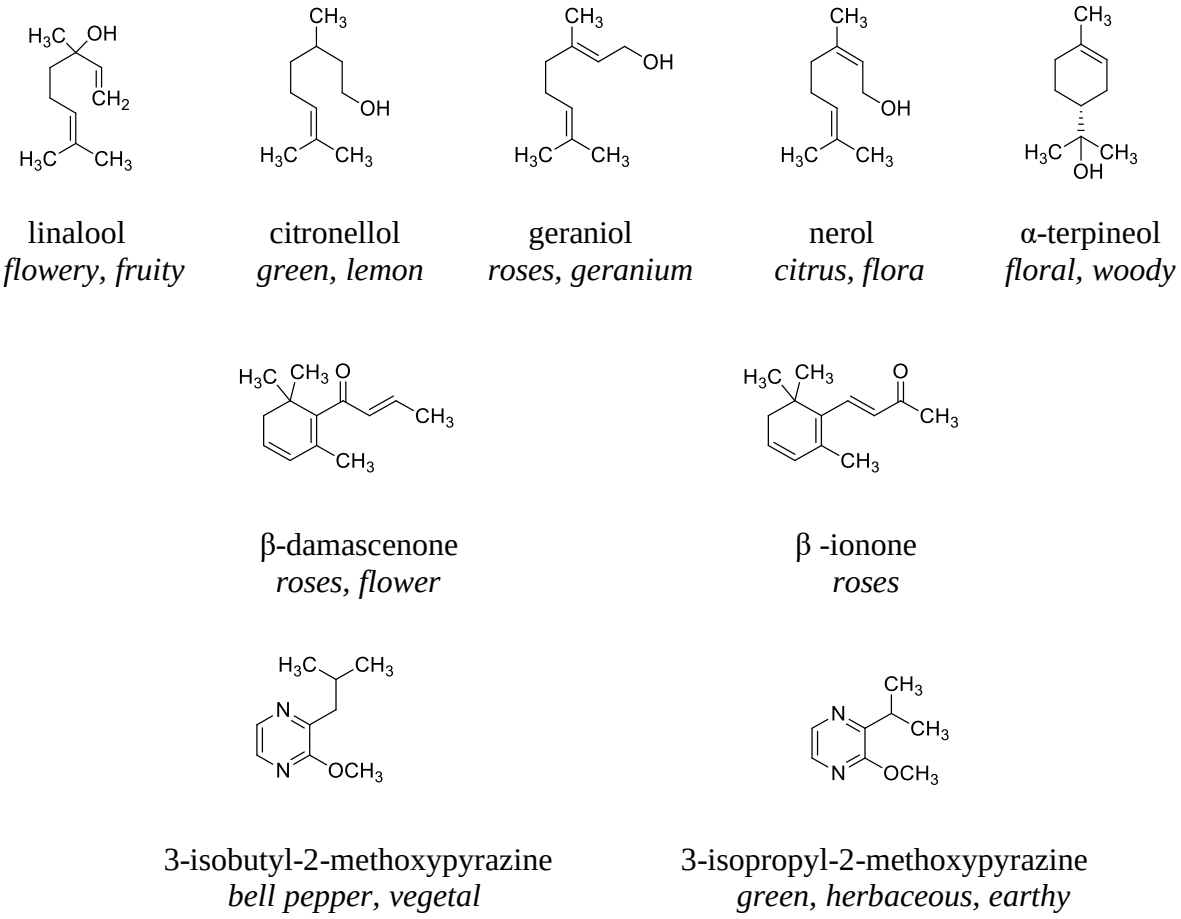


Figure 2. Main terpenes, norisoprenoids and pyrazines and their sensorial attributes.

Secondary aromas are compounds formed during the alcoholic fermentation as yeast by-products and are usually the ones present at highest concentrations. The volatile composition is highly dependent on fermentation and thus on the species and trains of selected yeasts. They belong to different classes of compounds, and are mainly alcohols, ethers, acids, aldehydes and esters and their biosynthesis involve different and complex pathways (**Figure 3**).

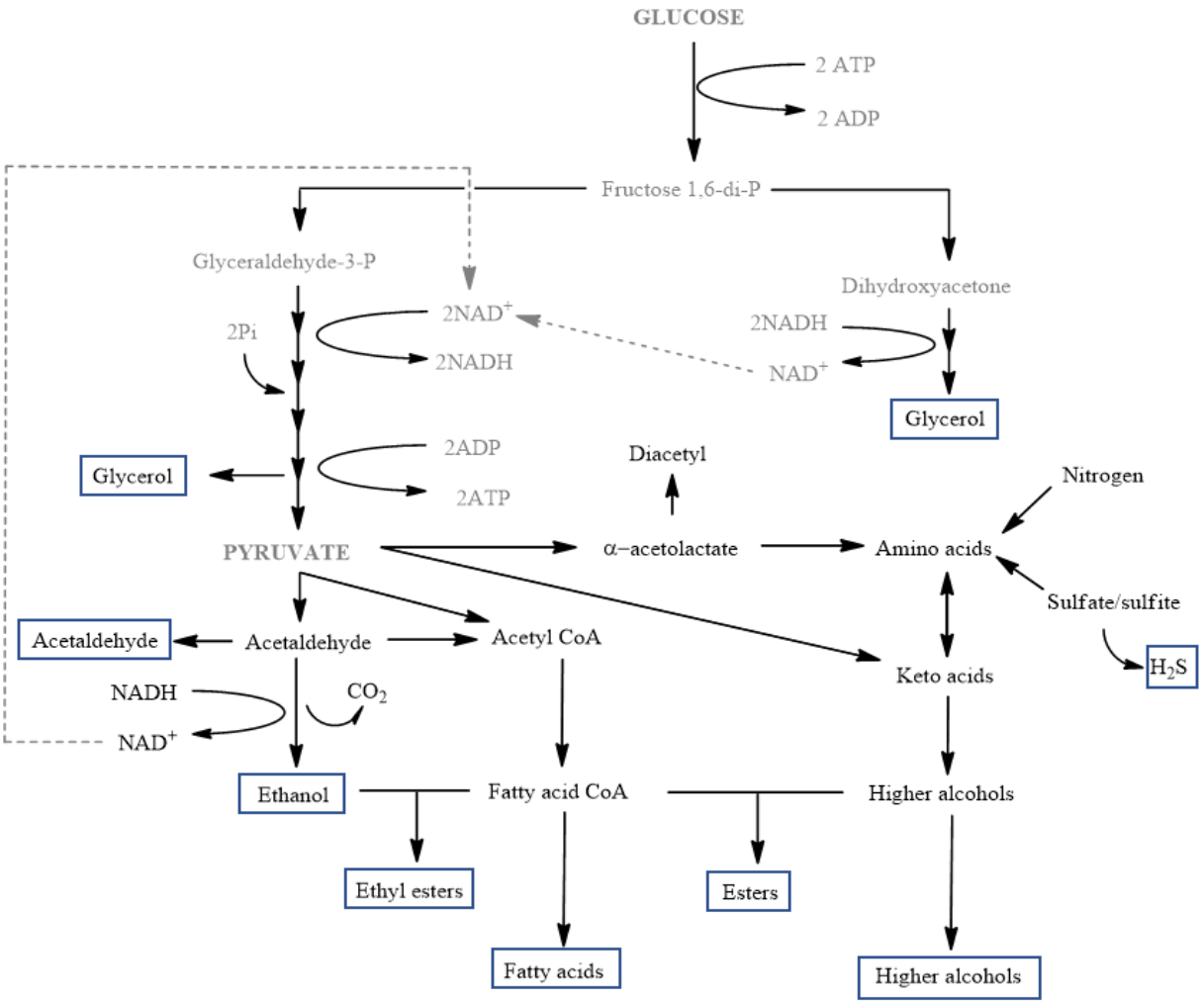
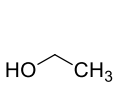
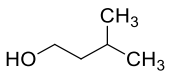


Figure 3. General representation of aroma compounds metabolism (Carpena *et al.*, 2021).

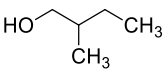
Among the compounds formed during yeast fermentation, the main product is obviously ethanol, which is produced by the exothermic degradation of sugars present in the grape, with the consequent release of this major component together with CO_2. It is considered a very important matrix component since it can affect the perception of volatile substances. Following, the most important alcohols are isoamyl alcohol, 2-methyl-1-butanol and 2-methyl-1-propanol (**Figure 4**). These compounds are known to contribute to the so-called complexity at concentrations below 300 mg/L.



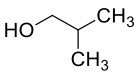
ethanol
alcoholic



isoamyl alcohol
pungent, alcoholic



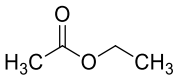
2-methyl-1-butanol
alcoholic



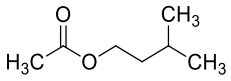
2-methyl-1-propanol
winey

Figure 4. Main alcohols and their sensorial attributes.

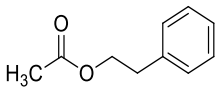
The characteristic fruity aroma is, instead, attributed to the presence of acetyl and ethyl esters of fatty acids formed during the fermentation by the reaction between organic acids and alcohols. The ones showing lower detection thresholds are ethyl acetate, isoamyl acetate, 2-phenyl ethyl acetate, ethyl butyrate, ethyl octanoate and ethyl hexanoate whose chemical structures are shown in figure **Figure 5** (Baumes *et al.* 1986).



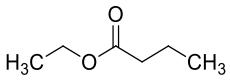
ethyl acetate
sweet



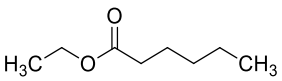
isoamyl acetate
banana



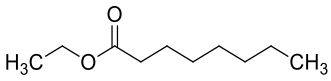
2-phenyl ethyl acetate
rose



ethyl butyrate
fruity



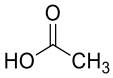
ethyl hexanoate
fruity, apple



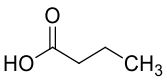
ethyl octanoate
fruity, pineapple

Figure 5. Main esters and their sensorial attributes.

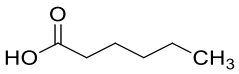
Concerning acids, 90% of the volatile acidity consists of acetic acid which is formed during the fermentation by the oxidation of acetaldehyde, but whose higher concentrations can also be attributed to the present of acetic acid and lactic bacteria. Other acids such as butyric, isopentanoic, hexanoic, octanoic and decanoic are also detected in wine (**Figure 6**).



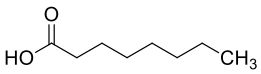
acetic acid
vinegar



butyric acid
butter



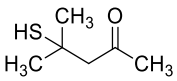
hexanoic acid
goaty, fatty



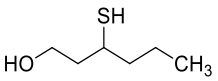
octanoic acid
goaty, fatty, cheese

Figure 6. Main acids and their sensorial attributes.

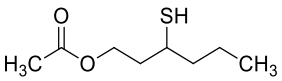
Sulphur compounds are also known to possess very low threshold levels and hence, even if present in traces are able to greatly contributes with their characteristic flavours. Some of the most known that can arise from alcoholic fermentation are reported in **Figure 7** (Swiegers & Pretorius, 2007).



3-methyl-3-mercapto-2-pentanone
fruity



3-methylhexanol
grapefruit



3-mercaptohexyl acetate
tropical fruit

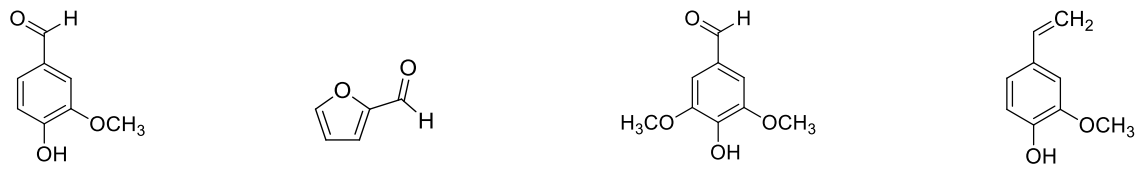
Figure 7. Main sulfur compounds and their sensorial attributes.

There are a huge number of factors able to influence the production and the concentrations of secondary aromas, such as grape variety, grape maturity, yeast growth and strain, ethanol production, skin contact time, etc. (Antonelli *et al.* 1999; Keyzers & Boss, 2010; Sánchez Palomo *et al.* 2006).

Tertiary aromas are compounds which can evolve during aging in oaks and/or bottles. During this period there is a general loss of grape and fermentation-derived characters which are replaced by aroma characterizing aged wines *bouquet*. The evolution of aged wine aroma is strictly dependent on the storage conditions, which influence the chemical reaction that may take place. Red wines are usually aged, since white wines lack in the presence of phenolic compounds making them less resistant to oxidation processes.

The aging in barrels is also called oxidative, since a low quantity of oxygen is able to come into contact with wine. In fact, oxygen can pass through the pas of barrel wood stave and wood micropores. During this step wine undergoes a controlled oxidation with the consequent transfer of volatiles and non-volatile compounds from the wine to the barrel and vice versa. This is the moment in which the unique aromatic outline is developed, which depends on type of wood used and on storage time (De Símon 2010; Sanz *et al.*, 2012). Moreover, also the barrel-making process is able to greatly affect the chemical properties of barrels, and thus the impact they can have on wine, even if the flavor contribution is not easy to predict. The most relevant compounds that can be transferred from wood to wine are ellagitannins, hydroxybenzoic acid and hydroxycinnamic acids, but also reactions, such as tannins and flavonols condensation and pigment polymerization, can take place (Oberholster *et al.*, 2015). Moreover, the different composition and concentrations of the oak constituents present in wine and that are extracted from the barrels vary a lot, depending on the aging times and toasting level of barrels.

Some examples of the main tertiary aromas that derive from aging processes are shown in **Figure 8**.



vanillin *vanilla* | furfural *woody, bread* | syringaldehyde *green* | 4-vinylguaiacol *spicy, clove-like*

Figure 8. Main tertiary aromas and their sensorial attributes.

Once the oxidative aging is completed, wine is placed in bottles and, as the stopper is generally gas-porous, it acts as a permeable barrier for gases such as volatiles alcohols, water vapours that may dissipate out of the wine bottle. At the same time, oxygen is present in wine in dissolved form and is also present in the headspace (HS) of the bottle, which is the volume that remains empty after the filling with wine (**Figure 9**). For this reason, the HS of the bottle, is usually replaced with inert gas, to minimize further unwanted oxidation, evaporation of water and alcohol processes and also to avoid pressure difficulties (Strobl, 2018).

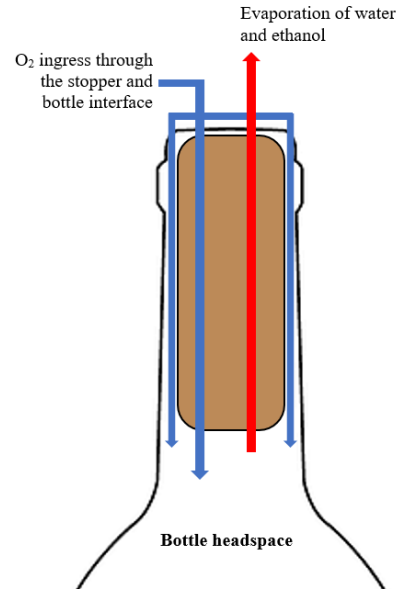


Figure 9. Oxygen passage through the stopper.

The huge amounts of different aromatic substances present in wine, together with the very low threshold levels, greatly affecting the *bouquet*, explain why the study of wine aroma is one of the most challenging topics for wine researchers and the booming trend in this research field in the last years (**Figure 10**) (Chen *et al.*, 2021).

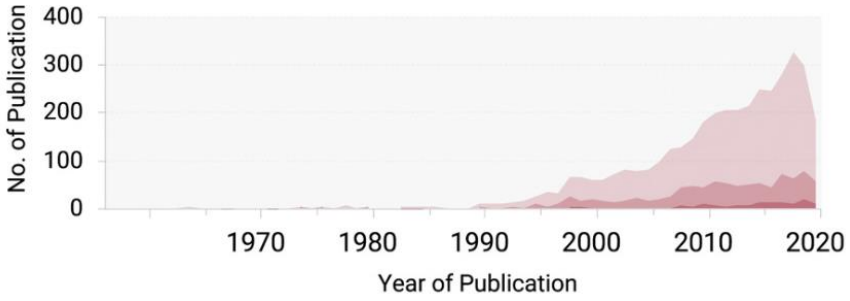


Figure 10. Publication per year retrieved from Scopus database (June, 2020).

1.1.2 Methods for the analysis of volatiles in wine

Because of the complexity of wine aroma and wine matrix, a pre-treatment of the sample is usually necessary in order to remove most of the impurities and to avoid problems which may shorten the life of analytical instruments or parts, such as chromatographic columns. Furthermore, pre-treatments are used also to isolate, in some case stabilize, and concentrate the analytes of interest that in the case of volatiles are found in quite low concentration of ng/L (Román *et al.*, 2020). Hence, the good choice of the sample preparation is essential for improving the analytical response, reliability and precision and thus helping method validations.

Different sample preparations are chosen based on the different physicochemical properties of analytes of interest, such as their solubility in organic solvents, volatility or capacity to be absorbed on particular materials. For this reason, it is very important to choose the suitable extraction method among all possible techniques. The table below lists advantages and disadvantages of the most common techniques used for the extraction of volatiles from grapes and wine (**Table 2**).

Table 2. Advantages and disadvantages of extraction techniques (Román *et al.*, 2020).

Method	Advantages	Disadvantages
LLE	Widely known Extraction of compounds with different polarity and volatility is allowed Low sample volume	Low reproducibility Low selectivity Analytes loss Difficult automatization Large amount of organic solvent High environmental pollution High cost Danger for workers
SPE	Reduce organic solvent consumption Faster than LLE Good reproducibility and repeatability Fares for workers Does not produce emulsions The analytes retained in the solid phase do not decompose Allows a wide range of analytes to be extracted simultaneously in the whole range of polarities	Low reproducibility Low selectivity Analyte loss Difficult automatization Large sample volume
SPME	Extraction and concentration in a single step No organic solvents Simplicity and speed Low handling and low sample volume Cheaper than LLE and SPE Best detection limits (DLs)	High extraction temperature Easy to break the fiber Coating stripping Expensive needles

	Can be used with solid, liquid and gaseous samples	
SBSE	Extraction and concentration in a single step No organic solvents More sensitive and robust than SPME Quick and easy	Requires a specific TDU Low number of absorbent available
TF-SPME	Extraction and concentration in a single step No organic solvents More sensitive and robust than SPME Quick and easy More absorbents available	Requires a specific TDU Few works in grapes and wine

LLE: liquid-liquid extraction; SPE: solid-phase extraction; SPME: solid-phase microextraction; SBSE: stir bar sorptive extraction; TF-SPME: thin film solid phase microextraction.

Among the different extraction techniques that can be exploited for the extraction of aromatic compounds from wine, the HS-SPME can be considered one of the most performing techniques (Román *et al.*, 2020). In fact, it is simple, it does not include the use of hazardous organic solvents and it also permits the extraction and concentration of analytes in a single step (Kataoka *et al.*, 2000). Moreover, this technique improves sensitivity since it is directly introduced into the GC injector for the thermal desorption of the analytes.

The technique was first Id by Pawliszyn and Arthur in the 90s and consists of a fused silica fiber coated with an absorbent polymeric material where analytes are transferred from the sample (**Figure 11**) (Pawliszyn & Arthur, 1990).

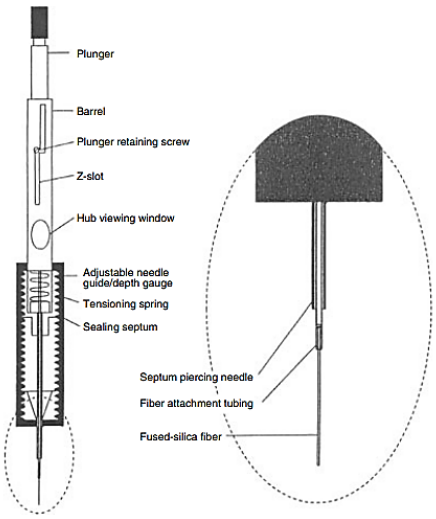


Figure 11. SPME device (Pawliszyn & Arthur, 1990).

The SPME requires two subsequent steps:

1) The fiber is firstly exposed in the flask where the sample is located. In this step the analytes are absorbed by the polymeric material of the fiber.
2) After the appropriate time the fiber is removed from the vial and introduced in the chromatograph desorption system for the identification of the analytes.

When the sample is liquid, the SPME extraction can be performed in two different ways: by exposing the fiber to the head space (HS) of the sample or by direct immersion (DI) of the fiber into the liquid sample (**Figure 12**).

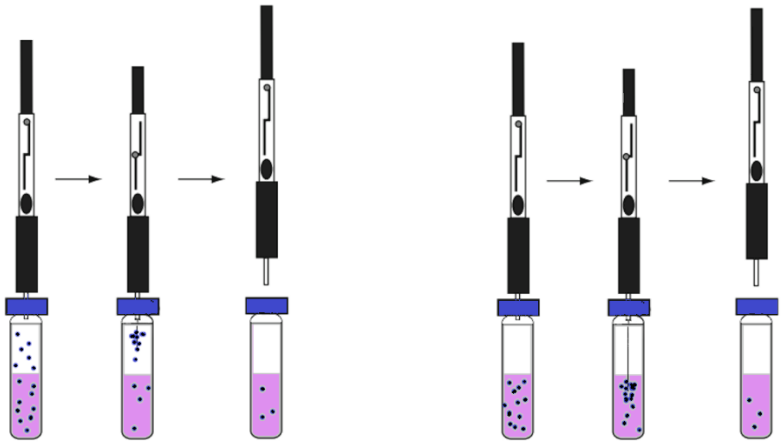


Figure 12. Two different ways to perform SPME extraction with liquid samples.

1.1.2.1 SPME in the analysis of volatiles in wine

The extraction through SPME technique has been largely used for the analysis of volatiles in wine and, mainly, with the purpose to characterize different grape varieties depending on the type and abundance of flavouring compounds. The first study regarding the wine volatile compounds with SPME was reported in 1996 and it had the aim to study the monoterpenes composition from wine of made with different grape varieties, after the optimization of extraction conditions with a polyacrylate (PA) fiber (De la Calle Garcia *et al.*, 1996). This study confirmed the simplicity by which the SPME could be applied to analyse wine volatiles and during the years a huge number of different studies have been performed on the SPME performances for the extraction of wine aromatic compounds.

Anyway, the majority of the works exploit the use of the HS-SPME extraction for the analysis of volatiles in wine. The direct immersion is in fact usually recommended only for simple matrices,

given that, otherwise, the degradation of the fiber coating can rapidly occur (Peñalver *et al.*, 2000; Möder *et al.*, 1998). Macromolecules contained in complex matrix, such as wine, could in fact irreversibly stick on the coating surface with a non-negligible decrease of the fiber performances and lifetime (Ridway *et al.*, 2007). For this reason, a new fiber was developed and introduced in the market which contains an extra thicker layer of polydimethylsiloxane (PDMS). This makes the fiber more robust and suitable to be used in DI in more complex matrices (Michel *et al*., 2015). After the introduction of this overcoated fiber (OC-fiber) several authors started to publish works reporting the performances of the fiber in the DI extraction from different matrices (Souza-Silva & Pawliszyn, 2012; Stenerson et al., 2016; Godage & Gionfriddo, 2019).

Few studies explored the combination of extraction mode, DI and HS, but performed with the more classical non-OC fibers. Bianchin *et al.* (2012) for example applied the combination mode for the analysis of pollutants in water samples (polycyclic aromatic hydrocarbons, toluene, benzene, ethyl benzene and xylene isomers), finding out that it was actually able to better extract the compounds, with very different volatilities, when compared to the DI or HS extraction only. Also, Merib *et al.* (2013) investigated the combination mode for the determination of pesticides in water samples. Even in this case the study confirmed that the DI-HS extraction mode was more performing and was a good compromise when there is the necessity to extract analytes with a wide range of volatilities.

Hence, starting from these premises, and given that the combination DI-HS has never been investigated on wine samples, a study was performed in order to assess the best extraction conditions by using the more promising OC-fiber and comparing its extraction performances in HS, DI and the combination DI-HS, considering also different extraction temperatures (25, 35 and 45 °C). To that purpose, a preliminary study was also performed to compare the commercial DVB/CAR/PDMS triphasic fiber (TP) performances in HS with the OC-fiber, since the TP has been reported in several studies as the best to perform HS extraction of volatiles from wine (e.g. Mitropoulou *et al.*, 2011; Fiorini *et al.*, 2014). This preliminary study on the method development, that will be presented in the next paragraphs, has been published (Lenti, L.; Scortichini, S.; Pacetti, D.; Cespi, M.; Fiorini, D. "Polydimethylsiloxane/divinylbenzene overcoated fiber and its application to extract and analye wine volatile compounds by solid-phase microextraction and gas chromatography coupled to mass spectrometry: direct immersion, headspace or both?" *Food Research International* **2021**, *148*, 110632. Available online at: https://doi.org/10.1016/j.foodres.2021.110632).

The optimized method was then applied for the characterization and comparison of volatile profiles of the 18 DOCG wines produced in Marche region.

1.2 Materials and method

1.2.1 Reagents and standards

All the standards used to prepare the wine model were purchased from Sigma-Aldrich (Milan, Italy). Sodium chloride and absolute ethanol were purchased by Carlo Erba (Milan, Italy). Deionized water was obtained from Milli-Q Reagent Water System (resistivity > 8 MΩcm obtained, Millipore, Bedford, MA, USA). The wine sample used for the method development was a red wine produced in 2018 in Morro d'Alba, (Ancona, Italy) and provided by the "Istituto Marchigiano di Tutela dei Vini" (IMT, Jesi, Italy).

1.2.2 Wine model

A hydro-alcoholic solution was prepared based on the macroscopic composition of a red wine sample used as reference. The model was prepared dissolving D-tartaric acid (0.531 g), D-glucose (0.260 g), D-fructose (0.260 g), absolute ethanol (14.16 mL) and glycerol (1 g) in deionized water to a final volume of 100 mL. The pH (3.0) was measured with the pH meter Jenway 3510 (Jenway, Staffordshire, UK) and the XS sensor (XS Instruments, Modena, Italy). The wine model was spiked with a mixture of volatile substances from four different chemical classes (organic acids, esters, alcohols and terpenes), preparing for each of them a stock solution. A series of preliminary tests was performed to adjust the analyte concentrations In the stock solutions and to finally obtain comparable results to the real wine sample in terms of chromatographic peak areas. In the organic acids stock solution, that was prepared in acetone, acetic acid had a concentration of 525 g/L, butyric acid of 48 g/L, hexanoic acid of 4.65 g/L, octanoic acid of 0.45 g/L and decanoic acid of 0.45 g/L. The esters stock solution was prepared in acetone with isoamyl acetate at a concentration of 43.5 mg/L, ethyl hexanoate at 43.5 mg/L, ethyl lactate at 51.5 g/L, ethyl octanoate at 43.1 mg/L, ethyl decanoate 50 at 43.1 mg/L, diethyl succinate at 523.5 mg/L, 2-phenylethyl alcohol at 516 mg/L and ethyl decanoate at 43.1 mg/L. The alcohols stock solution was prepared in ethanol with propanol at a concentration of 120 g/L, iso-butanol at 120.45 g/L, butanol at 121.5 g/L, 2-methyl-1-butanol at 122.25 g/L, hexanol at 1.22 g/L, 2-phenylethyl alcohol at 1.53 g/L and nerolidol at 131.4 mg/L. Terpenoids linalool, α-terpineol and citronellol were dissolved in ethanol at a concentration of 217.5 mg/L, 232.5 mg/L and 214.25 mg/L, respectively. To prepare the final sample to be analysed by SPME-GC coupled to mass spectrometry (SPME-GC-MS), 5 mL of wine model were spiked with 10 µL of each of the above stock solutions to obtain a sample with the concentrations reported in **Table 3**.

Table 3. Concentration of the volatile substances in the wine model.

	Compound	Concentration in wine model sample (mg/L)
organic acids	acetic acid	1050
	butyric acid	96
	hexanoic acid	9.30
	octanoic acid	0.91
	decanoic acid	0.89
esters	isoamyl acetate	0.09
	ethyl hexanoate	0.09
	ethyl lactate	103
	ethyl octanoate	0.09
	ethyl decanoate	0.09
	diethyl succinate	1.05
	2-phenylethyl acetate	1.03
	ethyl dodecanoate	0.9
alcohols	propanol	241.2
	iso-butanol	240.9
	butanol	243
	2-methyl-1-butanol	244.5
	hexanol	2.44
	2-phenylethyl alcohol	3.06
	nerolidol	0.26
terpenes	linalool	0.44
	α-terpineol	0.47
	citronellol	0.43

1.2.3 SPME extraction in headspace (HS), direct immersion (DI) or combination (DI:HS)

The extraction of the volatile compounds was performed by SPME using fibers purchased from Supelco (Bellefonte, PA, USA). Two different fibers were used: the TP fiber (DVB/CAR/PDMS, 50/30 μm, length: 1 cm) and the OC fiber (PDMS/DVB 75 μm, including 65 μm coating + 10 μm overcoating, length: 1 cm). The performance of the two fibers in HS extraction mode was initially compared. Then, the OC fiber was used to compare the different extraction mode (HS, DI, and combined DI-HS extraction) performances. Three SPME equilibration and extraction temperatures have been assessed: 25, 35 and 45 °C. The temperature of 35 °C was selected after having ascertained that 35 °C represents the best compromise, as will be discussed in the following sections. Results reported and discussed in the section refer to a equilibration and extraction temperature of 35 °C.

HS – SPME

In a 10 mL screw cap vial, 5 mL of wine and 1.7 g of NaCl were added. The sample was conditioned at 25, 35 or 45 °C for 15 min under magnetic stirring (500 rpm). The sample was analysed by GC–MS after exposing the TP or the OC fiber in HS for 30 min, according to our previous study (Fiorini *et al.*, 2014). The same procedure was applied for both the fibers, and the results compared by means of one-way analysis of variance (One-way ANOVA, $P < 0.05$). The software used was PAST (Hammer *et al.*, 2001).

DI – SPME

In a 10 mL screw cap vial, 5 mL of wine were added. The sample was conditioned at 25, 35 or 45 °C for 15 min under magnetic stirring (500 rpm) and then the OC fiber was directly soaked in the wine sample for 30 min, maintaining the magnetic stirring (and keeping the fiber at such a distance from the magnet sufficient to avoid their contact). Before the GC–MS analysis, the fiber was washed in ultrapure water for 30 sec under magnetic stirring.

DI-HS-SPME

In a 10 mL screw cap vial, 5 mL of wine were added. The sample was conditioned at 25, 35 or 45 °C, for 15 min under magnetic stirring (500 rpm). The OC fiber was directly soaked in the wine sample for 15 min, maintaining the magnetic stirring (and keeping the fiber at such a distance from the magnet sufficient to avoid their contact). Then, the fiber was washed in ultrapure water for 30 sec (under stirring) and then it was exposed again to the sample HS for 15 min, maintaining the sample stirring, before the thermal desorption in the GC–MS inlet liner.

1.2.4 GC-MS analysis

The analytes extracted from wine by SPME with the different procedures, were analysed using a 6890 N GC coupled with a 5973 N single quadrupole mass spectrometer detector (Agilent Technologies, Santa Clara, CA, USA). The desorption was performed by exposing the SPME fiber in the hot GC injector in splitless mode (4 min) at 260 °C, then leaving the fiber exposed for further 11 min for cleaning. If the SPME extraction was performed in DI or in DI-HS mode, a final washing of the fiber with a 1:1 MeOH:H_2O solution under stirring, was performed for 2.5 min in order to complete the removal of any possible carbon build-up (fouling) left on the coating surface (Souza-Silva *et al.*, 2016). The capillary chromatographic column used was a polyethylenglycol (DB-WAX,

length 60 m, i.d. 0.25 mm, film thickness 0.25 μm; Agilent Technologies, Santa Clara, USA). The oven temperature was held at 35 °C for 4 min and then raised at 2.5 °C/min until reaching 120 °C and then raised at 15 ◦C/min until 250 °C. The temperature was held for 2.6 min for a total run time of 50 min. The initial carrier gas (helium) flow rate in the column was 1.2 mL/min. The mass analysis was performed in scan mode in the range of 29–400 Da. A solvent delay of 10 min was set to avoid the overload due to ethanol content. The transfer line was maintained at 260 °C, ion source at 230 °C, quadrupole at 150 °C. The identification of the analytes was performed by means of their retention indices, calculated using straight-chain C10-C25 alkanes.

1.2.5 Design of experiments (DoE)

The effects of the experimental set-up, which included the extraction modality (HS, DI or DI:HS) and the extraction temperature (25, 35 and 45 °C) on the amount and extent of volatiles extracted, has been evaluated exploiting the design of experiments (DoE). The approach allows to optimize the number of experiments that need to be run with deeply understanding an investigated processes. This is done through a rational planning of experiments and with the application of mathematical and statistical modelling of the obtained data (Sahu *et al.*, 2018; Lewis *et al.*, 1999). For this purpose, a multilevel factorial deign (MFD) has been chosen since the investigated factors were qualitative (extraction mode) and quantitative (temperature); furthermore, they were both characterized by more than two levels. The number of experiments in the design (*N*) is equal to:

$$N = l^f = 3^2 = 9 \quad (1)$$

To provide an estimation of the error associated with each experimental condition, it has been decided to perform every condition combination in duplicate. The complete list with all the experimental run is reported in **Table 4**.

Table 4. Experimental runs necessary to be performed to evaluate the performances of OC fiber in different extraction modes and temperature.

Run	Factors	
	OC-Fiber extraction mode	Temperature (°C)
1	HS	25
2	HS	25
3	HS	35
4	HS	35
5	HS	45
6	HS	45
7	DI	25
8	DI	25
9	DI	35

10	DI	35
11	DI	45
12	DI	45
13	DI-HS	25
14	DI-HS	25
15	DI-HS	35
16	DI-HS	35
17	DI-HS	45
18	DI-HS	45

All the 18 experiment runs were performed both on the wine model and on the wine sample and all the extracted compounds were analysed as reported in the section 1.2.4 (GC-MS analysis). The concentration of each single analyte represents the responses of the design. The MFD has been obtained using Minitab 18 statistical software.

1.2.6 Statistical analysis

1.2.6.1 One-way ANOVA

One-way analysis of variance (ANOVA) was performed to assess significant differences ($P < 0.05$) between the abundances of analytes in the final volatile profile obtained from the GC-MS analysis of DOCG wine samples. The software used for this purpose was PAST (Hammer *et al.*, 2001).

1.2.6.2 Principal component analysis

The Principal Components Analysis (PCA) based on correlation matrix was performed on the GC-MS results of all MFD runs to identify homogeneous groups of data (clusters), as initial pattern recognition methods. The PCA was performed using Minitab 18 statistical. The PCA was also performed on the analytes in the final volatile profile obtained from the GC-MS analysis of DOCG wine samples, by using R-base software CAT (Leardi *et al.*, 2021).

1.2.6.3 Multilevel factorial design analysis

The identification of the relationships existing between the two parameters under investigation, (the extraction modes and the extraction temperatures), and the responses, which are proportional to the concentration of all the analytes, has been carried out by analysing the results of all the 18 runs of the MFD by the multilinear regression using a synergistic linear model (equation 2):

$$y = \beta_0 + \sum_{i=1}^{n} \beta_i \cdot x_i + \sum_{i<j}^{n} \beta_{ij} \cdot x_i x_j \quad (2)$$

Where y is the response, β_0 is the model constant, β_i is the coefficient corresponding to the variable xi (linear terms) and β_{ij} are the coefficients associated with the variable $x_i x_j$ (first-order interaction terms). All the obtained models were evaluated through ANOVA, coeffient and residual analyses and the MFD analysis has been carried out with Minitab 18 statistical software.

1.3 Results and discussion

Among the total compounds which are detected by GC-MS analysis, 40 analytes were selected and identified after excluding those detected with absolute areas below $1.5 \cdot 10^5$ and those which may arise from exogeneous contamination, as e.g. siloxanes. All compounds, with their linear retention indices, boiling points, and their signals in terms of absolute peak areas are listed in the table below, for all extraction fiber conditions comparisons (**Table 5**). Absolute areas do not allow a reliable comparison of the concentration of the different analytes within a sample, since each of them has its own response factor, but they provide a reliable tool to compare the concentration of a same analyte among different samples. Thus, for the purpose to compare the extraction efficiency of different fibers, fiber positions and temperatures, the absolute areas can be considered a very useful and reliable tool. Indeed, even for the further comparison of volatiles in the DOCG wine samples, the absolute areas were used to discuss the relative abundances of the analytes in the 18 different samples.

Table 5. Analytes detected in the real red wine sample, their boiling points (°C) at 760 mmHg (from NIST library), their experimental (LRI(exp)) and from literature (LRI(lit), NIST library) linear retention indices. Average peak areas and % relative standard deviation (RSD).

					HS-TP		HS		DI		DI-HS	
	Compound	**bp (°C)**	**LRI (exp)**	**LRI (lit)[a]**	**area[a]**	**RSD %**	**area[b]**	**RSD %**	**area[c]**	**RSD %**	**area[d]**	**RSD %**
1	ethyl butyrate	121	1028	1030	3.33E+06	21.5	3.13E+06	19.1	1.53E+06	67.3	1.85E+06	5.8
2	propanol	97	1037	1032	8.26E+05	19.2	1.17E+06	22,1	nd		7.02E+05	32.3
3	ethyl 2-methyl butyrate	133	1044	1042	9,26E+05	19.2	8.50E+05	7.4	5.36E+05	75.2	4.97E+05	16.3
4	isobutanol	108	1092	1087	5.68E+06	15	9.00E+06 *	29.4	1.35E+06	77.7	4.51E+06	11
5	isoamyl acetate	141	1113	1112	2.07E+07	5.5	1.84E+07 *	9.9	9.20E+06	5.1	1.13E+07 *	14.3
6	isoamyl alcohol	131	1213	1207	2.14E+08 *	7.6	2.53E+08 *	8.7	8.56E+07	5.8	1.20E+08 *	2.9
7	ethyl hexanoate	167	1230	1233	7.57E+07	15.3	6.33E+07 *	11	3.54E+07	4.5	4.21E+07 *	11
8	ethyl heptanoate	189	1329	1310	1.14E+06	16.9	1.18E+06 *	13.1	3.19E+05 *	10.9	6.38E+05 *	7.5
9	ethyl lactate	154	1338	1341	1.08E+07 *	3.5	1.38E+07 *	9.1	6.20E+06	5.1	8.41E+06 *	1.3
10	*trans*-rose oxide	197	1347	1350	7.79E+05	18.2	6.42E+05 *	11.3	1.74E+05	6.3	2.29E+05 *	7.9
11	hexanol	157	1355	1355	1.99E+06 *	4.8	2.21E+06 *	2.6	6.31E+05 *	18.2	9,62E+05 *	9.3
12	methyl octanoate	193	1384	1373	1.05E+06	34,8	8.97E+05 *	17	5.47E+05	2.8	7.39E+05	5.9
13	ethyl octanoate	206	1436	1429	2.10E+08	31.8	2.10E+08 *	14	1.59E+08	3.9	2.17E+08 *	1.8
14	acetic acid	118	1444	1442	1.52E+07	23.5	1.40E+07 *	7.9	8.19E+06 *	3.9	1.15E+07 *	4.1
15	furfural	162	1450	1457	1.64E+06 *	12.9	5.85E+05	16.3	4.91E+05	31.9	3.72E+05	7.8
16	isoamyl hexanoate	226	1457	1456	1.28E+06	21.4	1.30E+06 *	10.8	4.67E+05 *	27.4	1.01E+06	2.6
17	formic acid	101	1493	1492	5.08E+05 *	14.4	7.14E+05	5.1	6.01E+05 *	22.1	1.29E+06	40.4
18	ethyl nonanoate	227	1539	1535	1.22E+06 *	23,8	2.95E+05	20.1	2.91E+05	18.2	4.32E+05	9.9
19	linalool	199	1555	1549	1.53E+07	11.8	1.52E+07 *	5.6	7.25E+06	2.1	7.42E+06 *	7.9
20	octanol	195	1570	1573	1.31E+06	15.9	1.30E+06 *	16	8.77E+05	7.7	9.70E+05	0.1
21	isoamyl lactate	202	1575	1615	1.28E+06	1.8	1.39E+06 *	9.4	7.79E+05	22.6	1.12E+06	9.4
22	butyrolactone	204	1620	1610	4.33E+05 *	6.6	5.98E+05	12.2	7.01E+05	41.7	5.68E+05	10.1
23	ethyl decanoate	242	1643	1648	2.75E+07	34.2	3.39E+07	10.9	3.34E+07 *	5.4	4.88E+07 *	0.5
24	isoamyl octanoate	267	1663	1657	8.55E+05	35.2	1.02E+06	12.5	1.16E+06	8	1.38E+06 *	0.4
25	2-butyl-2-octenal	262	1669	1653	1.77E+06 *	7.5	2.08E+06	6.6	2.07E+06	14.5	1.94E+06	3.1
26	diethyl succinate	218	1678	1682	7.30E+07	5.1	7.12E+07 *	6.3	5.77E+07	3.1	6.59E+07	3
27	α-terpineol	217	1699	1705	1.29E+07 *	3.8	1.14E+07 *	8.5	8.58E+06	5.5	8.84E+06 *	0.7
28	1,2-dihydro-1,1,6-trimethylnaphthalene	242	1741	1751	6.56E+05	28.5	7.34E+05 *	17.9	1.28E+06	25.6	1.29E+06	16
29	citronellol	225	1772	1774	6.64E+06	20.5	7.54E+06 *	5.3	1.08E+07	5.9	9.79E+06 *	5.1
30	ethyl phenylacetate	228	1782	1781	5.65E+05	10.7	5.36E+05	20.2	5.65E+05	10.5	6.45E+05	0.7
31	2-phenylethyl acetate	233	1813	1813	3.43E+06	13.3	3.66E+06 *	13.4	5.17E+06	5.7	5.14E+06 *	6.1
32	hexanoic acid	204	1841	1842	5.32E+06	5.8	5.29E+06	13.4	4.15E+06	6.9	3.99E+06	2.5
33	benzyl alcohol	205	1884	1874	4.48E+05	2	3.71E+05	39.3	5.90E+05	30.9	4.39E+05	35.2
34	2-phenylethyl alcohol	220	1918	1910	1.00E+08	5.2	9.91E+07	8.5	9.37E+07	0.9	9.70E+07	1.4
35	nerolidol II	276	2069	2014	7.68E+05 *	12.1	1.17E+06	9.5	3.15E+06	48.4	1.30E+06	30.3

36	octanoic acid	237	2096	2083	1.32E+07 *	11.3	1.69E+07 *	8.6	4.42E+07 *	4.9	3.60E+07 *	1
37	decanoic acid	270	2298	2283	3.31E+06	17.2	4.43E+06 *	16.2	5.88E+07 *	3,2	3.60E+07 *	16.6
38	geranic acid	295	2360	2366	5.28E+05	22	1.48E+06 *	48.8	1.93E+07 *	1.7	1.31E+07 *	3
39	ethyl hydrogen succinate	248	2397	2395	4.52E+06	29.1	7.80E+06 *	31.1	1.88E+07	7.3	1.33E+07	22.6
40	dodecanoic acid	298	2478	2470	2.84E+05	12.4	5.28E+05 *	31.5	1.00E+07 *	16.7	3.94E+06 *	22.5

For HS-TP *n=3*, with OC fiber: for HS *n* =4, for DI *n* =2 and for DI-HS *n*=2. [a] The asterisk (*) in this column indicates significant differences between HS-TP. [b] The asterisk in this column indicate significant differences between HS and DI; [c] The asterisk (*) in this column indicate significant differences between DI and DI-HS, [d] The asterisk (*) in this column indicate significant differences between DI-HS and HS. Statistical significance at $P<0.05$, *t*-test. Nd: area $<1.5*10^5$.

1.3.1 Preliminary assessment of OC fiber performances

At first, the preliminary assessment of the OC was performed in order to compare both, its performances when used in HS with respect to the classical TP and its performances when used only in HS or DI.

1.3.1.1 HS-OC versus HS-TP

Volatile extraction from wine was performed in HS comparing the OC fiber to the TP fiber. Statistical analysis showed that among the total 40 compounds, 11 were extracted differently in a statistically significant way. In particular 8 compounds were better extracted by the OC fiber, while only 3 by the classical TP fiber. For the remaining 29 analytes no statistically significant differences were observed. This demonstrated that the OC fiber showed higher extracting performances with respect to the TP, and, furthermore, when taking a loot to the type of analytes extracted better by one fiber or another, interesting information about their possible specific behaviour can be highlighted. In order to do that, the analytes were divided in three groups, based on their boiling points (bp). The range of temperatures covered by all analytes was divided by into three equal parts and the analytes into the corresponding group. In this way 11 analytes belong to the first group of the most volatile ones (bp in the range 97-164 °C), 18 analytes in the second group with intermediate volatility (bp 165-232 °C) and finally, 11 analytes in the group of the less volatiles (bp 233-298 °C) as shown in **Table 6**.

Table 6. Number of analytes that are better extracted in a significant difference by paired comparison.

	TP-HS *vs* HS		HS *vs* DI		DI *vs* DI-HS		HS *vs* DI-HS	
Group	N° of analytes significantly better extracted by		N° of analytes significantly better extracted by		N° of analytes significantly better extracted by		N° of analytes significantly better extracted by	
	TP-HS	HS	HS	DI	DI	DI-HS	HS	DI-HS
1st – 11, bp 97-164°C	1	4	6	0	0	3	5	0
2nd -18, bp 165-232°C	2	1	11	1	0	2	5	2
3rd – 11, bp 233-298°C	0	3	0	7	4	1	0	7

Total – 40, bp 97-298°C	3	8	17	8	4	6	10	9

One-way analysis of variance was performed and differences were considered significant at $P < 0.05$.

Considering the distribution which results from this subdivision, a certain difference in the selectivity of the two fibers can be highlighted. In fact, while the TP fiber gives slightly lower performance especially in the extraction of less volatile compounds, the OC fiber seems to present a more homogenous behaviour all along the three groups. This finding can be explained by the different absorbent phase which characterise the two fibers. The presence in the TP fiber of a CAR inner layer, which is strongly polar, may interacts mostly with small and thus generally more volatile analytes that can migrate relatively fast through the DVB layer and be trapped in the CAR. By contrary, the larger molecules that are not able to migrate through the DVB layer, will remain on this one (Shirey, 2009). Furthermore, when it saturates, a displacement effect may occur where the most polar and volatile compounds, which have a higher affinity for the polar coating, displace the least volatile compounds. This effect was observed by Gionfriddo *et al.* (2015) when comparing TP and OC fibers used in HS, for the extraction of analytes having difference polarity and molecular weights, in starch matrix model, able to mimic plant-based matrices. They observed a minor displacement effect with the PDMS modified coating, that allowed a more representative extraction of the analytes. Hence, considering the excellent performance shown by the OC fiber also in the HS extraction mode, being slightly better and more homogenous than that given by the TP fiber, the further investigation was performed with the OC fiber, comparing its behaviour in HS with that in DI and inquiring also it the combined DI-HS mode could provide some improvements.

1.3.1.2 HS-OC versus DI-OC

Comparing the extraction performed in HS or DI by the OC fiber, among the 40 compounds, 17 were significantly better extracted in HS, while other 8 in DI mode, thus clearing pointing out that the HS gave the best results among the two modes. Anyway, by looking at the distribution of the analytes better extracted by one mode or another, even in this case a peculiar and specific feature can be highlighted. In fact, the analytes of the first and second groups, such as isoamyl alcohol and ethyl octanoate respectively, are better extracted in HS more, while analytes belong in the thirst group of the least volatile ones, such as decanoic acid, are better extracted in the DI mode, that theoretically should be the method of choice when the purpose are compounds having a bp above 230 °C. This result agrees with other studies. As an example, Demyttenaere *et al.* (2003) performed an investigation on whisky samples, containing both volatile and non-volatile flavouring compounds,

comparing the DI and HS use of three different non-OC fibers: PDMS, DVB/CAS/PDMS and polyacrylate (PA). PDMS and DVB/CAR/PDMS were the fibers able to give better results when used in DI for the extraction of least volatile compounds. On the other hand, the use in HS favoured the extraction of more volatile compounds. Kafkas *et al.* (2006) obtained similar results when using a 100 µm PDMS fiber and comparing its use in DI and HS for the extraction of volatiles from strawberry wine, highlighting that esters were preferentially enriched by HS, while fatty acids and, in particular, octanoic and decanoic acids, by DI. The OC-fiber was subject of investigation by Gionfriddo *et al.* (2015) which reported the comparison between the two extraction modes, while investigation the behaviour of analytes in complex mixture matrices. The best results in terms of compounds distribution were obtained by using the fiber in DI extraction mode, since the use of HS may lead to a saturation of the fiber by the most volatile compounds, arising in a lack for the less volatile ones. Hence, the DI mode was able to minimize the occurrence of artifacts due to fiber saturation, providing a more balanced extraction of compounds with different range of volatility. Considering the obtained results and on the findings reported from literature, it was hypnotised that a combined us of DI followed by HS in a same experiment, could improve the extraction of the most volatile substances compared to the use of the DI extraction mode only. Hence, the combine mode was evaluated for the first time in wine and with the use of the OC fiber.

1.3.1.3 DI-HS versus DI and DI-HS versus HS

Taking into consideration the obtained results obtained by the HS or DI extraction mode only (30 min), the combination DI-HS (15 min DI extraction followed by 15 min HS) was then evaluated. As expected, the combination mode was able to give slightly better overall extraction performance than that of DI, with a significant difference obtained only for 10 analytes, 6 of which were better extracted by the DI-HS mode, while 4 by DI (**Table 7**). Moreover, the compounds that were better extracted by the combined mode, showed also a better distribution among the three different bp groups, as compared to the analytes better extracted by DI group which belong all in the 3rd group of the less volatile compounds. Considering the HS vs DI-HS comparison, the number of compounds better extracted by one extraction mode over the other is similar, being 10 for HS and 9 for DI-HS, even though without a balanced distribution among the three groups. In fact, while the DI-HS favour the extraction of the least volatile compounds, the HS by contrary, showed a preference for highest and average volatile compounds.

In order to investigate the results more in detail, a different score was assigned to the three techniques (HS, DI and DI-HS) based on their relative extraction extend, measured in terms of peak

area percentage. Hence, for each analyte, a score was calculated for each of the three extraction modes, as the analyte peak area obtained with the specific extraction mode divided by the sum of the three peak area values obtained for the same analyte with the three different extraction modes investigated, then multiplying the result for a factor of 100. Then, the obtained scores by each technique and for all analytes was summed and divided for the number of compounds in the group, thus obtaining an average score for each of the three extraction modes and for each of the three groups of analytes, measuring the extraction performances of each extraction mode within each group of analytes (**Figure 13**).

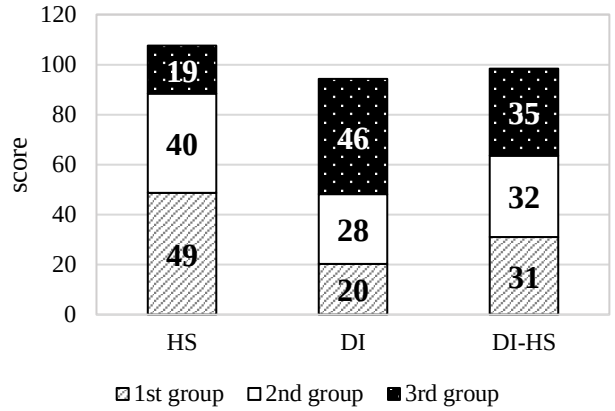


Figure 13. Extraction efficiency of HS, DI and DI-HS among the three groups (Lenti *et al*., 2021).

The total scores are 107, 94 and 98 for HS, DI and DI-HS, respectively and this suggested that none of them markedly overperformed over the others. Considering the scores for the different groups of analytes, DI and HS showed practically a complementary behaviour. In fact, HS has scores of 49, 40 and 19 for the 1st, 2nd and 3rd group, while DI has 20, 28 and 46. This suggest HS to be the extraction mode to be preferred for the extraction of volatile with boiling points up to 160°C, while DI for the ones with boiling points higher than 230 °C. For analytes with intermediate boiling points there is not an important difference between the different extraction modes. The DI-HS combination efficiency showed, instead, a much more homogeneous trend within the three classes of analytes, having scores of 31, 32 and 35 for the 1st, 2nd and 3rd group. This suggests again that this could be a good compromise when a broad range of volatility in the analytes is expected or cannot be excluded. These finding were in agreements with the stud reported by Bianchin *et al.* (2012) and Merib *et at.* (2013) where the combined mode was used for the extraction of pollutants from water samples by the non-OC PDMS/DVB and the DVB/CAR/PDMS fibers respectively, showing that a combination of the extraction condition favoured the extraction of compounds with different volatilities in a single procedure.

In order to clarify the different behaviours, specificity and extraction extent for the three different extraction modes, a statistical approach was used. Furthermore, a wine model was prepared (**Table 4**) containing selected compounds from the major classes of compound normally found in wine (alcohol, esters, acids and terpenes). The experiments were then performed on both wine model and real wine evaluating the three extraction modes (HS, DI and DI-HS and three temperatures (25, 35 and 45 °C). An example of a chromatogram obtained from the GC-MS analysis is reported below (**Figure 14**).

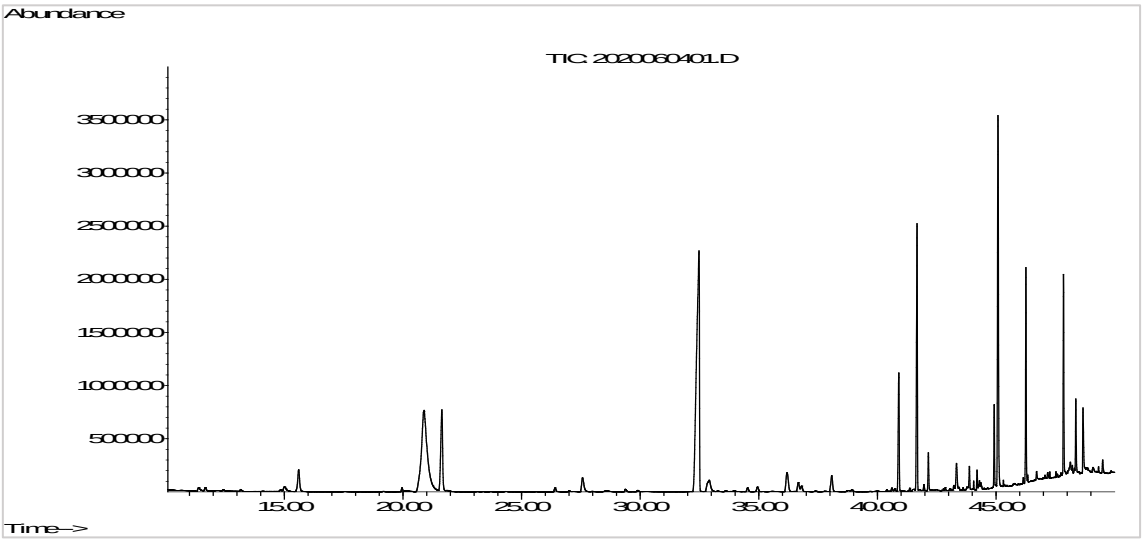


Figure 14. Example of a chromatogram obtained by the GC-MS analysis of wine sample in DI-HS at 35 °C.

1.3.2 Statistical analysis to assess the effect of SPME extraction mode and temperature

1.3.2.1 PCA

The principal component analysis model was applied as initial pattern recognition method in order to identify possible relationship between the extraction modes and the chemical profile obtained from wine sample or wine model (**Figure 15**). For both systems the two principal components explain mode than the 60% of the total variance, in particular the 78% for wine model and the 65 % for wine sample.

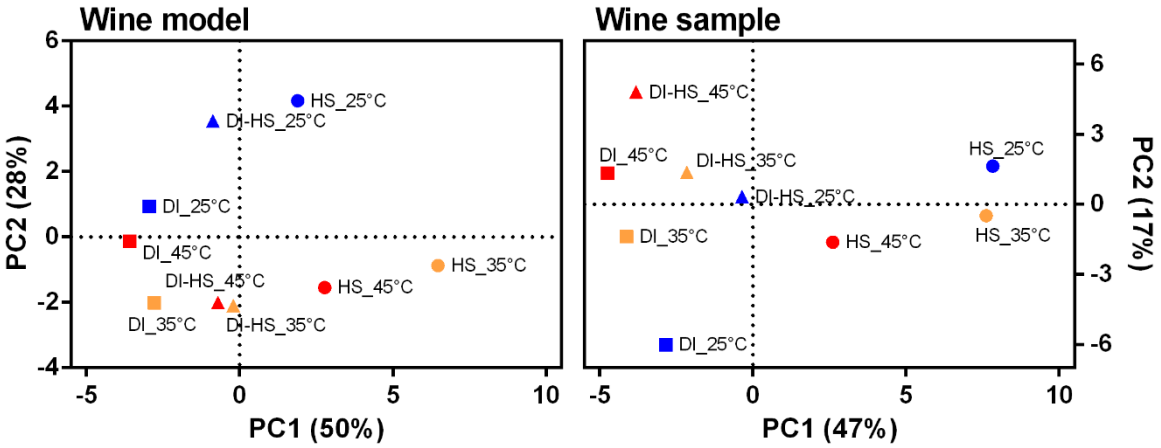


Figure 15. PCA for wine model and wine sample (Lenti *et al.*, 2021).

For the wine model the data appeared to be clustered in function of both the extraction mode and temperature. Specifically, PC1 seems to be more sensitive to the extraction mode, while PC2 to the temperature. Thus, for the wine model the extraction extent of the different identified analytes is strongly dependent on the experimental set up. By contrary, for wine sample the interpretation is more complex. In fact, while PC1 was still able to discriminate the effect of the extraction mode, the temperature did not show any influence. In order to investigate the results obtained by the preliminary PCA, a Pearson correlation analysis between the loadings of the variables of the first two components (Eigenvectors coefficient) and the boiling point was performed (**Figure 16**).

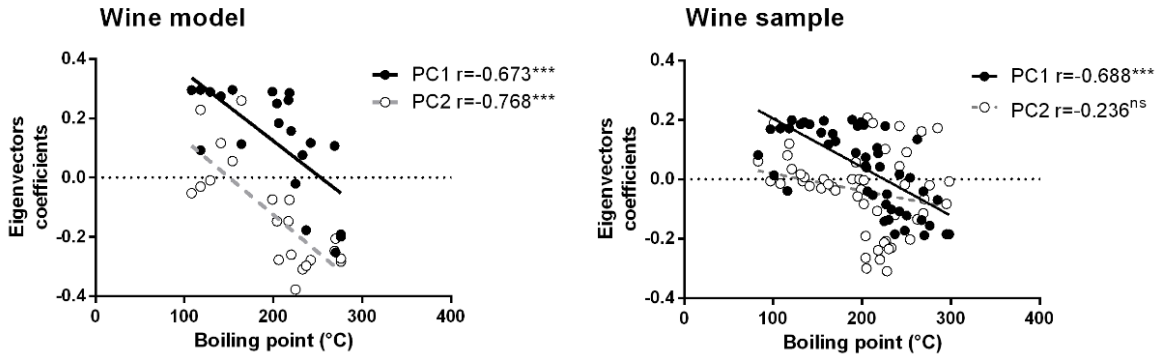


Figure 16. Pearson correlation (Lenti *et al.*, 2021).

The result showed that the PC1 was negatively correlated with the boiling point for both samples ì, and consequently, the compounds found with a high PC1 values generally possess lower boiling point. This agrees with the results previously shown and confirms that HS favours the

extraction of low boiling point compounds (more volatile) and DI the extraction of high volatile compounds (less volatile).

1.3.2.2 Multilevel factorial design

While the PCA provided a more general overview of the effect of the different extraction modes and temperature on the extent of analytes extraction, the regression analysis carried out for the 18 total runs provided a detailed picture of the effect of experimental conditions on the extraction of each single analytes. The regression results for both wine model and sample are reported in **Table 7** and **8**.

Table 7. Summery regression the results on the wine model.

Compounds	R^2	R^2_{adj}	R^2_{pred}	Variability (%)				*P*-value Regression
				Temperature	Extraction mode	Interaction	Error	
butanol	0.962	0.928	0.847	8.8**	78***	9.3*	3.8	***
2-methyl-1-butanol	0.963	0.931	0.853	5.7*	78.5***	12**	3.7	***
2-phenylethyl acetate	0.853	0.772	0.411	38.2*	8.1^{ns}	39.0*	14.7	**
2-phenylethyl alcohol	0.778	0.58	0.111	60.0**	3.0^{ns}	14.7^{ns}	22.2	*
acetic acid	0.805	0.631	0.218	16.2^{ns}	24.0*	40.2*	19.5	*
α-terpineol	0.965	0.934	0.86	36.3***	36.2***	24.0***	3.5	***
butyric acid	0.789	0.601	0.154	21.8*	19.2^{ns}	37.8*	21.1	*
citronellol	0.853	0.723	0.413	40.6**	10.8^{ns}	34.0*	14.7	**
decanoic acid	0.979	0.961	0.916	12.2***	81.5***	4.2*	2.1	***
diethyl succinate	0.924	0.856	0.694	26.2**	48.7***	17.4*	7.6	***
ethyl decanoate	0.979	0.961	0.917	54.2***	17.5***	25.6***	2.1	***
ethyl dodecanoate	0.987	0.975	0.946	58.0***	21.7***	19.0***	1.3	***
ethyl lactate	0.852	0.72	0.407	2.5^{ns}	70.7***	12^{ns}	14.8	**
ethyl octanoate	0.929	0.866	0.716	42.9***	33.9***	16.1***	7.1	***
hexanoic acid	0.855	0.726	0.42	34.9**	30.2**	20.4^{ns}	14.5	**
isobutanol	0.983	0.967	0.93	13.0***	73.0***	12.3***	1.7	***
isoamyl acetate	0.941	0.888	0.763	6.4*	80.2***	7.5^{ns}	5.9	***
linalool	0.977	0.957	0.909	11.9***	80.5***	5.3*	2.3	***
nerolidol I	0.939	0.884	0.755	42.9***	47.7***	3.3^{ns}	6.1	***
nerolidol II	0.944	0.894	0.776	39.4***	51.5***	3.4^{ns}	5.6	***
octanoic acid	0.925	0.858	0.7	25.3**	58.5***	8.7^{ns}	7.5	***

The statistical significance of columns temperature, extraction mode, interaction and P-value Regression are reported as follows: ns = p>0.05; * 0.05<p<0.01; ** 0.01<p<0.001; *** p<0.001.

Table 8. Summery regression the results on the wine sample.

Compounds	R^2	R^2_{adj}	R^2_{pred}	Variability (%)[A]				*P*-value Regression
				Temperature	Extraction mode	Interaction	Error	
ethyl butyrate	0.988	0.977	0.952	5**	85.7***	8.2***	1.2	***
propanol	0.948	0.902	0.792	2.5 ns	88.9***	3.4 ns	5.2	***
ethyl 2-methylbutyrate	0.94	0.867	0.76	23.9**	55.1***	14.9*	6	***
isoamyl ethyl ether	0.982	0.967	0.929	23.8***	62***	12.5***	1.8	***
isobutanol	0.974	0.95	0.895	1.6 ns	89.5***	6.2*	2.6	***
isoamyl acetate	0.97	0.943	0.979	36.9***	50***	10**	3	***
isoamyl alcohol	0.989	0.998	0.957	0.3 ns	98.5***	0.2 ns	1.1	***
ethyl hexanoate	0.946	0.898	0.784	55.9***	21.5**	17.2**	5.4	***
ethyl heptanoate	0.949	0.903	0.795	5.8*	85.1***	4 ns	5.1	***
ethyl lactate	0.943	0.893	0.773	2.4 ns	81.8***	10*	5.7	***
trans-rose oxide	0.949	0.904	0.797	16.2**	64.4***	14.3**	5.1	***
hexanol	0.988	0.977	0.952	1.6*	96.2***	0.9 ns	1.2	***
methyl octanoate	0.817	0.655	0.269	35**	8.7 ns	38.1*	18.2	*
ethyl octanoate	0.934	0.875	0.734	25**	40.2***	28.1**	6.6	***
acetic acid	0.936	0.879	0.743	3.1ns	70.2***	20.2**	6.4	***
furfural	0.626	0.294	<0.001					ns
isoamyl hexanoate	0.947	0.901	0.79	6.7*	72***	16**	5.3	***
formic acid	0.802	0.626	0.207	12.3 ns	27.2*	40.2*	19.8	*
ethyl nonanoate	0.46	<0.001	<0.001					ns
ethyl 2-hydroxycaproate	0.356	<0.001	<0.001					ns
linalool	0.98	0.962	0.919	7**	83.7***	7.3**	2	***
octanol	0.961	0.926	0.843	44.4***	43.7***	8*	3.9	***
isoamyl lactate	0.95	0.906	0.8	34.2***	55***	5.8 ns	5	***
butyrolactone	0.687	0.409	<0.001					ns
ethyl decanoate	0.881	0.776	0.525	26.1**	43.3**	18.7 ns	11.9	**
isoamyl octanoate	0.827	0.673	0.308	32.6**	36.1*	14 ns	17.3	*
2-butyl-2-octenal	0.975	0.953	0.901	0.3 ns	89.5***	7.7*	2.5	***
diethyl succinate	0.947	0.9	0.787	14.7**	15**	65***	5.3	***
ethyl 9-decenoate	0.872	0.758	0.487	52.1**	16.6*	18.4 ns	12.8	**
α-terpineol	0.932	0.872	0.73	15.9**	47.2***	30.2**	6.8	***
1,2-dihydro-1,1,6-trimethyl-naphthalene	0.656	0.35	<0.001					ns
citronellol	0.962	0.927	0.846	1.5 ns	58.8***	35.8***	3.8	***
ethyl phenylacetate	0.925	0.859	0.7	11.2*	30.8**	50.6***	7.5	***
2-phenylethyl acetate	0.981	0.963	0.922	12.7***	34.1***	51.2***	1.9	***
β-damascenone	0.25	<0.001	<0.001					ns
hexanoic acid	0.936	0.878	0.742	8.5*	39.4***	45.7***	6.5	***
benzyl alcohol	0.897	0.805	0.586	6.9 ns	57.1***	25.7*	10.3	**
2-phenylethyl alcohol	0.941	0.889	0.764	9.1*	24.2**	60.9***	5.9	***

nerolidol II	0.949	0.904	0.797	30.2***	56.3***	8.4*	5.1	***
octanoic acid	0.993	0.987	0.972	0.7*	83.1***	15.5***	0.7	***
decanoic acid	0.982	0.966	0.929	0.1 [ns]	97.2***	0.9 [ns]	1.8	***
geranic acid	0.995	0.99	0.979	0.7*	96.9***	1.9**	0.5	***
ethyl hydrogen succinate	0.896	0.803	0.583	37.9**	40.2**	11.5 [ns]	10.4	**
dodecanoic acid	0.965	0.934	0.86	4.2*	89.7***	2.6 [ns]	3.5	***

The statistical significance of columns temperature, extraction mode, interaction and *P*-value Regression are reported as follows: ns = *P*>0.05; * 0.05< *P* <0.01; ** 0.01< *P* <0.001; *** *P* <0.001. [A] when the regression was not statistically significant the variability values were not reported (NR).

Interestingly, all the 21 compounds of the wine model were described in an almost satisfactory manner by the synergic model (lowest R^2 adj was around 0.58) and the regression was always significant. Concerning wine sample, 4 compounds out of the 40 total analytes could not be efficiently described (regression not significant, Table 9) because the model was not adequate, or the studied parameters did not affect the extraction efficiency. In order to allow a more immediate visualization of the effect due to experimental parameters on each single analytes, the results of the ANOVA analysis of variance are reported both in terms of significance and variability. The significance indicated that a certain factor affects the response not by chance, the variability represents the extent of the factor influence on the response and it has been expressed in terms of the eta quare (η^2, proportion of the total variation of the dependent variable associated with each individual effect or source of variation) (Richardson, 2011).

$$Variability = \eta^2 = \frac{adjSS_F}{adjSS_R} \cdot 100 \quad (3)$$

$adjSS_F$: adjusted regression sum of squares for a certain factor F (temperature, extraction mode or interaction): $adjSS_r$: sum of regression sum of squares (the response variability explained by the model) and the sum of squares and sum of squares of the residual error (response variability that cannot be estimated by the model). All the compounds extracted from the wine model and the wine sample were grouped according to the variability values. Specifically, analytes with η^2 for a certain factor higher than 40 % were identified as strongly affected (large effect), those with values between 10 and 40% as affected (medium effect) and those with values lower than 10% as mildly affected (small effect). It is worth noting that no benchmarks have been suggested in the literature for η^2 values, which should not be confused with the measures called partial η^2 (for which instead, benchmark values are proposed).

The Figure below shows the results of the frequency distribution of the number of analytes in wine model and wine sample according to the analytes sensitiveness to the experimental factors (**Figure 17**).

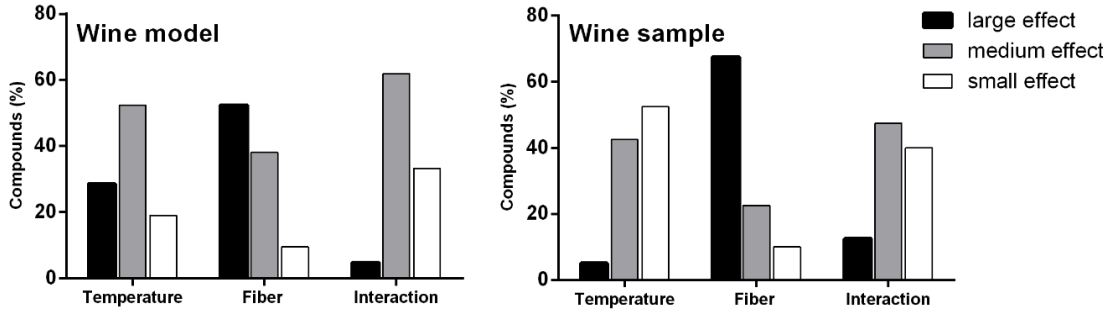
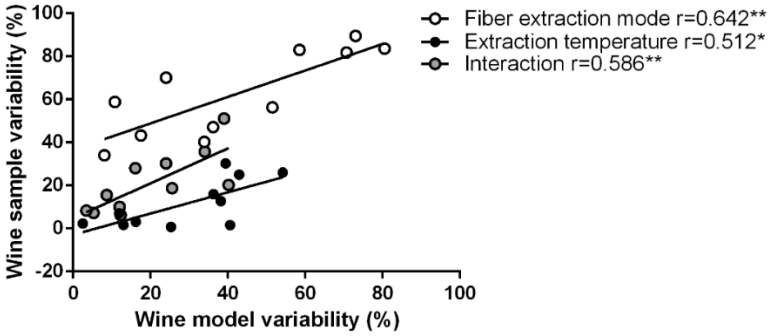


Figure 17. Frequency distribution of analytes (Lenti *et al.*, 2021).

Large effect is referred to the analytes having a variability $\eta^2 > 4\%$, medium for variability $10 < \eta^2 < 40\%$ and small for variability value $\eta^2 < 10\%$. The results clearly indicate that the extraction mode represents the most relevant factor being able to explain most of the variability of more than half of the extracted compounds, both in wine model and sample. The other two factors, being temperature and interaction, play a similar role only for a limited number of compounds.

Compared to the extraction mode and the interaction, the temperature seems to produce a different effect in the wine model and in the wine samples. However, this could be due to the different numbers of analytes, being 21 and 40, respectively. For this reason, in order to better compare the results obtained in both samples, the Pearson correlation analysis was performed between the variability % η^2 values for the analytes extracted both in the wine model and sample, hence, considering only the compounds extracted from both matrices. The results obtained are shown in **Figure 18**.



In the legend are reported the Pearson correlation coefficient I and the statistically significant differences (* $0.05 < P < 0.01$; ** $0.01 < P < 0.001$; *** $P < 0.001$).

Figure 18. Pearson correlation analysis (Lenti *et al.*, 2021).

A positive correlation, statistically significant for all the factor, suggesting that the amount of explained variability follow the same trends for most of the compounds in both matrices, was obtained. However, the absolute values of the correlation coefficients (from 0.51 to 0.64) also suggested that the results are not completely overlapping, probably due to the intrinsic variability of the measurements and/or to a certain matrix effect. The analysis of the variability provided a general overview of the effect off the investigated factor affecting the extraction performances for wine model and wine sample. A more detailed assessment was performed by taking into consideration the single analyte.

Due to the high number of compounds, it was decided to present the result only for those compounds strongly affected by the studied factor that is all the analytes having a % variability defined as "large effect", meaning the ones with $\eta^2 > 40\%$. For the other cases, with a medium or small effect, even if the extraction mode, temperature or interaction possess a certain relevance (in most of the cases statistically significant), the absolute variation of the responses was relatively low and consequently not very relevant. As an example, the choice of the most performing extraction mode for the compounds having a "large effect" variability in the wine sample assures an average increase of the extracted amount equal to 3.54 times. On the other hand, the same choice for the compounds with a "medium effect" variability, in the same matrix, determines an average increase of 1.27 times only. The factor effect results for each compound are reported in the main effect plots (**Figure 19**). This was done by using the relative values in order to compare all analyte in a single plot.

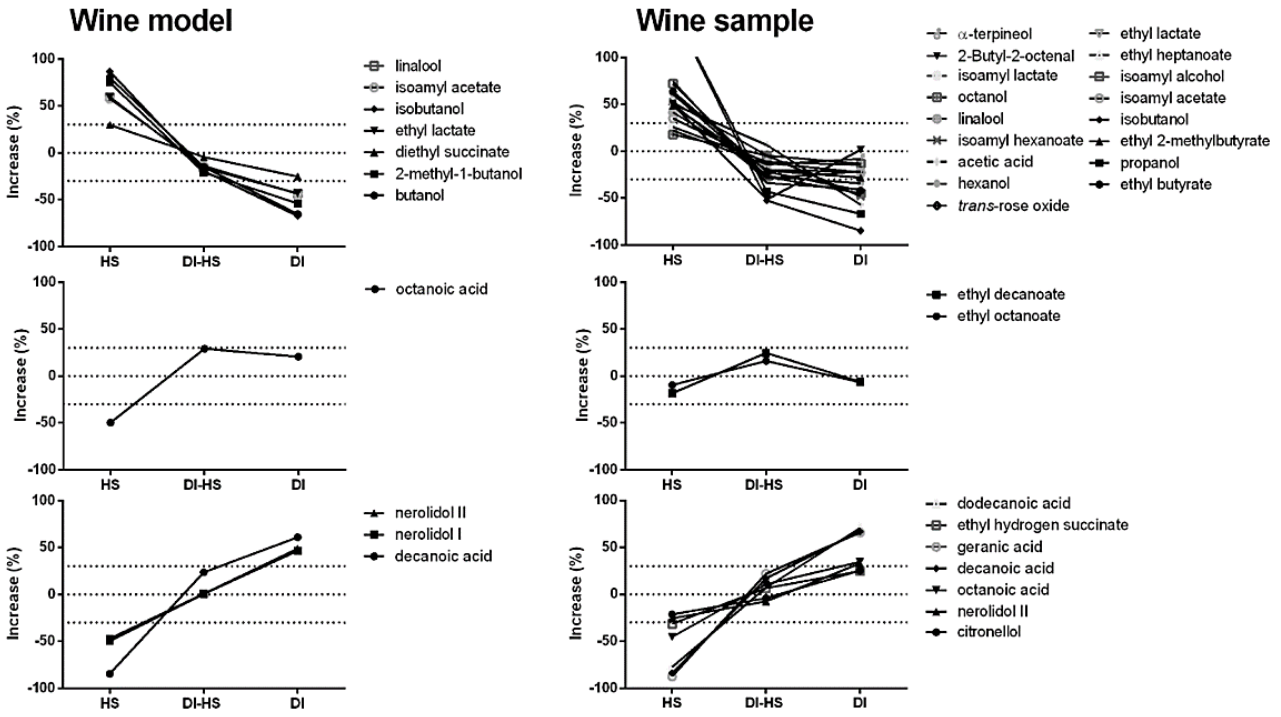


Figure 19. Main effect plot reporting the effect of the extraction mode for all the analytes extracted from the wine model and sample, for the "large effect" extraction mode. All data were grouped according to their favourite extraction mode. HS preferring compounds in the upper panels, while de DI preferring compounds in the lower ones (Lenti *et al.*, 2021).

With this approach, the average gran mean for all extraction runs is zero and the effect of each single level for a certain factor is reported as the percentage increase/decrease, with respect to the mean value. By looking at the results, it is clear that most of the compounds are in the upper panels, the ones where the compounds showed a preference for HS extraction. In fact, the 64% of the analytes were more sensitive to this extraction mode, while the 27% preferred the DI mode. Only the 1-2 % showed a preference for the combination DI-HS.

The same plot was also construct for the temperatures, for the compounds that were significantly affected by this parameter (**Figure 20**).

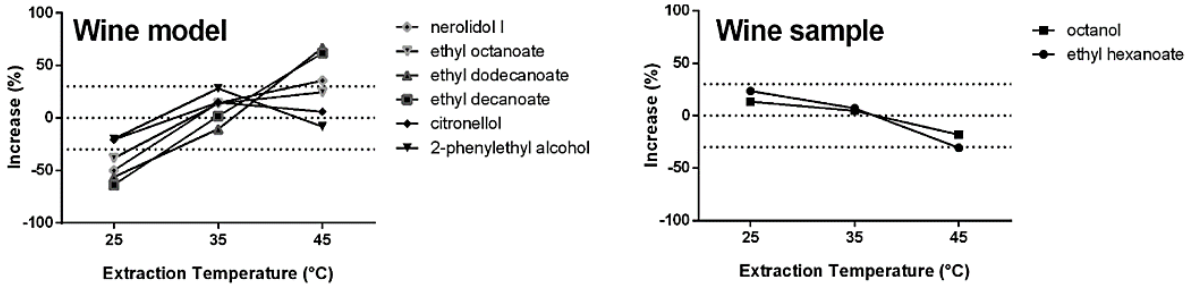


Figure 20. Main effect plot reporting the effect of the extraction temperature for the analytes which were significantly affected ("large effect") (Lenti *et al.*, 2021).

In general, the temperature had a lower influence on the responses with compared to the extraction modality, in fact, the results obtained from the statistical analysis showed that each analyte, or in some cases a class of analytes, has a specific preference in the extraction conditions that are able to maximize their extraction extent. Hence, an overall ideal condition for all analytes cannot be designed, as it results for both wine model and real sample. Moreover, in this context, the DI-HS extraction mode seems to prove the worst performance since the majority of the compounds are better extracted by the HS od DI mode only. Anyway, in order to provide a fair assessment of the combined mode performance, it should be compared to the other two extraction modes not only from the number of analytes better extracted by one mode or the other, but also considering the relative extraction extent obtained with each extraction mode, for each analyte.

This comparison was made as percentage variation of the extracted compound in DI-HS, *vs* HS and *vs* DI. The positive or negative values represent an increase or decrease in the extraction extent for the analyte using DI-HS compared to the other two extraction modes (**Figure 21**).

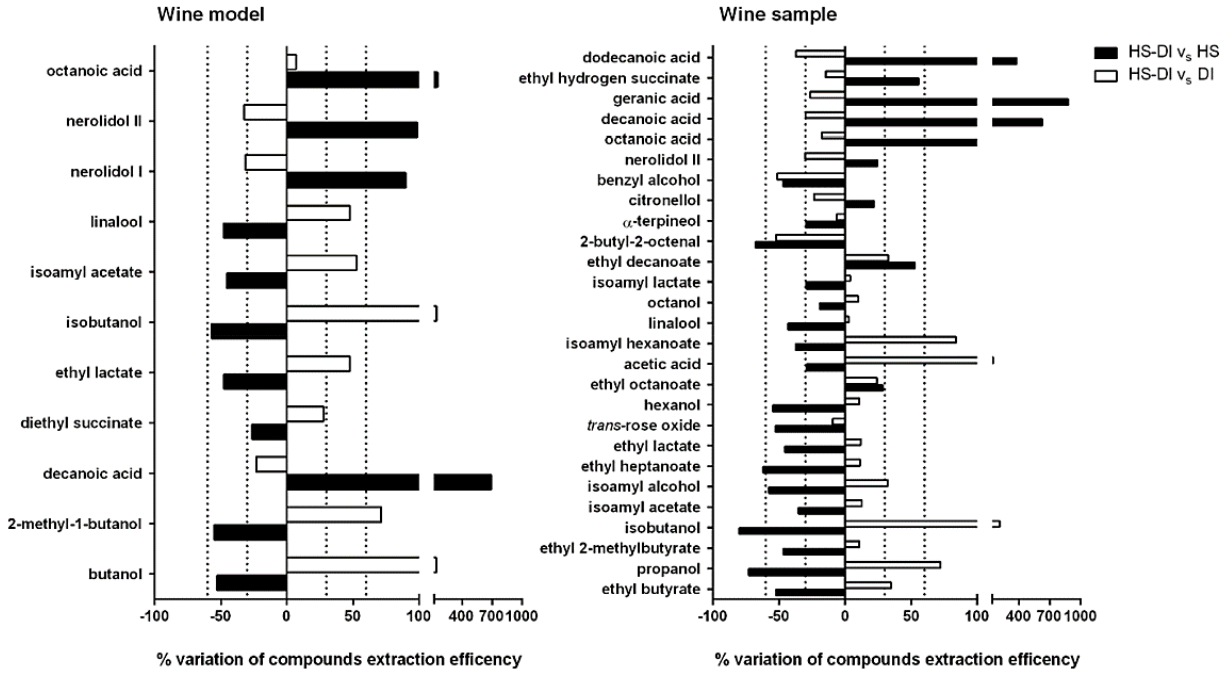


Figure 21. % Variation of the amount of extracted compounds switching the extraction mode from HS to DI-HS (black bars) or from DI to DI-HS (white bars). For both, model and wine sample (Lenti *et al.*, 2021).

Interestingly, these results pointed out that the combination mode is surely a better option with respect to DI only. In fact, it performed with a slightly worst results for the compounds that preferred the DI extraction mode only (isobutanol and 2-butyl-2-octenal, with a reduction of 50%), while is

showed an improvement for all other analytes which in 9 cases, was over 50% and in three cases was even greater, reaching an improvement of about 140%. Similar consideration can be highlighted when comparing DI-HS to HS only, bearing in mind that most of the compounds were HS preferring. In fact, even if in most of the cases the HS gave the best results, the use of the combined DI-HS mode showed a poor performance only for 2-butyl-2-octenal, isobutanol and propanol (with an important worsening of 70%). On the other hand, it gave outstanding improvements for 7 analytes (higher than the 90%) and in some cases even higher than the 350%.

1.4 Conclusions

It can be concluded that, the DI-HS extraction performed with the commercially available PDMS/DVB SPME OC fiber, mitigates the lack in the extraction efficiency performed in HS or DI only. In fact, while the first is suitable for the extraction of more volatile compounds, the second is more appropriate for least volatile ones. Hence, the DI-HS extraction represents a good compromise, and it is especially useful to obtain a more reliable and general overview of the chemical profile for matrices, such as wine, containing a huge number of compounds and with a wide range of volatilities.

1.5 Application of the method for the volatile profile characterization of DOCG wines of Marche region

1.5.1 DOCG wines sampling

To proceed with a representative sampling of DOCG wines of Marche region, a precise strategy was followed. To standardise the sampling, it was decided to take into consideration the last vintage produced that was legally marketable in 2021, the year of the sampling. Moreover, to choose the wineries which were more representative of the wine market for Marche region, we decided to consider the ones which achieved higher sales. The sales statistical analysis was done by the count of status flags asked by wineries and which are representative of the effective sales.

For “Vernaccia di Serrapetrona” both, the sweet and dry typologies were considered. For the “Offida” DOCG, in the same way, “Offida Pecorino”, “Offida Passerina” and “Offida Rosso” were considered.

Finally, 18 samples were collected, given that for the eight DOCGs, from 2 to 3 samples from different wineries were obtained (**Figure 22**). The samples are listed below, together with details

about grape variety, winery, harvest year, bottling year and the identification code that was used all along the thesis text (**Table 9**).

Figure 22. Bottles of DOCG wine samples.

Table 9. List of DOCG wine samples.

	D.O.C.G.	winery	grape variety	harvest year	bottling year	CODE
1	Verdicchio di Matelica Riserva	*Belisario – Cantina sociale di Matelica e Cerreto d'Esi*	Verdicchio 100%	2018	2021	VM01
		PRO.VI.MA – Società Agricola Cooperativa	Verdicchio 100%	2019	2021	VM02
2	Castelli di Jesi Verdicchio Riserva	*Vitivinicoltori Azienda Agricola – Giovanni e Francesca Marotti Campi S.S.*	Verdicchio 100%	2018	2021	VJ01
		Azienda Agricola Fratelli Bucci S.S.	Verdicchio 100%	2017	2019	VJ02
		Azienda Vinicola -Umani Ronchi s.p.a.	Verdicchio 100%	2019	2021	VJ03
3	Offida Pecorino	*Cantine di Castignano Società Cooperativa Agricola Comunanza*	Pecorino 100%	2020	2021	PE01
		Terre Cortesi Moncaro – Società Cooperativa Agricola	Pecorino 100%	2020	2021	PE02
4	Offida Passerina	*La cantina dei colli Ripani – Società Cooperativa*	Passerina 100%	2020	2021	PA01
		Terre Cortesi Moncaro – Società Cooperativa Agricola	Passerina 100%	2020	2021	PA02
5	Offida Rosso	*La cantina dei colli Ripani Società Cooperativa*	Montepulciano Cabernet Sauvignon	2017	2020	RO01
		Velenosi Vini – Azienda Vitivinicola Ascoli Piceno s.r.l.	Montepulciano 85% Cabernet Sauvignon 8% Merlot 7%	2017	2019	RO02
6	Cònero	*Azienda Agricola – Moroder Alessandro*	Montepulciano 100%	2017	2021	CO01

		Azienda Vinicola -Umani Ronchi s.p.a.	Montepulciano 100%	2017	2021	CO02
		Terre Cortesi Moncaro – Società Cooperativa Agricola	Montepulciano 100%	2018	2021	CO03
7	Vernaccia di Serrapetrona sweet	*Alberto Quacquarini – Società Agricola Semplice*	Vernaccia Nera 100%	2020	2021	VD01
		Rocchi Paris e C. Società Agricola S.S.	Vernaccia Nera 100%	2020	2021	VD02
8	Vernaccia di Serrapetrona dry	*Alberto Quacquarini – Società Agricola Semplice*	Vernaccia Nera 100%	2020	2021	VS01
		Serboni Massimo Azienza Agricola	Vernaccia Nera 100%	2020	2021	VS02

The optimized and validated method, and thus the extraction of volatile substances performed in the combination mode (DI-HS, 15 min-15 min) at 35 °C, followed by GC-MS analysis, was then applied to determine the volatile profile of the 18 different samples of DOCG wines of Marche region. This was performed to characterize the volatile profile of the samples and to possibly find those substances which may be used as markers to assess the authenticity of a given DOCG. The analysis was also performed to try to correlate the wine volatile profile and the sensorial olfactory attributes by which these wines are descried.

1.5.2 Results and discussion

From the GC-MS analysis of the 18 DOCG wine samples, performed in duplicate, a total of 50 compounds were detected, identified and finally selected after excluding those compounds that could derive from exogenous contamination, as e.g. siloxanes. The obtained results in terms of absolute mean peak areas are reported in **Tables 10-11** followed by an example of a chromatogram of the GC-MS analysis, performed in scan mode (**Figure 23**).

Table 10. Volatile compounds of DOCG white wine samples detected by DI-HS-SPME GC-MS. Ret time is referred to the retention times for every compound obtained with the chromatographic method used. Match quality (%) is referred to the comparison of experimental mass spectra with those found in NIST Library (2017), their experimental linear retention indices (LRI(exp) and from literature LRI(lit), NIST library) on a DB-WAX column. Their abundances are reported in terms of peak areas . RSD% range is referred to the range of percentage relative standard deviations obtained for every analyte among all white samples (RSD% range, *n*=2).

N.	ret time	Compound	Ric (%)	LRI (exp)	LRI (lit)	VM01	VM02	VJ01	VJ02	VJ03	PE01	PE02	PA01	PA02	RSD% range
1	13,993	ethyl butyrate	95	1040	1037	2,02E+06	6,55E+05	1,73E+06	4,70E+05	1,83E+05	3,41E+05	2,30E+05	5,18E+05	3,26E+05	6.5-37.1
2	14,806	ethyl 2-methyl butyrate	94	1056	1042	2,22E+05	1,59E+05	1,99E+05	1,68E+05	6,94E+04	n.d.	n.d.	n.d.	n.d.	5.5-70.7
3	15,590	isoamyl ethyl ester	96	1072	1062	5,27E+05	3,58E+05	6,28E+05	3,61E+05	9,61E+04	n.d.	4,85E+04	n.d.	6,71E+04	10.8-81.4
4	17,620	isobutanol	94	1110	1087	4,24E+05	5,21E+05	1,50E+06	3,64E+05	2,98E+05	2,76E+05	4,51E+05	3,75E+05	5,64E+05	1.4-29.7
5	18,225	isoamyl acetate	95	1123	1112	1,07E+06	2,85E+06	2,36E+07	1,73E+05	5,66E+05	9,76E+06	5,89E+06	7,98E+06	5,38E+06	1.3-23.6
6	23,538	2-methyl-1-butanol	85	1224	1212	1,05E+07	9,40E+06	2,50E+07	4,11E+06	4,96E+06	3,49E+06	4,78E+06	2,79E+06	4,73E+06	4.5-27.9
7	23,684	isoamyl alcohol	98	1220	1207	6,74E+07	5,70E+07	1,69E+08	2,83E+07	2,12E+07	2,98E+07	2,84E+07	3,24E+07	2,11E+07	1.2-27.7
8	24,534	ethyl hexanoate	97	1237	1233	8,08E+07	4,25E+07	1,72E+08	1,89E+07	1,69E+07	2,16E+07	1,04E+07	1,89E+07	1,10E+07	0.8-25.9
9	26,640	hexyl acetate	95	1273	1274	9,95E+05	2,65E+06	1,33E+07	n.d.	1,53E+05	4,04E+06	3,63E+06	3,08E+06	3,34E+06	6.4-34.9
10	30,142	ethyl heptanoate	84	1336	1310	1,54E+05	1,34E+05	n.d.	n.d.	8,30E+04	1,28E+05	n.d.	n.d.	n.d.	5.5-16.4
11	30,819	ethyl lactate	97	1350	1341	7,02E+06	5,50E+05	7,38E+06	1,14E+06	8,16E+05	4,24E+05	3,83E+05	2,22E+05	7,23E+05	3.6-45.6
12	31,428	hexanol	96	1361	1355	1,91E+06	2,08E+06	6,19E+06	1,33E+06	6,48E+05	6,46E+05	1,60E+06	6,60E+05	1,34E+06	3.3-127.8
13	31,919	4-hydroxy-4-methyl-2-pentanone	93	1370	1376	8,28E+04	n.d.	n.d.	n.d.	1,38E+06	n.d.	7,99E+05	n.d.	n.d.	0.2-5.7
14	32,074	3-octanol	93	1373	1368	n.d.	1,79E+05	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
15	33,033	methyl octanoate	91	1392	1373	9,97E+05	6,98E+05	1,25E+06	n.d.	1,72E+05	2,63E+05	2,49E+05	6,33E+05	1,57E+05	3.7-30.5
16	35,586	ethyl octanoate	97	1442	1429	3,60E+08	1,08E+09	1,12E+09	1,07E+08	1,12E+08	1,26E+08	8,56E+07	1,47E+08	1,21E+08	1.5-16.9
17	36,323	acetic acid	84	1455	1442	1,45E+06	1,27E+06	5,90E+06	7,93E+05	1,22E+06	4,69E+05	7,05E+05	6,65E+05	9,64E+05	1.0-23.5
18	36,683	isopentyl hexanoate	95	1456	1456	6,54E+05	1,06E+06	1,82E+06	2,25E+05	1,28E+05	2,64E+05	1,92E+05	4,66E+05	3,62E+05	2.9-101.9
19	37,019	furfural	97	1469	1457	1,07E+05	1,02E+05	3,29E+05	n.d.	n.d.	n.d.	1,88E+05	n.d.	1,98E+05	1.9-18.6
20	39,885	ethyl nonanoate	85	1548	1535	2,21E+05	2,54E+05	6,24E+05	1,13E+05	8,28E+04	1,97E+05	1,84E+05	1,24E+05	1,93E+05	0.4-115.0
21	40,296	linalool	70	1561	1549	n.d.	1,33E+06	n.d.	n.d.	n.d.	n.d.	3,06E+05	5,68E+05	1,38E+06	7.1-55.9
22	40,609	octanol	94	1572	1573	5,22E+05	n.d.	8,22E+05	n.d.	1,29E+05	n.d.	n.d.	n.d.	n.d.	2.7-4.4
23	40,897	isoamyl lactate	94	1582	1615	2,91E+05	2,26E+05	3,49E+05	7,26E+04	6,37E+04	6,78E+04	5,05E+04	n.d.	n.d.	0.1-35.5
24	41,109	2,3-butanediol	75	1589	1580	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
25	41,366	methyl decanoate	93	1598	1586	8,32E+05	3,50E+05	n.d.	n.d.	6,20E+05	n.d.	n.d.	1,48E+05	n.d.	7.9-27.8

26	42,279	ethyl decanoate	98	1648	1648	1,81E+09	1,42E+08	5,92E+08	3,39E+07	8,25E+07	7,22E+07	5,34E+07	9,30E+07	7,50E+07	
27	42,373	hexadecene	90	1655	1645	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.0-27.7
28	42,554	isoamyl octanoate	93	1665	1675	1,47E+06	2,62E+06	7,04E+06	5,98E+05	4,21E+05	5,31E+05	3,17E+05	6,30E+05	5,66E+05	0.6-14.5
29	42,601	isobutanoic acid	91	1688	1682	n.d.	3,23E+05	6,08E+05	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.1-29.9
30	42,921	diethyl succinate	97	1688	1682	3,18E+07	1,75E+07	4,09E+07	3,05E+07	1,06E+07	2,73E+06	3,14E+06	1,67E+06	7,56E+06	0.1-20.8
31	43,122	ethyl 9-decenoate	81	1697	1688	4,41E+05	1,06E+06	9,02E+06	1,42E+05	1,72E+06	2,69E+06	1,57E+06	2,90E+06	1,39E+06	1.2-60.1
32	43,328	α-terpineol	95	1712	1705	n.d.	n.d.	n.d.	n.d.	n.d.	5,70E+04	3,40E+05	1,65E+05	7,22E+05	6.3-23.8
33	43,589	(3-methylthio)-propanol	79	1732	1708	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
34	44,174	citronellol	96	1777	1774	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
35	44,424	methyl salicylate	95	1796	1794	6,89E+06	6,09E+05	6,25E+06	9,16E+05	1,12E+06	n.d.	4,10E+06	n.d.	5,11E+05	5.2-110.0
36	44,503	ethyl phenyl acetate	95	1802	1781	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
37	44,848	2-phenylethyl acetate	93	1839	1813	2,57E+06	7,12E+06	1,44E+07	2,63E+05	1,28E+06	1,51E+07	1,02E+07	8,02E+06	2,12E+07	3.1-26.6
38	44,898	isopropyl dodecanoate	96	1841	1822	1,83E+06	3,23E+05	4,31E+06	1,66E+05	8,96E+05	n.d.	4,11E+05	n.d.	n.d.	0.8-96.5
39	45,019	hexanoic acid	83	1854	1842	1,85E+07	7,87E+06	4,90E+07	3,39E+06	4,13E+06	4,21E+06	4,73E+06	6,82E+06	5,65E+06	2.7-32.1
40	45,104	(*E*)-geraniol	92	1861	1852	1,63E+07	8,23E+05	6,78E+06	7,97E+05	9,49E+05	3,51E+05	1,18E+06	n.d.	n.d.	4.2-113.1
41	45,487	benzyl alcohol	97	1898	1891	4,93E+05	2,32E+05	1,93E+05	1,43E+05	4,21E+05	1,14E+06	2,51E+06	n.d.	n.d.	6.2-119.2
42	45,854	phenyl ethyl alcohol	91	1942	1932	3,65E+07	4,69E+07	1,04E+08	3,07E+07	2,54E+07	2,72E+07	3,07E+07	2,45E+07	3,00E+07	0.1-19.1
43	46,726	isopropyl myristate	84	2045	2040	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
44	46,397	nerolidol	89	2003	2014	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
45	46,859	4-ethyl-2-methoxy phenol	92	2063	2057	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
46	46,901	octanoic acid	95	2068	2083	1,02E+08	7,80E+07	2,42E+08	3,92E+07	3,82E+07	5,48E+07	4,04E+07	5,51E+07	5,38E+07	0.3-24.3
47	47,924	3-ethyl phenol	97	2150	2171	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
48	48,448	ethyl hexadecanoate	91	2184	2207	1,19E+06	7,73E+05	2,25E+06	5,96E+05	5,25E+05	9,77E+05	1,09E+06	7,29E+05	5,46E+05	1.3-33.3
49	48,574	decanoic acid	95	2192	2244	1,38E+08	8,55E+07	4,04E+08	8,26E+08	1,54E+09	5,52E+07	5,11E+07	7,22E+07	5,55E+07	2.7-53.1
50	49,560	ethyl hydrogen succinate	97	2347	2350	5,61E+06	3,36E+06	4,98E+06	4,81E+06	3,21E+06	1,04E+06	1,14E+06	1,54E+06	1,63E+06	1.0-30.2

n.d.: not detected, area < 1.5E+05. VM: “Verdicchio di Matelica Riserva”; VJ: “Castelli di Jesi Verdicchio Riserva”; PE: “Offida Pecorino”; PA: “Offida Passerina”.

Table 11. Volatile compounds of DOCG red wine samples detected by DI-HS-SPME GC-MS. Ret time is referred to the retention times for every compound obtained with the chromatographic method used. Match quality (%) is referred to the comparison of experimental mass spectra with those found in NIST Library (2017), their experimental linear retention indices (LRI(exp) and from literature LRI(lit), NIST library) on a DB-WAX column. Their abundances are reported in terms of peak areas . RSD% range is referred to the range of percentage relative standard deviations obtained for every analyte among all red samples (RSD% range, *n*=2).

N.	ret time	Compound	Ric (%)	LRI (exp)	LRI (lit)	RO01	RO02	CO01	CO02	CO03	VD01	VD02	VS01	VS02	RSD% range
1	13,993	ethyl butyrate	95	1040	1037	1,44E+05	1,28E+05	2,39E+05	9,95E+04	1,48E+05	1,62E+05	1,35E+05	1,57E+05	1,80E+05	3.5
2	14,806	ethyl 2-methyl butyrate	94	1056	1042	7,82E+04	8,35E+04	1,96E+05	1,74E+05	8,93E+04	2,24E+05	7,25E+04	2,37E+05	1,09E+05	6.1-54.6
3	15,590	isoamyl ethyl ester	96	1072	1062	1,61E+05	8,57E+04	2,14E+05	1,91E+05	1,09E+05	1,56E+05	1,25E+05	1,72E+05	1,12E+05	0.1-41.3
4	17,620	isobutanol	94	1110	1087	6,46E+05	7,09E+05	1,06E+06	6,10E+05	8,21E+05	6,50E+05	8,78E+05	8,60E+05	9,30E+05	1.0-18.3
5	18,225	isoamyl acetate	95	1123	1112	8,37E+05	8,86E+05	7,02E+05	5,18E+05	9,27E+05	1,72E+06	1,45E+06	8,88E+05	1,37E+06	0.4-58.5
6	23,538	2-methyl-1-butanol	85	1224	1212	6,84E+06	5,07E+06	6,15E+06	6,22E+06	6,65E+06	8,69E+06	8,01E+06	8,32E+06	2,95E+06	4.4-70.3
7	23,684	isoamyl alcohol	98	1220	1207	3,16E+07	2,57E+07	3,46E+07	2,87E+07	2,29E+07	4,84E+07	3,19E+07	3,94E+07	1,67E+07	3.5-32.8
8	24,534	ethyl hexanoate	97	1237	1233	4,68E+06	2,98E+06	6,49E+06	7,29E+06	5,51E+06	1,22E+07	5,26E+06	n.d.	2,28E+06	1.8-34.0
9	26,640	hexyl acetate	95	1273	1274	5,31E+04	7,00E+04	n.d.	n.d.	5,17E+05	2,73E+06	1,85E+05	9,49E+04	n.d.	1.2-67.9
10	30,142	ethyl heptanoate	84	1336	1310	2,18E+05	5,47E+04	n.d.	5,51E+04	8,81E+04	1,24E+05	7,14E+04	2,11E+05	3,83E+05	1.2-78.0
11	30,819	ethyl lactate	97	1350	1341	3,10E+06	2,47E+06	2,01E+06	1,79E+06	2,57E+06	4,26E+05	4,13E+06	9,72E+06	1,72E+06	0.9-31.4
12	31,428	hexanol	96	1361	1355	8,52E+05	8,08E+05	6,26E+05	5,27E+05	6,71E+05	1,85E+06	1,12E+06	1,90E+06	1,57E+06	7.9-25.9
13	31,919	4-hydroxy-4-methyl -2-pentanone	93	1370	1376	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
14	32,074	3-octanol	93	1373	1368	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1,07E+05	1,01E+05	70.0-76.7
15	33,033	methyl octanoate	91	1392	1373	1,39E+05	5,86E+04	1,36E+05	3,05E+04	1,12E+05	1,32E+05	1,26E+05	1,60E+05	n.d.	3.0-42.7
16	35,586	ethyl octanoate	97	1442	1429	4,57E+07	2,55E+07	5,40E+07	4,41E+07	4,91E+07	1,55E+08	4,10E+07	5,04E+07	3,57E+07	4.8-47.3
17	36,323	acetic acid	84	1455	1442	2,70E+06	2,90E+06	2,07E+06	2,51E+06	5,96E+06	1,54E+06	1,18E+06	8,43E+06	4,20E+05	0.3-62.2
18	36,683	isopentyl hexanoate	95	1456	1456	1,56E+06	1,01E+05	1,76E+05	1,21E+05	1,24E+05	1,88E+05	n.d.	n.d.	n.d.	3.7-129.5
19	37,019	furfural	97	1469	1457	1,12E+05	9,03E+04	1,80E+05	2,98E+04	3,99E+04	n.d.	4,15E+05	n.d.	n.d.	4.9-33.5
20	39,885	ethyl nonanoate	85	1548	1535	4,15E+05	1,45E+05	1,05E+05	9,73E+04	1,50E+05	1,48E+05	1,07E+05	7,55E+04	5,79E+05	1.5-86.7
21	40,296	linalool	70	1561	1549	1,37E+05	1,78E+05	2,12E+05	1,35E+05	1,34E+05	2,54E+05	1,80E+05	1,93E+05	1,82E+05	2.5-56.3
22	40,609	octanol	94	1572	1573	2,42E+05	1,96E+05	2,31E+05	1,83E+05	1,63E+05	2,29E+05	2,51E+05	4,10E+05	6,43E+05	1.8-59.4
23	40,897	isoamyl lactate	94	1582	1615	3,16E+05	3,01E+05	2,13E+05	1,29E+05	3,01E+05	6,08E+05	4,10E+05	7,14E+05	2,77E+05	0.2-31.9
24	41,109	2,3-butanediol	75	1589	1580	n.d.	2,75E+05	n.d.	n.d.	n.d.	n.d.	n.d.	4,02E+05	2,45E+05	5.6-99.9
25	41,366	methyl decanoate	93	1598	1586	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	

26	42,279	ethyl decanoate	98	1648	1648	1,95E+07	1,15E+07	1,99E+07	2,33E+07	3,75E+07	1,25E+07	1,18E+07	1,22E+07	1,17E+07	0.9-56.7
27	42,373	hexadecene	90	1655	1645	n.d.	n.d.	1,09E+06	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
28	42,554	isoamyl octanoate	93	1665	1675	2,49E+05	4,74E+05	4,20E+05	2,20E+05	1,41E+05	2,26E+05	1,92E+05	9,10E+05	n.d.	2.4-70.3
29	42,601	isobutanoic acid	91	1688	1682	2,95E+05	3,21E+05	n.d.	1,28E+05	2,70E+05	2,18E+05	n.d.	3,29E+05	n.d.	0.1-7.0
30	42,921	diethyl succinate	97	1688	1682	3,13E+07	1,85E+07	4,61E+07	3,84E+07	8,29E+06	2,56E+07	1,73E+07	3,85E+07	1,77E+07	3.2-15.1
31	43,122	ethyl 9-decenoate	81	1697	1688	1,31E+05	1,63E+05	4,31E+05	1,16E+05	7,09E+05	7,86E+05	1,37E+06	6,50E+05	8,02E+05	3.5-43.6
32	43,328	α-terpineol	95	1712	1705	4,78E+05	7,06E+04	1,60E+05	1,44E+05	n.d.	4,61E+05	3,21E+05	2,75E+05	1,21E+05	2.2-59.2
33	43,589	(3-methylthio)-propanol	79	1732	1708	2,05E+05	1,24E+05	1,60E+05	1,07E+05	5,24E+05	2,21E+05	2,81E+05	n.d.	n.d.	0.7-42.2
34	44,174	citronellol	96	1777	1774	2,21E+05	2,78E+05	3,62E+05	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.5-5.7
35	44,424	methyl salicylate	95	1796	1794	7,80E+05	3,03E+05	1,08E+06	5,23E+05	n.d.	5,38E+05	2,21E+05	3,96E+05	9,26E+04	1.1-56.8
36	44,503	ethyl phenyl acetate	95	1802	1781	1,22E+06	n.d.	n.d.	n.d.	n.d.	n.d.	1,84E+06	n.d.	n.d.	8.8
37	44,848	2-phenylethyl acetate	93	1839	1813	1,63E+06	9,22E+05	8,46E+05	9,37E+05	2,14E+06	1,81E+06	2,74E+06	2,02E+06	9,36E+05	4.7-61.2
38	44,898	isopropyl dodecanoate	96	1841	1822	6,58E+05	5,56E+05	3,15E+05	n.d.	9,88E+05	n.d.	3,23E+05	1,85E+05	n.d.	6.7-63.0
39	45,019	hexanoic acid	83	1854	1842	1,84E+06	1,27E+06	1,63E+06	1,21E+06	2,35E+06	1,15E+06	1,60E+06	2,33E+06	1,52E+06	4.4-119.9
40	45,104	(*E*)-geraniol	92	1861	1852	4,54E+05	3,91E+05	8,07E+05	1,04E+06	1,24E+06	8,18E+05	8,40E+05	2,10E+06	n.d.	18.3-59.4
41	45,487	benzyl alcohol	97	1898	1891	4.77E+05	3,54E+05	n.d.	n.d.	n.d.	n.d.	n.d.	7,45E+05	3,44E+06	0.0-27.2
42	45,854	phenyl ethyl alcohol	91	1942	1932	4,77E+07	2,96E+07	3,76E+07	3,62E+07	2,62E+07	4,37E+07	3,45E+07	4,25E+07	3,39E+07	0.8-61.6
43	46,726	isopropyl myristate	84	2045	2040	n.d.	n.d.	2,40E+05	n.d.	n.d.	n.d.	n.d.	n.d.	1,09E+06	13.9-17.0
44	46,397	nerolidol	89	2003	2014	7,85E+05	5,95E+05	n.d.	n.d.	n.d.	n.d.	7,55E+05	n.d.	n.d.	0.4-8.2
45	46,859	4-ethyl-2-methoxy phenol	92	2063	2057	1,66E+06	4,67E+05	1,26E+06	9,48E+05	n.d.	n.d.	n.d.	2,66E+06	2,04E+06	2.8-21.6
46	46,901	octanoic acid	95	2068	2083	9,43E+06	7,74E+06	1,27E+07	8,19E+06	1,40E+07	2,49E+06	1,07E+07	1,12E+07	8,63E+06	0.2-63.6
47	47,924	3-ethyl phenol	97	2150	2171	4,80E+06	7,13E+05	1,96E+06	2,30E+06	4,07E+05	8,01E+06	9,85E+05	1,19E+07	4,46E+06	0.6-71.0
48	48,448	ethyl hexadecanoate	91	2184	2207	5,22E+05	4,09E+05	n.d.	4,23E+05	1,35E+05	9,44E+05	n.d.	8,65E+05	4,98E+05	4.1-41.6
49	48,574	decanoic acid	95	2192	2244	1,16E+07	7,93E+06	1,44E+07	1,40E+07	2,71E+07	9,22E+06	1,06E+07	1,34E+07	1,05E+07	0.5-58.2
50	49,560	ethyl hydrogen succinate	97	2347	2350	4,88E+06	6,29E+06	6,48E+06	1,40E+07	2,03E+06	3,32E+06	2,84E+06	5,16E+06	3,28E+06	0.8-33.8

n.d.: not detected, area < 1.5E+05. RO: “Offida Rosso”; CO: “Cònero”; VD: “Vernaccia di Serrapetrona” sweet; VS: “Vernaccia di Serrapetrona” dry.

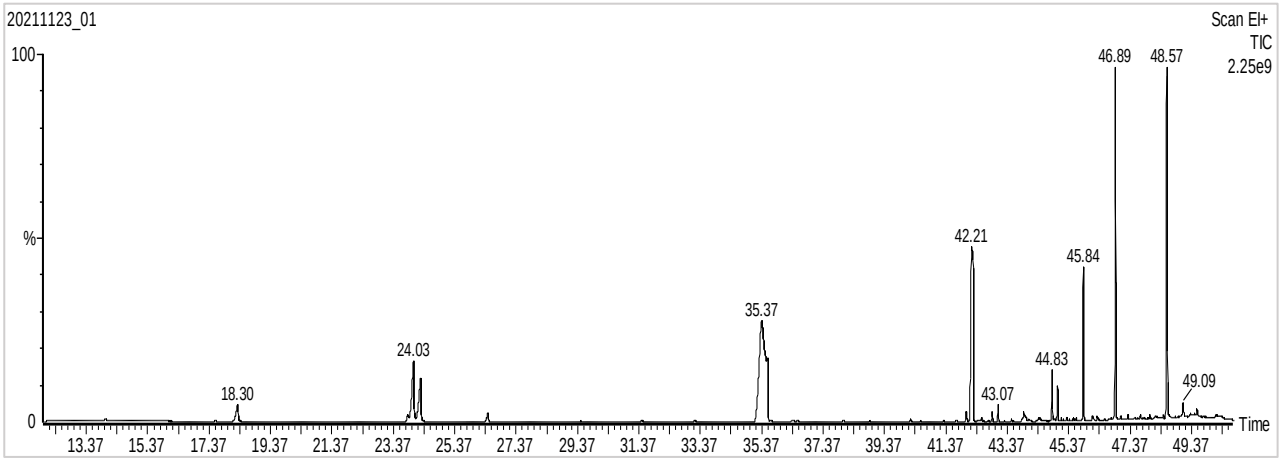


Figure 23. Example of a chromatogram obtained by the GC-MS analysis of a DOCG wine sample after DI:HS SPME.

To analyse the results from different points of view, the relative percentage areas for all 50 compounds and for each wine sample were calculated and the results reported in **Table 12** and graphically represented in **Figure 24**. The compounds were also divided depending on their chemical class into six groups: ethyl esters, other esters, alcohols, terpenes, acids and others.

Table 12. Mean percentage areas calculated for each compound, with the relative standard deviation range (RSD% range, *n*=2).

		VM01	VM02	VJ01	VJ02	VJ03	PE01	PE02	PA01	PA02	RO01	RO02	CO01	CO02	CO03	VD01	VD02	VS01	VS02	RSD % range
	ethyl esters																			
1	ethyl butyrate	0.07	0.04	0.1	0.04	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.1	0.0	0.1	0.1	0.1	2.4-48.4
2	ethyl 2-methyl butyrate	0.01	0.01	0.0	0.01	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.0	0.1	0.0	0.1	0.1	0.4-56.3
3	isoamyl ethyl ester	0.02	0.02	0.0	0.03	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.1	0.1	0.0	0.1	0.1	0.1	1.4-65.7
4	ethyl hexanoate	2.97	2.64	5.7	1.7	1.0	4.9	3.0	3.9	2.6	1.9	1.9	2.5	3.1	2.6	3.5	2.7	0	1.3	7.3-59.3
5	ethyl heptanoate	0.01	0.01	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.2	7.0-40.6
6	ethyl lactate	0.26	0.03	0.2	0.1	0.0	0.1	0.1	0.0	0.2	1.3	1.6	0.8	0.8	1.2	0.1	2.1	3.6	1.0	0.6-26.9
7	ethyl octanoate	13.24	67.64	36.9	9.65	6.6	28.9	24.4	30.3	28.4	19.0	16.2	21.1	18.7	22.0	42.4	20.9	18.5	21.3	0.7-45.6
8	ethyl nonanoate	0.01	0.02	0.0	0.01	0.0	0.0	0.1	0.0	0.0	0.2	0.1	0.0	0.0	0.1	0.0	0.1	0.0	0.3	0.3-115.0
9	ethyl decanoate	66.84	8.86	19.4	3.05	4.8	16.5	15.3	19.2	17.5	8.1	7.3	7.8	9.9	16.4	3.7	6.0	4.5	7.0	0.8-34.5
10	diethyl succinate	1.17	1.09	1.3	2.75	0.6	0.6	0.9	0.3	1.8	13.0	11.8	18.0	16.3	4.2	8.3	8.8	14.3	10.6	1.2-47.6
11	ethyl 9-decenoate	0.02	0.07	0.3	0.01	0.1	0.6	0.4	0.6	0.3	0.1	0.1	0.2	0.0	0.3	0.2	0.7	0.2	0.5	0.1-36.8
12	ethyl phenylacetate	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.9	0.0	0.0	13.8
13	ethyl hexadecanoate	0.04	0.05	0.1	0.05	0.0	0.2	0.3	0.2	0.1	0.2	0.3	0.1	0.2	0.1	0.3	0.0	0.3	0.3	2.2-141.4
14	ethyl hydrogen succinate	0.21	0.21	0.2	0.42	0.2	0.2	0.3	0.3	0.4	2.0	4.0	2.5	6.0	0.9	1.0	1.4	1.9	2.0	1.0-60.3
	other esters																			
15	isoamyl acetate	0.04	0.18	0.8	0.02	0.0	2.2	1.7	1.6	1.3	0.3	0.6	0.3	0.2	0.5	0.5	0.7	0.3	0.8	2.3-25.9
16	hexyl acetate	0.04	0.16	0.4	0.0	0.0	0.9	1.0	0.6	0.8	0.0	0.0	0.0	0.0	0.2	0.8	0.1	0.0	0.0	0.0-58.2
17	methyl octanoate	0.04	0.04	0.0	0.0	0.0	0.1	0.1	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.1	0.0	2.1-48.3
18	isopentyl hexanoate	0.02	0.07	0.1	0.02	0.0	0.1	0.1	0.1	0.1	0.7	0.1	0.0	0.1	0.1	0.1	0.0	0.0	0.0	6.7-130.2
19	isoamyl lactate	0.01	0.01	0.0	0.01	0.0	0.0	0.0	0.0	0.0	0,1	0.2	0.1	0.1	0.1	0.2	0.2	0.3	0.2	13.9-37.7
20	methyl decanoate	0.03	0.02	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.6-74.2
21	isoamyl octanoate	0.05	0.16	0.2	0.05	0.0	0,1	0.1	0.1	0.1	0.1	0.3	0.2	0.1	0.1	0.1	0.1	0.3	0.0	2.6-74.2
22	methyl salicylate	0.25	0.04	0.2	0.09	0.1	0.0	1.2	0.0	0.1	0.3	0.2	0.4	0.2	0.0	0.2	0.1	0.1	0.1	4.5-98.5
23	2-phenylethyl acetate	0.09	0.44	0.5	0.02	0.1	3.5	2.9	1.7	5.0	0.7	0.6	0.3	0.4	0.9	0.5	1.4	0.8	0.6	0.5-21.3
24	isopropyl dodecanoate	0.07	0.02	0,1	0.01	0.1	0.0	0.1	0.0	0.0	0.3	0.2	0.1	0.0	0.5	0.0	0.2	0.0	0.0	9.9-97.0
25	isopropyl myristate	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.7	22.9-26.1
	alcohols																			
26	isobutanol	0.02	0.03	0.0	0.03	0.0	0.1	0.1	0.1	0.1	0.3	0.5	0.2	0.3	0.4	0.2	0.4	0.3	0.6	0.1-38.4
27	2-methyl-1-butanol	0.39	0.58	0.8	0.37	0.3	0.8	1.4	0.6	1.1	2.8	3.2	2.3	2.6	3.1	2.7	4.1	3.0	1.8	0.6-49.9
28	isoamyl alcohol	2.48	3.54	5.6	2.55	1.2	6.8	8.1	6.7	5	13.1	16.4	13.4	12.1	10.7	14.3	16.3	14.5	9.9	0.5-35.8
29	hexanol	0.07	0.13	0.2	0.12	0.0	0.1	0.5	0.1	0.3	0.4	0.5	0.2	0.2	0.3	0.6	0.6	0.7	0.9	0.8-128.0
30	3-octanol	0.0	0.01	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	62.7-67.7
31	octanol	0.01	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.1	0,1	0,1	0,1	0.2	0.4	4.3-59.7
32	2,3-butandiol	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.1	0.1	3.8-100.0

33	(3-methylthio)-propanol	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.0	0.3	0.1	0.1	0.0	0.0	8.4-595
34	benzyl alcohol	0.02	0.01	0.0	0.01	0.0	0.3	0.7	0.0	0.0	0.0	0.2	0.0	0.0	0	0	0.0	0.3	2.1	6.8-141.4
35	phenyl ethyl alcohol	1.34	2.92	3.4	2.76	1.5	6.3	8.7	5.1	7.1	19.9	18.8	14.7	15.4	11,3	13.1	17.6	15.8	20.3	0.3-35.5
36	4-ethyl-2-methoxy phenol	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.7	0.1	0.5	0.4	0.0	0.0	0.0	1.0	1.2	6.6-141.4
37	3-ethyl phenol	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.0	0.5	0.7	1.0	0.2	2,4	0.5	4.4	2.7	4.6-46.6
	terpenes																			
38	linalool	0.0	0.08	0.0	0.0	0.0	0.0	0.1	0.1	0.3	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1-52.5
39	α-terpineol	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.2	0.2	0.0	0.1	0.1	0.0	0.1	0.2	0.1	0.1	0.84-85.1
40	citronellol	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.2	0.1	0.0	0.0	0.0	0.0	0.0	0.0	5,4-7.1
41	I-geraniol	0.6	0.05	0.2	0,07	0.0	0.1	0.3	0.0	0.0	0.2	0.2	0.3	0.4	0.6	0.2	0.4	0.8	0.0	0.1-50.6
42	nerolidol	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.4	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.2-6.2
	acids																			
43	acetic acid	0.05	0.08	0.2	0.07	0.1	0.1	0.2	0.1	0.2	1.1	1.8	0.8	1.1	3,4	0,5	0,6	3	0.2	0.6-74.8
44	isobutanoic acid	0.0	0.02	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.2	0.0	0.1	0.1	0.1	0.0	0,1	0.0	5.1-42.8
45	hexanoic acid	0.68	0.49	1,6	0.31	0.2	1.0	1.4	1.4	1.3	0.8	0.8	0.6	0.5	1.2	0.4	0.8	0.9	0.9	1-62.7
46	octanoic acid	3.74	4.86	7.9	3.5	2.2	12.6	11.5	11.4	12.6	3.9	4.9	5.0	3.5	6.0	0.4	55	4.2	5.2	0.3-141.4
47	decanoic acid	5.08	5.34	13.2	72.1	80.5	12.7	14.6	14.9	13.0	4.8	5.0	5.6	6.0	11.8	2.8	5.4	4.9	6.3	2.3-45
	others																			
48	4-hydroxy-4-methyl pentanone	0.0	0.0	0.0	0.0	0.1	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.4-41.3
49	furfural	0.0	0.01	0.0	0.0	0.0	0.0	0,1	0.0	0.0	0.0	0.1	0.1	0.0	0.0	0.0	0.2	0.0	0.0	1.5-38.2
50	hexadecane	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	107.9

RSD% range: range of the percentage standard deviations obtained for a given analytes, along the eighteen DOCG wine samples. VM: “Verdicchio di Matelica Riserva”; VJ: “Castelli di Jesi Veridicchio Riserva”; PE: “Offida Pecorino”; PA: “Offida Passerina”; RO: “Offida Rosso”; CO: “Cònero”; VD: “Vernaccia di Serrapetrona” sweet; VS: “Vernaccia di Serrapetrona” dry

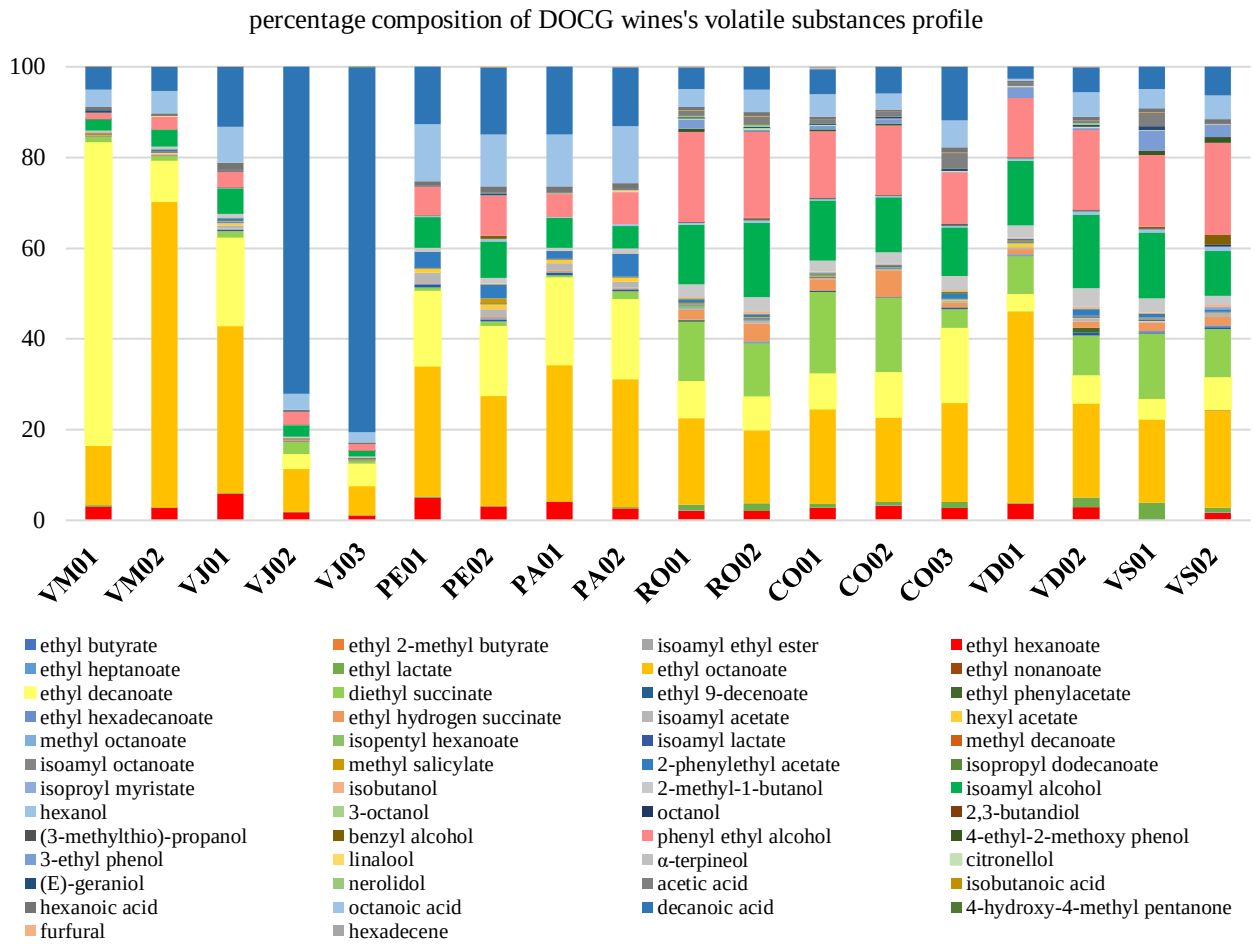


Figure 24. Percentage composition of volatile compounds in DOCG wine samples.

Interesting information can be immediately pointed out. Indeed, in some samples, a single compound was able to characterize more than the 50% of the total volatile fraction. Among the different DOCGs, clear differences were observed also within the same DOCG, from samples provided by different wineries.

Indeed, the volatile profiles of the two "Verdicchio di Matelica Riserva" (VM01 and VM02) were quite different. "Verdicchio di Matelica Riserva" VM01 showed the highest percentage of ethyl decanoate (66.8%), while the other (VM02) was characterized by the highest content of ethyl octanoate (67.6%,) and these percentages were also the highest when compared to all the other samples, in a statistically significant way (P <0.05). Hence, the two wines resulted to be enriched by different volatile compounds.

Differences were also observed within the "Castelli di Jesi Verdicchio Riserva" DOCG, since VJ01 showed higher percentages of ethyl octanoate (36.7%) and ethyl decanoate (19.5%) with respect to the other two Verdicchio samples VJ02 and VJ03 (13 and 4.13% and 7.8 and 5.8%, respectively).

By contrary, VJ02 showed a very high percentage of decanoic acid 44.2%, but that was even higher for VJ03, being 49.3%.

Concerning the samples of "Offida Pecorino" (PE01 and PE02) and "Offida Passerina" (PA01 and PA02), at a first sight a clear distinction on the volatile fraction composition did not arise and the samples were characterized by relatively high percentages of ethyl octanoate (24.4-30.4%), ethyl decanoate (15.2-19.2%), octanoic (11.4-12.5%) and decanoic acid (12.6-14.9%).

Regarding red wines, the overall pattern of the volatile fraction composition was very different when compared to white wine samples. They generally showed a higher percentage of compounds such as diethyl succinate (3.7-17.9%), isoamyl alcohol (10-16.3%), phenyl ethyl alcohol (11.9-20.3%), which were almost negligible, in terms of percentage composition, for white wines. Among the four red DOCGs, the composition was quite similar when looking to the compounds with highest percentages composition. Anyway, even in this case some differences raised also within samples of the same DOCG. Indeed, a sample of "Vernaccia di Serrapetrona" sweet (VD01) contained a very high amount of ethyl octanoate (44.2%), compared to the other red wine samples where this analyte ranged from 16.3 to 22.3 %. Again, ethyl hexanoate was not detected at all in one sample of "Vernaccia di Serrapetrona" dry (VS01).

The percentage composition of volatile compounds grouped into the six chemical classes was then calculated and shown in **Figure 25**.

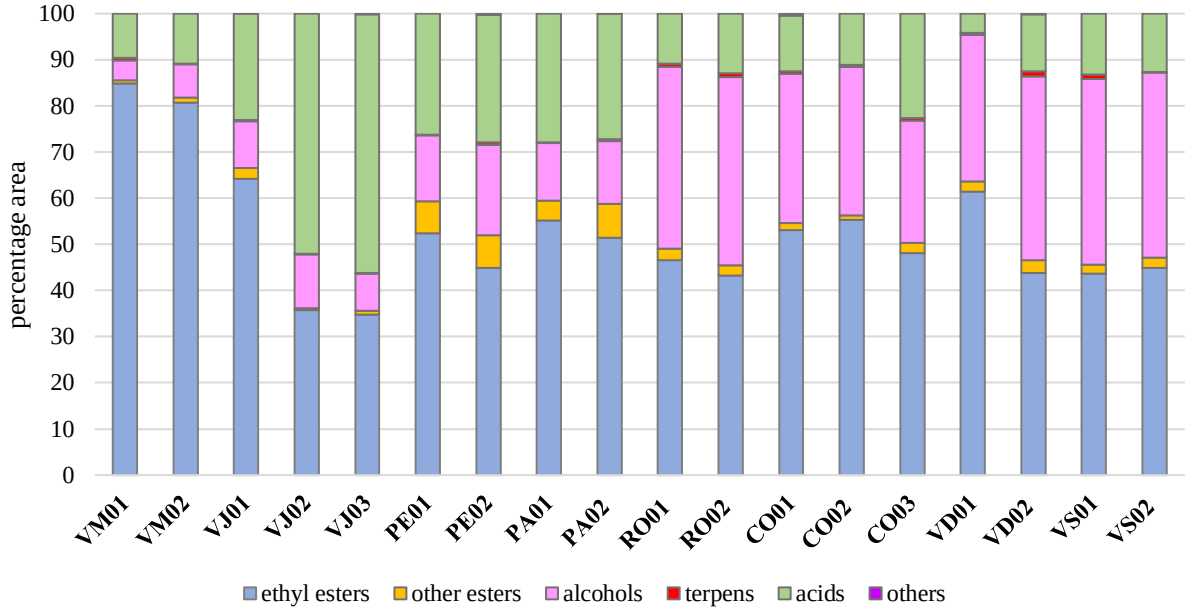


Figure 25. Percentage composition of the different classes of compounds in the volatile fraction of the wines under investigation.

The predominant class of compounds were generally ethyl esters (34.8-84.9%), followed by alcohols (8.1-40.7%) and acids (4.2-55.9%), while the other classes of compounds were characterized by much lower amounts. These percentages were quite variable among the 18 different samples and the main differences seemed to be correlated to the wine typology. In fact, "Verdicchio di Matelica Riserva" samples (VM01 and VM02) showed a similar pattern, that however was different compared to "Castelli di Jesi Verdicchio Riserva" samples (VJ01, VJ02 and VJ03). The two typologies of Offida white wines Pecorino and Passerina (PE01, PE02 and PA01 and PA02) were closer for the chemical class component profile. In red samples the pattern seemed to be more homogenous.

The one-way analysis of variance (ANOVA) and the Tukey's test for pairwise comparison was then performed to identify significant differences ($P < 0.05$) in the chemical classes' percentage composition, among the different DOCGs. The 18 wine samples were divided into eight groups, given that Offida comprises three distinct DOCGs (Pecorino, Passerina and Rosso) and "Vernaccia di Serrapetrona" two DOCGs (sweet and dry) and the obtained results are reported in **Table 13**.

Table 13. ANOVA performed among the percentage composition of the six chemical classes to compare the eight DOCGs.

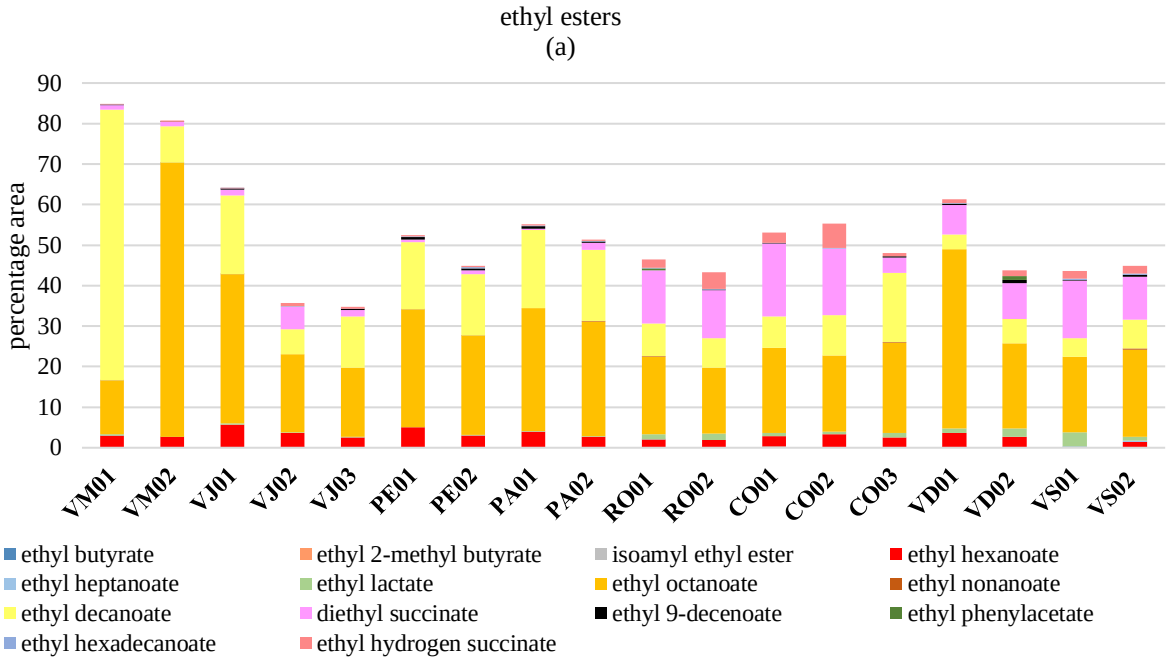
	Verdicchio di Matelica Riserva	**RSD %**	**Castelli di Jesi Verdicchio Riserva**	**RSD %**	**Offida Pecorino**	**RSD %**	**Offida Passerina**	**RSD %**	**Offida Rosso**	**RSD %**	**Cònero**	**RSD %**	**Vernaccia di Serrapetrona sweet**	**RSD %**	**Vernaccia di Serrapetrona dry**	**RSD %**
ethyl esters	82.8^{a}	3.6	44.9^{b}	37.2	48.7^{b}	10.9	$53.3^{a,b}$	4.9	44.9^{b}	7.1	$52.2^{a,b}$	7.1	$52.5^{a,b}$	23.7	44.3^{b}	2.0
other esters	0.9^{a}	39.7	1.2^{a}	86.6	7.0^{b}	2.6	$5.8^{b,c}$	37.1	2.4^{c}	9.4	1.6^{a}	40.7	2.5^{c}	16.6	2.1^{c}	8.7
alcohols	5.8^{a}	35.5	9.9^{a}	18.4	$16.9^{a,b}$	21.6	13.0^{b}	5.3	40.1^{c}	2.4	30.4^{d}	11.2	$35.8^{c,d}$	15.8	$40.2^{c,d}$	0.3
terpenes	0.4^{a}	89.6	0.2^{a}	26.8	0.3^{a}	103.6	0.3^{a}	74.8	0.9^{a}	9.3	0.5^{a}	15.5	0.8^{a}	59.5	0.6^{a}	75.3
acids	10.2^{a}	8.5	43.7^{b}	41.1	27.0^{b}	3.5	27.5^{b}	1.9	11.8^{a}	12.5	15.2^{a}	42.1	8.2^{a}	70.0	12.9^{a}	2.8
others	0.0^{a}	0.0	0.1^{a}	160.0	0.1^{a}	141.4	0.0^{a}	141.2	0.0^{a}	0.0	0.2^{a}	173.0	0.1^{a}	141.4	0.0^{a}	0.0

Letters indicate significant differences between the signals (in terms of percentage areas) among the six chemical classes and the eight DOCGs (One-way ANOVA, $P < 0.05$, Tukey's test for pairwise comparison).

While the percentage compositions for the class of terpenes were comparable among the DOCGs, the other chemical classes showed some interesting divergences. Indeed, statistically significant differences (P <0.05) were obtained even between DOCGs made with same grape variety or produced in relatively close geographical areas. For example, the percentage compositions of ethyl esters and acids were very different between "Verdicchio di Matelica Riserva" and "Castelli di Jesi Verdicchio Riserva". While the former was characterized by the highest abundance of ethyl esters (82.8%) in the latter this class of compounds was much lower (44.9%) and comparable to the percentage covered by acids (43.7 %). The class of other esters did not show a clear discrimination between DOCGs even if "Offida Pecorino" and "Offida Passerina" showed slightly higher percentages when compared to the other DOCGs and, in particular to the other white DOCGs ("Verdicchio di Matelica Riserva" and "Castelli di Jesi Verdicchio Riserva").

Moreover, the alcohols percentage in the "Offida Rosso" wines was different to the Offida Pecorino and Passerina, being higher and almost identical in the white Offida DOCGs (27 and 27.5 %, respectively). "Offida Rosso" and "Cònero", which are wines produced with the same minimal amount of Montepulciano grape variety (minimal amount of 85 %), but produced in distinct geographical area, showed comparable compositions. Finally, "Vernaccia di Serrapetrona" sweet and dry, showed comparable percentage composition along the classes of compounds.

Each class of compounds was then analysed more in detail, by considering the contribution of every compound within each class. This was performed with two different approaches as shown in **Figure 26 a** and **b,** for the class of ethyl ester, which comprises 14 compounds.



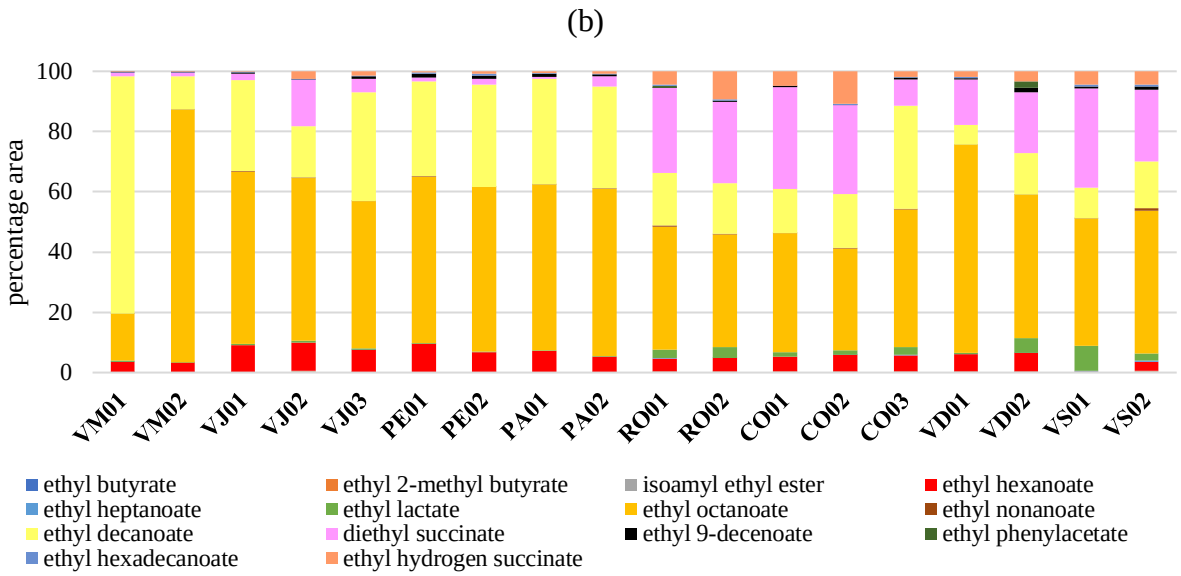


Figure 26. (a) Absolute percentage composition of ethyl esters in the wine samples under investigation, (b) Relative percentage composition of every ethyl ester within the class of ethyl esters in the wine samples under investigation.

While **Figure 26 a** represents the percentage of every ethyl ester calculated over the 50 total substances detected and identified, **Figure 26 b** shows the percentages calculated within the class of ethyl ester and thus considering only the compounds belonging to the chemical class under investigation. The two ways to investigate data are both important since they give different information about the volatile composition of the studied DOCG wines.

While some ethyl esters such as ethyl hexanoate, octanoate and decanoate, normally associated to fruity attributes, were present in all samples with different percentages, other compounds, were detected only in white or red samples. Diethyl succinate which can impart fruity and cooked apple scents, for example, was present in white wines with smaller percentages (0.6-5.5 %) when compared to red ones (3.7-18%) (**Figure 26**, **a**). This difference can be attributed to the fact that this compound is formed during malolactic fermentation, a transformation which is desirable in red wines while in white ones should be avoided or limited (Soufleros *et al.*, 1998). As expected, also ethyl lactate was detected with higher percentages in red wines (on average 0.11 % in white wines and 1.4% in red wines). The one-way analysis of variance (ANOVA, $P < 0.05$) together with Tukey's test for pairwise comparison was performed on the absolute areas obtained by the GC-MS analysis to identify substances which showed significant differences in terms of abundances in the investigated wine samples. In particular, the statistical analysis underlined that a sample of "Verdicchio di Matelica Riserva" (VM01) was enriched in ethyl butyrate, while a sample of "Vernaccia di

Serrapetrona" sweet (VS01) by ethyl nonanoate. Finally, a sample of "Cònero" (CO02) was characterized by the highest amount of ethyl hydrogen succinate.

The most abundant class of compounds were alcohols (excluding ethanol). The difference in the total amount of alcohols between white and red wines is evident, as showed in **Figure 27 a**. By contrary, when looking at the percentage composition of the alcohols, there is not a clear difference between the samples (**Figure 27, b**).

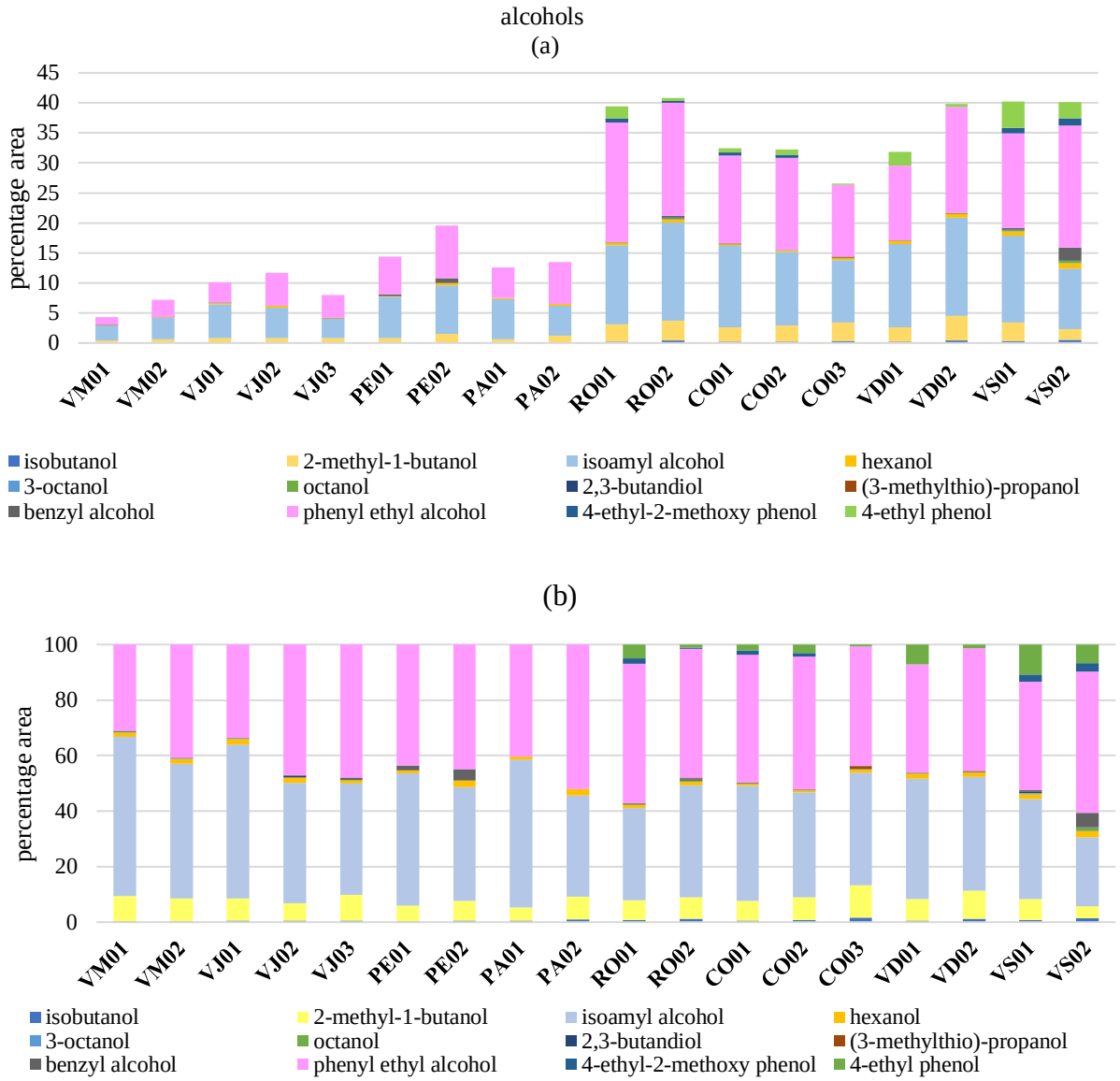
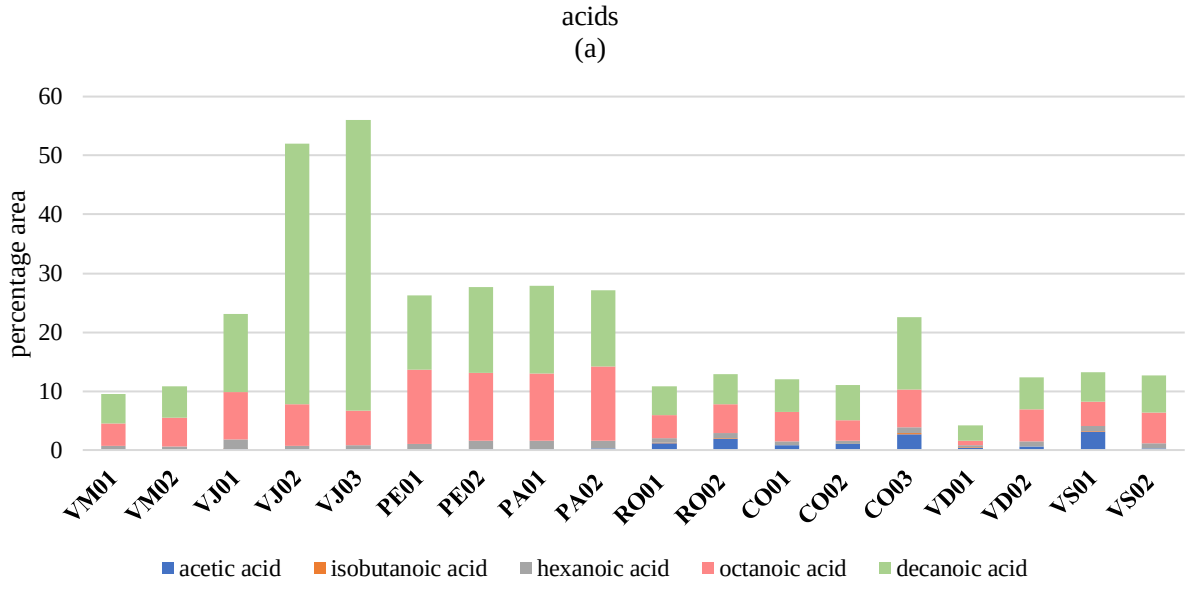


Figure 27. (a) Absolute percentage composition of alcohols in the DOCG wine samples (b) Relative percentage composition of compounds within the class of alcohols in the DOCG wine samples.

The most abundant detected analyte was generally ethyl phenyl alcohol, which can impart a rose-like aroma, and was found in a range between 1.3 and 8.7 % for white wines and 11.9 and 20.3% for red wine samples, followed by isoamyl alcohol, attributed to fruity and banana fragrances (10.3-

16.3% for red samples and 1.5-8.1% for white ones) (**Figure 27, a**). The others were present in very low percentages. Some of these minor compounds were only detected in red wines. 4-Ethylphenol, for example, is an aromatic compound characterized by smoky scents and it was not detected in the white wine samples. This is given that this compound derives from the decarboxylation of hydroxycinnamic acids, of which red wines are more abundant, followed by the reduction of the intermediate vinylphenol (Carpinteiro *et al.*, 2012). The ANOVA performed on absolute areas pointed out that some alcohols were present in a distinct 64veralt among the samples. Indeed, phenyl ethyl alcohol and isoamyl alcohol were present in a significant higher way in one sample of "Verdicchio di Jesi Riserva" (VJ01). A "Cònero" wine sample (CO03) was instead characterized by the higher quantity of 3-methylthio propanol. Finally, a sample of "Vernaccia di Serrapetrona" dry (VS01) was characterized by the statistically highest abundances of 2,3-butanediol and 3-ethylphenol.

Volatile acids, together with ethyl esters, were the most abundant compounds in the wine samples under investigation. Among the five identified volatile acids, decanoic acid reached very high percentage areas of 44.2 and 49.4%, being the most abundant acid (P <0.05) for VJ02 and VJ03 "Castelli di Jesi Verdicchio Riserva" samples comparted to the others, as can be also observed from **Figure 28**.



(b)

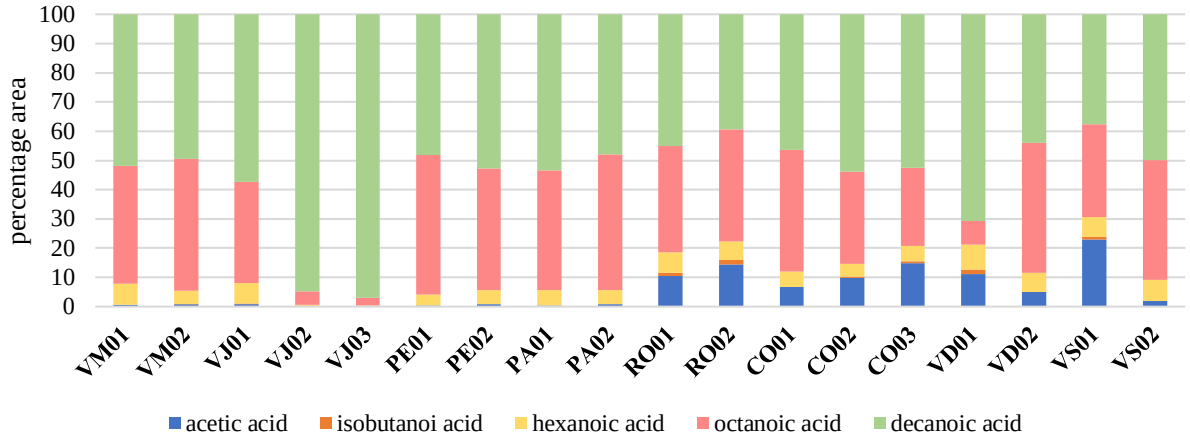


Figure 28. (a) Absolute percentage composition of acids in the DOCG wine samples (b) Relative percentage composition of compounds within the class of acids in the DOCG wine samples.

In the other samples it ranges from 2.6 to 14.9%. At the same time in the same two wine samples and it was found to be generally more abundant in white wines that in red ones (with mean values of 19.2 and 5.9%, respectively). For acetic acid the trend was the opposite, with higher percentages in red wines. The second most abundant acid was octanoic acid, with a range 2.6-12.6% and that did not show a particular pattern among the samples. Octanoic and decanoic acids are usually associated to waxy and oily scents when present in concentrations higher than their relative OTHs. Acetic acid showed its higher abundance in a sample of "Vernaccia di Serrapetrona" dry (VS01), where it was present in a significant different way when compared to the other samples (P <0.05).

Together with ethyl esters, other minor esters were also detected and the percentages within the class are reported in **Figure 29**.

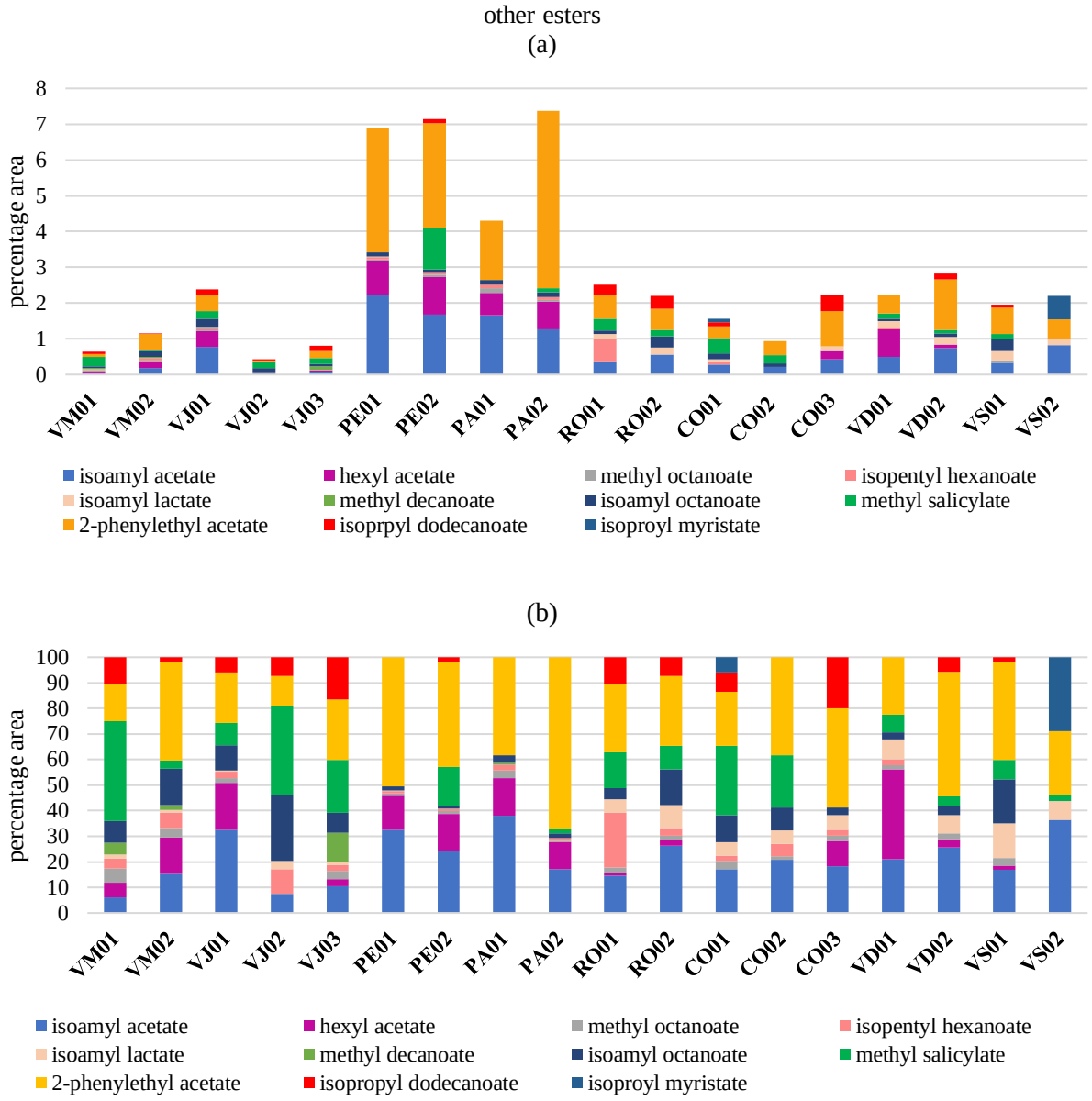


Figure 29. (a) Absolute percentage composition of "other esters" in the DOCG wine samples (b) Relative percentage composition of compounds within the class of "other esters" in the DOCG wine samples.

A total of eleven esters different from ethyl were detected in the range 0.6-7.4%, thus composing a relatively small percentage of the volatile fraction.

The highest percentages were observed only for four wine samples which, interestingly, belong to the same wine typology and production geographical area (PE01, PE02 and PA01, PA02). Interestingly, among these four samples, methyl salicylate was only detected in one sample (PE02).

Differently from the other classes of compounds considered by now, in this case the composition is variable among the samples, without showing a trend between samples belonging to the same DOCG.

For white Offida DOCGs 2-phenyl acetate was found to be the most abundant ester (with the highest abundance in the sample PA02, (P <0.05), showing a percentage area of 3.5, 2.9, 1.7 and 4.9% respectively, and lower in the others (in the range 0.05-1.6%). This compound is usually associated to floral fragrances, with honey or pear notes. These samples were also characterized by the higher values for isoamyl acetate (that can contribute with a typical banana aroma) and hexyl acetate (associated to green apple scents) with respect to the other samples (P <0.05).

Other compounds were instead found only in few samples. Isopentyl hexanoate, for example, characterized by fruity/pineapple scents, was found with a very small percentage area (0.65 %) in one sample of "Offida Rosso" (RO01), even if in a significant higher amount with respect to the other wine samples (P < 0.05). The same can be underlined for isopropyl myristate in one sample of "Vernaccia di Serrapetrona" (VS02), which showed the highest percentage (of only 0.66 %) compared to the other samples (P <0.05).

Differently from the previous compounds, which are formed as by-products of yeast metabolism during alcoholic fermentation, terpenes are varietal aromas whose presence is associated to the grape variety where they are already present as non-odorant precursors. Moreover, these compounds are associated to very distinct and characteristic aromas.

The vines used to produce DOCGs wines in Marche region (Verdicchio, Pecorino, Passerina, Montepulciano and Vernaccia Nera) belong to the class of neutral vines, hence to grape varieties that do not contain relevant quantities of varietal aroma and indeed. This can explain the very low percentage areas in which the analytes were found in this class of compounds.

Anyway, five different terpenes were detected and identified in the DOCG wines investigated, with a high variability among the samples (**Figure 30**).

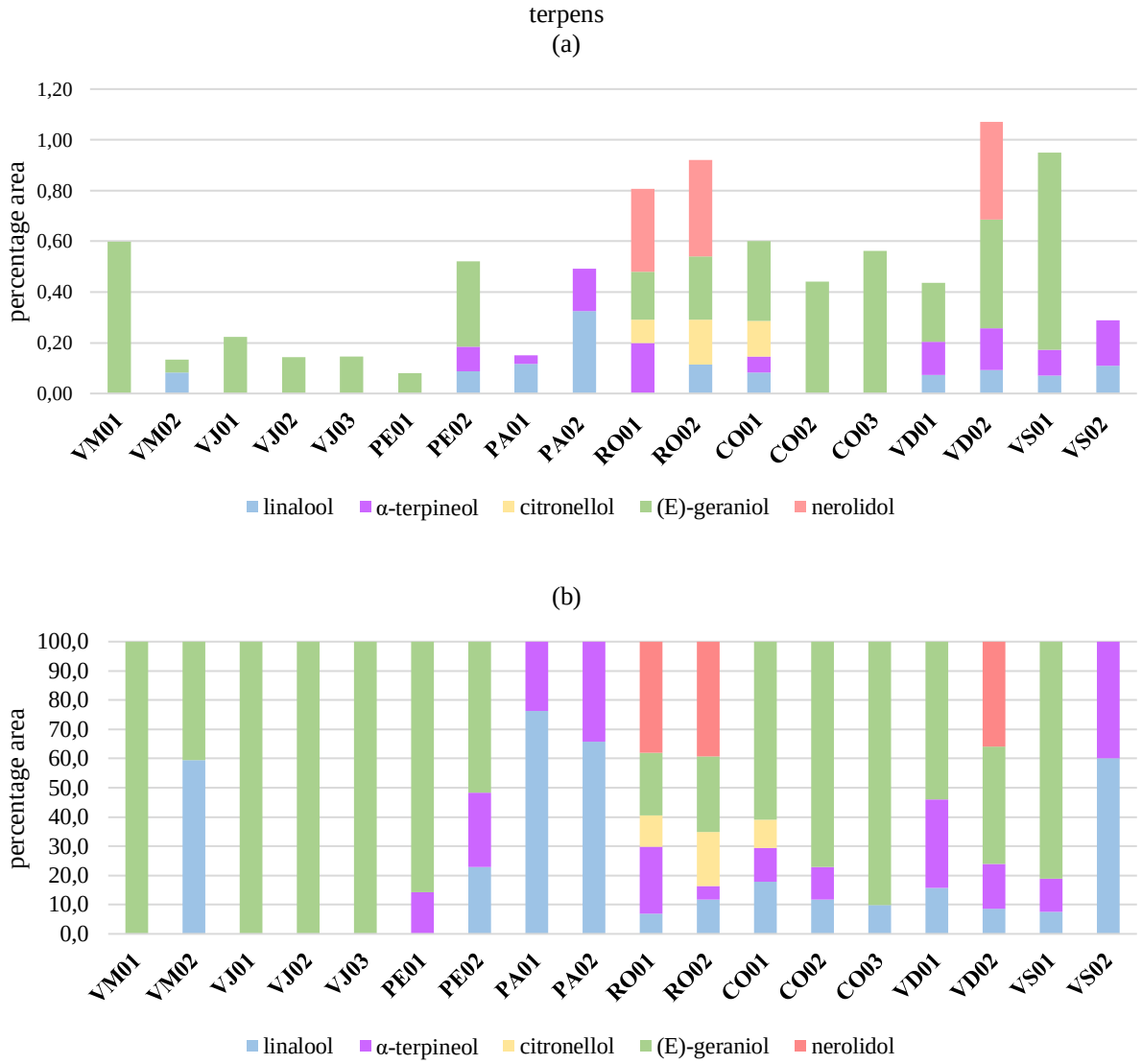


Figure 30. (a) Absolute percentage composition of terpenes in the DOCG wine samples (b) Relative percentage composition of compounds within the class of terpenes in the DOCG wine samples.

Beyond their poor levels in the investigated DOCGs, some relevant differences raised between white and red wine samples, the second being generally more enriched in terpenes in terms of both total abundance and number of compounds. Beyond this difference, every sample seems to have a unique terpenes composition apart from few samples, such as PE01 and the samples of "Castelli di Jesi Verdicchio Riserva" (VJ01, VJ02 and VJ03) which showed very similar amount for I-geraniol, the only terpen detected for these samples. On the other hand, compounds such as nerolidol or citronellol were found only in few samples. Interestingly, while citronellol was found in RO01, RO02 and CO01 samples, which are wines made with the same grape variety (Montepulciano, in percentages higher than 85%), nerolidol was found in two samples only, still made with Montepulciano (RO01 and RO02) and one sample of "Vernaccia di Serrapetrona" sweet (VD02)

which is made with Vernaccia Nera grape variety. The on-way analysis of variance, coupled with Tukey's test for pairwise comparison, pointed out that the sample of "Offida Passerina" PA02 was the one exhibiting the highest amounts of linalool, when compared to all other samples.

Finally, the class of other compounds ("others") was made up of only 3 analytes (hexadecane, 4-hydroxy-4-methylpentanone and furfural) and reported in **Figure 31**.

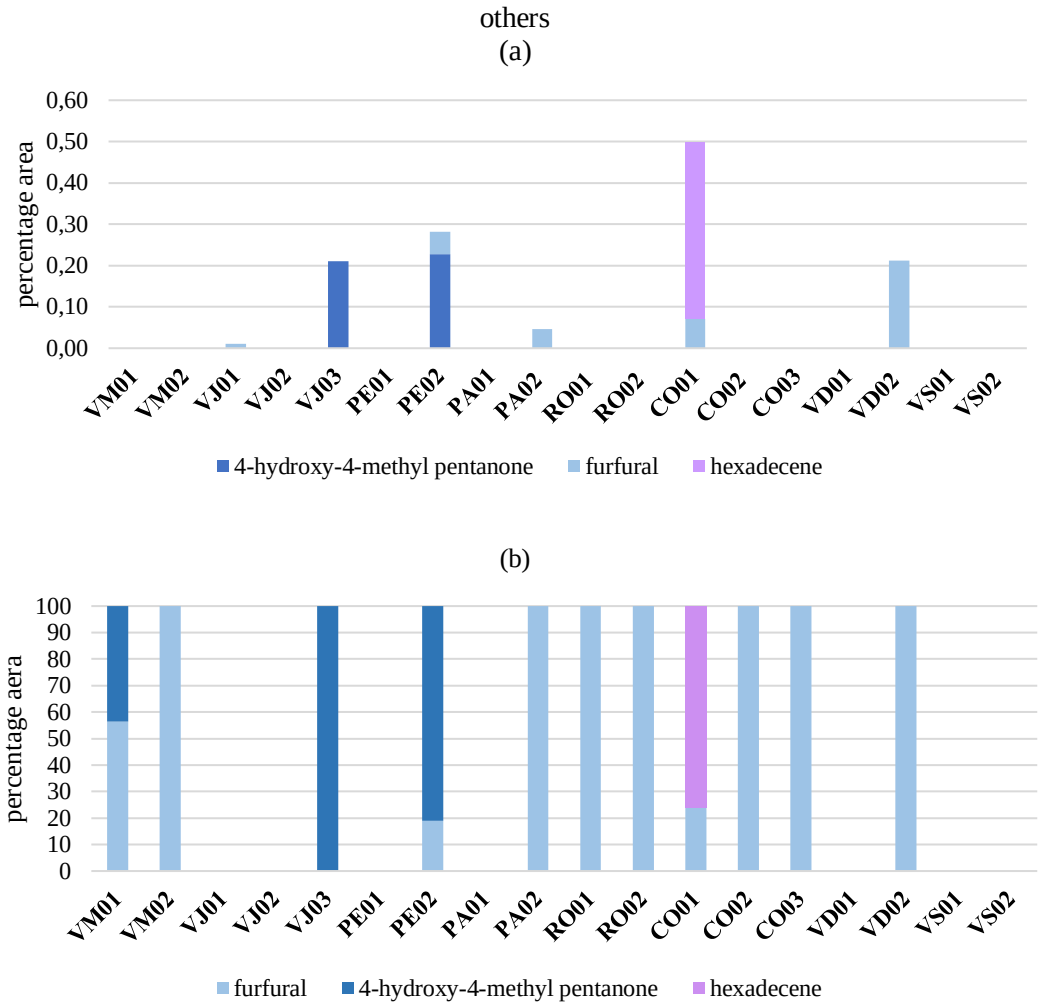


Figure 31. (a) Absolute percentage composition of "other" in the DOCG wine samples (b) Relative percentage composition of compounds within the class of "others" in the DOCG wine samples.

These compounds were present in very low amounts and in some wine samples their were not detected at all (VJ01, VJ02, PA01, VD01, VS01 and VS02). Hexadecene was only present in one sample of "Cònero" wine (CO01, P <0.05). Furfural is a compound released by toasted staves and it was detected as the most abundand in the class of "other compounds" in one sample of "Vernaccia di Serrapetrona" sweet (VD02, P <0.05) (Blanchard *et al.*, 2001).

The compositional data reported clearly pointed out that while for some compounds a general different trend was observed between wine typologies or white and red wines, other volatiles are present only in few samples or they were present with significant different quantities. These substances may have an important role in the authenticity and could also impact the wine aroma characterizing them in a unique way.

"Verdicchio di Matelica Riserva" wines, for example, were characterized by two different major compounds, being ethyl decanoate for VM01 with a percentage of 67.5% and ethyl octanoate for VM02 with a percentage of 68.2%. VM01 was the sample containing ethyl decanoate in the highest amount. Ethyl decanoate was also present as major compound in "Castelli di Jesi Verdicchio Riserva" (VJ01) even if with a much lower percentage of 37.3%.

Some works were reported concerning wines produced with Verdicchio grape variety. Carlin *et al.* (2019) for example, performed a study focused on the aroma complexity of Verdicchio wines on samples of "Castelli di Jesi Classico", differing for harvest years and production areas. They investigated the correlation between some volatile compounds and determined sensorial attributes also by gas-chromatography-olfactometry (GC-O) (Carlin *et al.*, 2019). Interestingly, they were able to detect the presence of methyl salicylate, a compound associated to the distinct balsamic, anise and liquorice notes characteristic of this non-aromatic grape variety, as earlier reported by Versini *et al.* (2005). This is quite in accordance with our results. Indeed, this compound was detected in 2 samples, both of Verdicchio wines (VM01 and VJ01), and it was present in a statistically significant higher amount (P <0.05) with respect to the others. Moreover, it was detected in all samples of Verdicchio wines, while, in other samples (such as PE01, PA01, CO03 and VS09) it was not detected at all. At the same time, it was also detected in one of the two samples of "Offida Pecorino" (PE02), in a relatively high amount.

Concerning "Offida Pecorino" and Passerina, the two samples of "Offida Pecorino" (PE01 and PE02) and the two of "Offida Passerina" (PA01 and PA02) showed a similar trend in terms of volatile composition. Indeed, in all the four samples, the most abundant compound was ethyl octanoate, followed by ethyl decanoate. They were also characterized by a relatively high percentage of the corresponding acids. The two wine typologies were the subject of study of Biancolillo *et al.* (2022) which performed a study with the aim to discriminate between Trebbiano d'Abruzzo Pecorino and Passerina monovarietal wines produced in Abruzzo region. The most abundant compounds in terms of percentage areas for Pecorino and Passerina wines were 3-methyl-1-butanol, ethyl hexanoate, ethyl octanoate (with the highest percentages) and ethyl decanoate. In the same way, also

for DOCG wine samples under investigation, the most abundant compounds were the esters and in particular ethyl octanoate, followed by ethyl decanoate and hexanoate.

Concerning red samples, "Cònero" and "Offida Rosso", there is still a lack in literature concerning the study and characterization of volatiles for these wines of Marche region. At the same time, studies were performed on wines made with the same grape variety, Montepulciano. Hence, some observations can be done based on same varietal wines. In 2012 an interesting study aimed to distinguish between monovarietal Montepulciano wines produced in Marche and Abruzzo regions, based on their volatile and phenolic profile (Sagratini *et al.*, 2012). Results showed that esters were the most abundant compounds, as obtained also for DOCG wine samples under investigation. The most abundant compounds obtained for the wines produced in Marche region were ethyl-9-decenoate, ethyl hexanoate, ethyl decanoate and ethyl octanoate and the winey and fresh aroma profile of these wines was attributed to these compounds. This is quite in accordance with the results obtained for the DOCG wine samples under investigation since they were characterised by a higher amount of ethyl hexanoate, octanoate and decanoate, together with diethyl succinate and ethyl hydrogen succinate. The principal class of compounds that seem to discriminate between these red wine samples appear to be terpenes, since the other major compounds does not point out relevant differences.

Finally, the volatile fraction of "Vernaccia di Serrapetrona" sweet was the subject of study of Fiorini *et al.* (2014). The study was performed though the HS-SPME-GC-FID/MS analysis on 22 samples from different vintages. In particular, eight volatiles were detected and quantified, being the most abundant. They were ethyl lactate, hexanol, 3-methylbutyl acetate, ethyl hexanoate, phenyl ethyl alcohol, diethyl succinate, ethyl octanoate and ethyl decanoate. The results are quite in accordance with the ones obtained by the analysis of DOCG wine samples here investigated. In fact, the samples seem to be characterized by a high percentage areas of ethyl octanoate, ethyl decanoate and diethyl succinate.

1.5.3 Principal component analysis

The PCA was performed to identify homologous groups of data (clusters), taking into consideration the 50 variables, hance all the identified volatile substances, and the 18 samples. The first two principal components were able to explain the 51.2% of the total variance among samples.

At first, the score plot in **Figure 32** was used to identify clusters, outliers and trends and thus, to identify samples with a similar volatile profile.

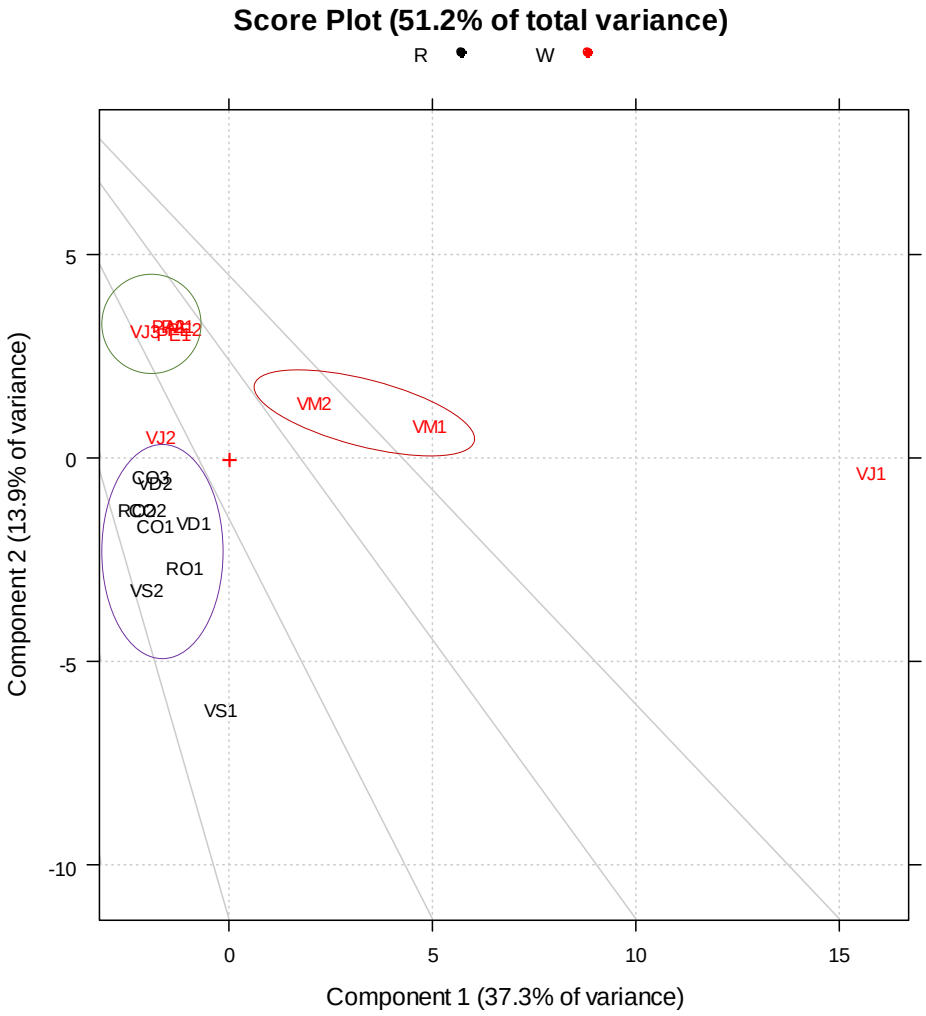


Figure 32. Principal component analysis and related score plot, obtained using as variable all DOCG wines investigated. The different colours distinguish white (in red) and red (in black) wine samples. VM: "Verdicchio di Matelica Riserva"; VJ "Castelli di Jesi Verdicchio Riserva"; PE: "Offida Pecorino"; PA: "Offida Passerina"; RO: "Offida Rosso"; CO: "Cònero"; VD: "Vernaccia di Serrapetrona" sweet; VS: "Vernaccia di Serrapetrona" dry. Violet, green and red clusters indicate the different groupings of wine samples.

The plot underlined the presence of different clusters, even if they were relatively close each other. Starting from the Offida white wines (PE01, PE02, PA01 and PA02) they were completely overlapped, together also with a "Castelli di Jesi Verdicchio Riserva" (VJ03) which formed a distinct cluster (green circle). For the other white samples, VM01 and VM02 due to their proximity can be considered as another cluster (red), while VJ01 showed a more peculiar behaviour, being far from all other samples. Red wines were closer, appearing as a unique cluster for both PC1 and PC2 (violet circle), with the only exception of a "Vernaccia di Serrapetrona" dry sample (VS01) that showed a distinguishing behaviour in the PC2 axis, even if relatively close to the other sample of same wine typology (VS02). Interestingly, one sample of "Castelli di Jesi Verdicchio Riserva" (VJ02) showed proximity to the cluster of red wines.

Finally, the biplot was used to assess which variable (volatile substance) had the largest effect on each component and to correlate the variables to the samples (**Figure 33**).

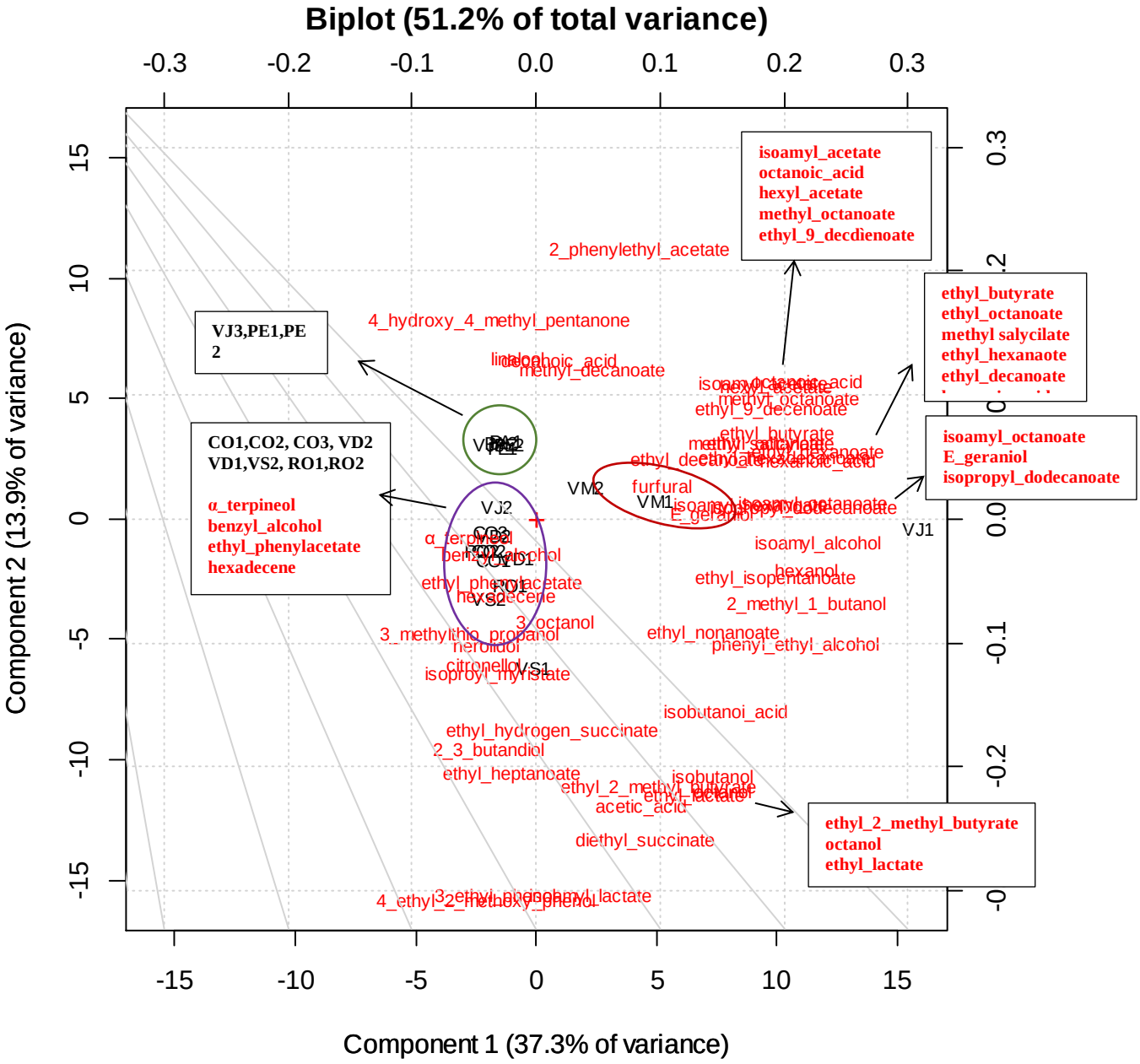


Figure 33. Principal component analysis and related biplot, obtained using all the analysed volatile substances (in red) as variables and the DOCG wines investigated (in black) as scores. VM: "Verdicchio di Matelica Riserva"; VJ "Castelli di Jesi Verdicchio Riserva"; PE: "Offida Pecorino"; PA: "Offida Passerina"; RO: "Offida Rosso"; CO: "Cònero"; VD: "Vernaccia di Serrapetrona" sweet; VS: "Vernaccia di Serrapetrona" dry.

By looking at the biplot obtained for all analytes detected, it can be underlined that some substances were the most important in explaining the variability among samples. These substances were the ones in the right part of the biplot, comprising most of the ethyl esters and other compounds

such as isoamyl alcohol or phenyl ethyl alcohol. The others were distributed around the zero, hence they were not relevant to explain the PC1 variability.

As expected, while the PC1 was able to discriminate depending on the wine samples, the second component was able to clearly distinguish between white and red wine samples as also supported by the ANOVA analysis. 2-Phenylethyl acetate, for example, was a compound significantly more abundant in white wines, compared to the red ones. Also, 4-hydroxy-4-methyl pentanone was observed only in two white wines (VJ03 and PE02). By contrary, isoamyl lactate, except for CO02, was detected only in red wines and 4-ethyl-4-methoxy phenol was only detected in few red wine samples.

Furthermore, the cluster comprising "Verdicchio di Matelica Riserva" DOCG samples was close to ethyl esters such as ethyl butyrate, ethyl hexanoate, ethyl octanoate and ethyl decanoate confirming that this DOCG seems to be characterized by a higher content of this class of compounds, as supported also by the ANOVA (**Table 13**).

At the same time, the different position of wines of the same typology along PC1 underlines that, even if they are produced relatively close and with the same grape variety, they were able to show a peculiar volatile profile.

1.6 Conclusions

A method for the solid-phase microextraction of volatile compounds from wine was optimized. It exploits the DI of a PDMS OC-fiber in the wine sample for 15 min, followed by HS-SPME for 15 min at a temperature of 35 °C, followed by the GC-MS analysis. In this way the huge number of substances, characterized by different volatilities can be investigated, filling the lack that the use of HS or DI only would cause. The optimized method was then applied for the volatile fraction characterization of 18 wine samples of the five DOCG wines produced in Marche region.

In order to study the results an initial investigation was performed by dividing the 50 detected and identified analytes into six chemical classes, namely ethyl esters, other esters, alcohols, acids, terpenes and others and the analysis of variance (ANOVA) was performed to study the different DOCGs chemical classes' percentage compositions.

This statistical analysis underlined some interesting peculiarities. Indeed "Verdicchio di Matelica Riserva" and "Castelli di Jesi Verdicchio Riserva", even if produced with the same grape variety (Verdicchio) were characterized by a very different profile, being the former more enriched in ethyl esters and the latter showing a more homogenous distribution of ethyl esters and volatile acids. By contrary Offida DOCGs, which comprise white ("Offida Pecorino" and "Offida Passerina") and red ("Offida Rosso") wines made with different vines, were characterized by comparable amounts of ethyl esters. White Offida DOCGs showed comparable profile also for the other chemical classes, being the class of volatile acids the second for abundance. By contrary, "Offida Rosso" showed a higher percentage of the class of alcohols, thus more in accordance with the profile of other red DOCGs wines and in particular to the one of "Vernaccia di Serrapetrona" DOCG. These differences were partially supported by the principal component analysis performed by considering all the compounds and all the wine samples, even if the total variance explained by the first two principal components is low.

Beyond the differences in the chemical classes composition of the DOCGs, some analytes were found to be more abundant in one DOCG (or sample) with respect to the others. These are analytes which may contribute to the characteristic flavours of the distinct DOCGs, if present above their OTHs. The method exploited for the determination of the volatile substances profile is qualitative, thus the concentrations in which these analytes are present in the samples is not determined. Anyway, it can be supposed that, due to the high percentage abundances of some compounds, they may actively participate to the wine aroma.

The DOCG “Verdicchio di Matelica Riserva”, for example, was overall characterized by high abundances of ethyl butyrate, ethyl octanoate and ethyl decanoate, compounds associated to yellow pulp fruits scents. “Castelli di Jesi Verdicchio Riserva” and white Offida DOCG (“Offida Pecorino” and “Offida Passerina”) showed more comparable composition even if “Castelli di Jesi Verdicchio Riserva” was characterized by a higher amount of decanoic acid, while “Offida Pecorino” and “Offida Passerina” of 2-phenyl ethyl acetate, isoamyl acetate and hexyl acetate, which are compounds associated to fruit or floral olfactory attributes. Concerning red DOCGs, “Offida Rosso” was found to be enriched in nerolidol but this DOCG showed also relatively high abundances of compounds such as isopentyl hexanoate, while “Cònero” of ethyl hydrogen succinate and diethyl succinate. Finally, in “Vernaccia di Serrapetrona” ethyl nonanote in the sweet typology was found with relatively high percentage abundances when compared to the other DOCGs.

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2: Development and validation of a new GC-FID method for the analysis of short and medium chain free fatty acids, and its application for the characterization of DOCG wines of Marche region

2.1 Introduction

2.1.1 Short and medium chain free fatty acids in wine

Short and medium chain fatty acids (SCFAs and MFCAs) are volatile organic acids containing from 2-6 and from 7-12 carbon atoms, respectively. They have the structure of fatty acids, but with shorter carbon chains that provides them with higher polarity, volatility and hence different properties compared to long chain fatty acids commonly constituting food lipids. These compounds are known to have an important impact in foods and in several biological samples and their variable amount and composition contribute to the characteristic flavour of some food, thus being in relation with their quality (Noronha *et al.*, 2008).

Fermentation processes, leading to the production of short and medium chain free fatty acids, have been exploited to increase the shelf-life of perishable food, but recently the scientific research has focused more on the beneficial effects of fermented foods and beverages. Indeed, emerging evidence suggests the positive effects on human health by their consumption, demonstrating that they are able to ameliorate several metabolic outcomes, such as glycemia, lipidemia and oxidative stress (Marazza *et al.*, 2013; Li *et al.*, 2016). Hence in this context, fermentation has been indicated as a tool to enhance the nutritional values of some foods and beverages, since it seems to increase bioavailability of bioactive compounds and to produce health-promoting end-products (Annunziata *et al.*, 2020).

In wine, the volatile acids that can influence the aroma are those showing low olfactory threshold levels, together with a relatively high concentrations in wine and also a sufficient volatility (Pérez Olivero, 2011). They are bio-synthetized during alcoholic fermentation by the action of both yeast and bacteria and they can arise from different origins. Acids such as propionic, isobutyric and isovaleric are produced by protein metabolism, while linear and saturated fatty acid that contain an even number of carbon atoms, such as hexanoic, octanoic and decanoic acids derive from the catabolism of long-chain fatty acids (Etiévant, 1991; Ribéreau-Gayon, 2002).

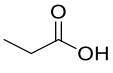
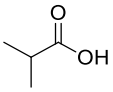
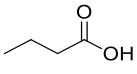
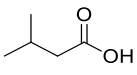
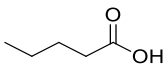
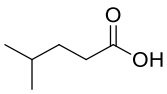
The final concentration of short and medium chain fatty acids in wine can be very variable since it depends on different factors. For example, Torija *et al.* (2003) reported a very interesting study assessing the correlation between the fermentation temperature on the yeasts cell fatty acid composition and the final presence of volatiles in wine. They observed that the action of yeast was

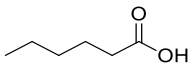
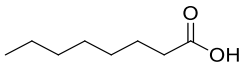
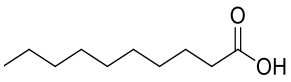
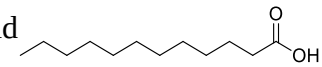
greatly influenced by environmental factors which could modify their plasma membrane and consequently their metabolism, concluding that the concentration of medium chain fatty acids was higher at lower fermentation temperatures.

Moreover, the presence of some free fatty acids was found to be critical during fermentation processes. In fact, when hexanoic, octanoic and decanoic acids were present in concentrations magnitude of mg/L they were able to inhibit fermentation by the inhibition of yeast metabolism and hence, they are directly involved in the interruption of fermentation processes during the production of certain sweet wines. By contrary, long-chain fatty acids (such as oleic and linoleic acids) act as fermentation activators, especially when in anaerobic conditions (Ribéreau-Gayon, 2002). Some authors also reported the importance of medium chain fatty acids, and in particular of C6-C12, in the foam formation for sparkling wines (Gallart *et al.* 2002; Kemp *et al.*, 2019).

When short and medium chain fatty acids are present at concentration equal or below their sensory thresholds, they can contribute to the aroma with the so-called complexity of wine *bouquet*. On the other hand, when in higher concentrations, they may contribute negatively with their characteristic unpleasant flavours associated to vinegar-like aroma, in the case of acetic acid, and with buttery, fatty and goaty scents, for the other volatile acids (Jackson, 1994). The names, chemical structures, and some physicochemical and sensorial attributes for SCFAs and MCFAs are listed in **Table 1**.

Table 1. Principal short and medium chain free fatty acids, physicochemical characteristics and sensory attribute.

Traditional name	IUPAC name (abbreviation)	Structure	Boiling point (°C) (P=1 atm)	Olfactory threshold (mg/L)	Sensory attribute
acetic acid	ethanoic acid (C2)		118	200[a]	vinegar
propionic acid	propanoic acid (C3)		141	20[b]	pungent, rancid
isobutyric acid	2-methylpropanoic acid (*i*C4)		155	0.23[c]	rancid, butter, cheese
butyric acid	butanoic acid (C4)		164	0.17[c]	cheese, pungent
isovaleric acid	3-methylbutanoic acid (*i*C5)		177	0.03[d]	cheese, rancid, sweety
valeric acid	pentanoic acid (C5)		186	3	unpleasant
isocaproic acid	4-methylpentanoic acid (*i*C6)		200	-	goaty, unpleasant

caproic acid	hexanoic acid (C6)		206	0.4[a]	goaty, cheese, fatty
caprylic acid	octanoic acid (C8)		237	0.5[e]	cheese, goaty, soapy
capric acid	decanoic acid (C10)		269	1[f]	rancid, fatty
lauric acid	dodecanoic acid (C12)		298.9	-	soapy, waxy

[a]Guth, 1997; [b]Kelebek & Selli, 2011; [c]Revel *et al*., 1999; [d]Antalick et al., 2012; [e]Izquierdo-Cañas *et* al. 2008; [f]Delequis *et al.*, 2000.

Beyond the negative olfactory attributes that are associated to short and medium chain free fatty acids, the presence in wine of a significant amount of ethanol, bring to the chemical formation of the relative ethyl esters, originated by the alcoholysis of the corresponding acids, activated as acyl-S-CoA by yeasts. Acetic acid in the same way can also form several acetate esters. These esters, by contrary, give to wine very pleasant fruity/floral attributes and indeed, some authors have found a positive correlation between the presence of these free fatty acids and wine quality (Ribéreau-Gayon, 2002). Bertuccioli *et al.* (1983), for example, found a positive relation particularly between hexanoic, octanoic and decanoic acids in the quality of white wines, assessed and rated by a tasters panel. Also, Marais & Pool (1980), reported a study that confirmed the positive correlation for hexanoic and octanoic acids and the corresponding ethyl esters in Chenin blanc, Colombar and Riesling wines.

While ethyl esters of straight-chain fatty acids containing an even number of carbons (i.e. hexanoic, octanoic and decanoic ethyl esters) and some fusel alcohol acetates (2-methylbutyl, 2-phenylethyl or isopentyl acetates), were immediately found to be important contributors to wine aroma in young wines, ethyl esters of branched-chain fatty acids, which were previously considered as negligible contributors to wine aroma, are now recognized as important odorants (Aznar *et al.*; 2001). The odour descriptors of the most significant ethyl esters originated from S and MCFAs are listed in **Table 2**.

Table 2. Odor description of SCFAs and MCFAs ethyl esters found in Rioja wines (Aznar *et al.*, 2001).

Compound	Odor description
ethyl isobutyrate	fruity
ethyl butyrate	fruity, peach
ethyl isovalerate	sweet, fruit
ethyl pentanoate	green, mint
ethyl hexanoate	fruity, apple

ethyl octanoate	sweet, fruity, pineapple

These premises show the importance of the analysis of short and medium chain free fatty acids and why the development of new analytical methods providing improvements for the quantification of these analytes, could be useful to better understand wine quality.

2.1.1.1 The importance of acetic acid

The acidity of wine can be described as the combination of non-volatile (or fixed) and volatile acidity. Fixed acidity is given by non-volatile acids, such as malic, tartaric, citric, succinic and lactic acids, while the volatile acidity arises from all the acids having low boiling points (comprising also carbon dioxide or sulfur dioxide) where acetic acid plays a fundamental role, being the most abundant (Zoecklein *et al*., 1990). Indeed, the volatile acidity is expressed in terms of acetic acid g/L.

As the other SCFAs and MCFAs, acetic acid is produced by yeasts during alcoholic fermentation, but it can be produced also by lactic acid bacteria during the eventual malolactic fermentation, at low concentrations (below its sensorial threshold).

Precise limits are set by the normative, concerning the maximum quantity of acetic acid that can be found in wine, with different values for white and rosé (18 mEq/L, corresponding to 1.08 g/L) and red wines (20 mEq/L, corresponding to 1.2 g/L) (Regulation (CE) N.606/2009). If present in concentrations above these limits it is generally considered to negatively affect their sensorial quality with the characteristic vinegar off-flavour.

This is associated to wine spoilage, as the results of the presence and uncontrolled proliferation of acetic acid bacteria (AAB), microorganisms belonging to the *Acetobacteraceae* family that are well adapted to resist to various sugar and ethanol environments. They act by efficiently converting ethanol into acetic acid, through acetaldehyde, reaction that is catalysed by two different membrane-bound enzymes: alcohol dehydrogenase (ADH) for the first step and acetaldehyde dehydrogenase (ALDH) for the second (**Figure 1**).

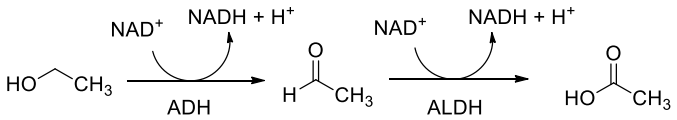


Figure 1. Conversion of ethanol into acetic acid. ADH and ALDH are the membrane-bound enzymes. ADH: alcohol dehydrogenase and ALDH: acetaldehyde dehydrogenase.

The intermediate acetaldehyde can also contribute to spoilage sensorial characteristic by imparting sherry-like bruised apple and/or nutty aromas, with a low threshold level of 0.5 mg/L (Francis & Newton, 2005).

When wine has been spoiled by AAB, it presents the characteristic vinegar-like sourness on the palate while the aroma consists of a loss of fruity character and an enrichment of unpleasant acetic, nutty, sherry-like, solvent flavours (Bartowsky *et al.* 2003). The defect can sometime be seen as the characteristic ring of bacterial biomass in the bottle neck at the wine/air interface (**Figure 2**).

Figure 2. Bacterial ring-shaped deposit in the bottle neck at the interface between tine and headspace (Bartowsky & Henschke, 2008).

However, wine can undergo AAB spoilage, not only in the final bottled product, but also in the previous vinification steps (Drysdale & Fleet, 1988). In fact, physical damages or fungi infection on grapes can be infected by AAB, but the infection can also occur subsequently in grape must and during stuck fermentation, due to air exposure and/or during storage.

2.1.2 Methods for the analysis of SCFAs and MCFAs in wine

The analysis of short and medium chain free fatty acids from different matrices can be performed by using techniques such as high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), nuclear magnetic resonance (NMR) or gas-chromatography (GC) (Zeppa *et al.*, 2001; Arellano *et al.*, 2000; Jacobs *et al.*, 2008). Anyway, GC is among the most used, due the volatility of the analytes.

Before the injection of a sample into the analytical instrument a pre-treatment is usually performed to purify, isolate and concentrate the analytes of interest. This is an important step since a simple filtration, dilution or centrifugation do not always allow to remove impurities which may cause irreversible column contaminations, shortening its life span. When taking into consideration the complex matrix which is wine, different extraction and purification techniques are reported in literature, with the choice of one technique over the other being dictated by the different physicochemical properties of the analytes of interest (volatility, solubility in organic phase, ability

to be absorbed on materials, and others). Thus, different extraction techniques can be classified according to analytes properties as reported in **Table 3** (Marìn-San Roman *et al*., 2020):

Table 3. List of some extraction techniques divided depending on the exploited analyte properties (Marìn-San Roman *et al*., 2020).

Property	**Extraction technique**
Solubility	liquid-liquid extraction (LLE)
	solid-phase extraction (SPE)
Volatility	distillation
	head space
	purge and trap
Absorption	purge and trap
	SPE
	solid-phase microextraction (SPME)
	stir bar sorptive extraction (SBSE)
	solid-phase dynamic extraction (SPDE)

The LLE is based on the different solubility of analytes between the aqueous and a water-immiscible organic solvent. The schematic representation of LLE is shown in **Figure 3**.

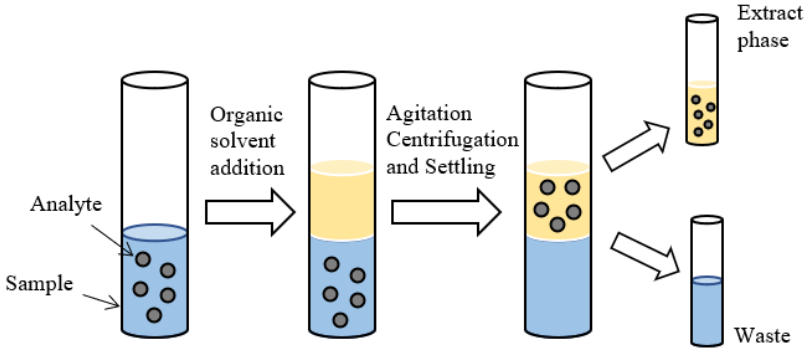


Figure 3. Schematic representation of LLE (Soares *et al*., 2021).

This extraction technique has some important advantages such as the possibility to extract compounds with different polarities and volatilities. At the same time, it presents some disadvantages, arising from the large amount of solvent usually necessary, making this technique non environmentally friendly (**Table 4**).

Table 4. List of some advantages and disadvantages of LLE.

Advantages	Disadvantages
Widely known	Low reproducibility
	Low selectivity
Extraction of compounds with different polarity and volatility is allowed	Analyte loss
	Difficult automatization
	Large amount of organic solvent
Low sample volume	High environmental pollution
	High cost
	Danger for workers

This extraction technique has been reported to be a valid method for short and medium chain free fatty acids due to their different polarities, permitting to use different solvents or mixture of solvents. Moreover, the obtained extract solution can be directly used for the quantitative analytical measurements in gas or liquid chromatography, without the need of desorption steps that are necessary in extraction techniques such as SPE or SPME (Cantwell & Losier, 2002).

Concerning the choice of extraction solvent, this is an important parameter that needs to be well evaluated, since it may affect in a sensible manner the extraction of the analytes when they have different polarities and partition coefficients. Reported studies suggest that dichloromethane is not the best choice for wine, due to poor recoveries for acetic acid in particular (Perestrelo *et al.*¸ 2006). Diethyl ether, by contrary has been widely exploited for the extraction of volatile fatty acids from different matrices (Sheveleva & Ramenskaya, 2010; Manny & Caron, 1995; Siedleckas *et al.*, 2007; Garcia-Villalba *et al.*, 2012).

One of the first studies concerning the LLE applied for the extraction and analysis of medium chain free fatty acids (in particular, C6-C10) in wine, investigated samples from Japan and from other countries, performing LLE with a mixture of ethyl acetate-*n*-pentane (2:1, v/v), followed by GC analysis (Shinohara, 1985). Calleja & Falqué (2005) reported a study on the volatile composition, including volatile acids, of two Mencìa wines with different "Appellation of Origin Controlled" (AOC) to assess possible significant differences, performing the LLE using a mixture of diethyl ether-hexane (1:1, v/v) followed by GC-FID analysis.

Gallart *et al.* (1997) performed the quantification of C6-C18 acids and their related ethyl ester, by extracting the analytes through LLE with hexane, evaluating the effect of the presence of fatty acids in bound or free form on sparkling wines foaming.

Furkídová, Makyšová, & Špánik (2017) reported the quantification of the major volatile alcohols, esters and acids in white Gewürztraminer wines treated with three different yeast strains. The extraction was performed through LLE with hexane followed by GCxGC-TOF-MS analysis. Another work was reported by Ivanova *et al.* (2012) for the analysis of aroma compounds, including octanoic

and decanoic acids, in red wines made with Kékfrankos grape variety, produced in Uruguay. The extraction was performed by LLE using dichloromethane as solvent and analysing the extract through GC-MS.

2.1.3 Aim of the work

Due to the importance of short and medium chain free fatty acids as contributors to define wine quality and to the limited number of works reporting methods specifically developed for their quantitative GC analysis, the scope of this work was to optimize and validate a new method for the direct analysis of acids from acetic to octanoic in wine, avoiding any step of derivatisation.

For this purpose, the LLE was exploited by investigating both the number of extractions and the impact of different salts as salting out agents. The extract was then analysed by GC coupled with a flame ionization detector (GC-FID). After the development and the validation of the method, it was applied for the analysis of 18 different DOCG wines produced in Marche region, with the aim to characterised them in terms of short and medium chain free fatty acids composition.

The odor active value (OAV) is also an important parameter when the analysis and quantification of volatile compounds in wine is performed. It estimates the contribution of each compound to the entire wine aroma. Particularly, when a compound has an OAV ≥ 1 it is considered to actively participate in the overall olfactory perception (Gil *et al.*, 2006). At the same time, it is important to keep in mind that these values serve as a first approximation to the potential contribution of a compounds to the total aroma, since it does not consider the depressive or synergic odor effects that result from the interaction of the huge number of compounds present in wine. Hence, after the application of the method for the quantification of short and medium chain free fatty acids in the 18 DOCG wine samples, the relative OAV values for each compound were also calculated, to obtain a first approximation of the role that these analytes could play in the aroma of the wine samples under investigation.

This preliminary study regarding the method development, that will be presented in the next paragraphs, has been recently published (Lenti, L.; Nartea, A.; Orhotohwo, O.L.; Pacetti, D.; Fiorini, D. “Development and Validation of a New GC-FID Method for the Determination of Short and Medium Chain Free Fatty Acids in Wine” *Molecules* **2022**, *27*, 8195. Available at: https://doi.org/10.3390/molecules27238195).

2.2 Materials and method

2.2.1 Reagents and standards

The analytical standards acetic acid (C2, purity ≥ 99%), propionic acid (C3, purity ≥ 99,5%), isobutyric acid (*i*C4, purity ≥ 99%), butyric acid (C4, purity ≥ 99%), isovaleric acid (*i*C5, purity ≥ 98%), pentanoic acid (C5, purity ≥ 99%), isohexanoic acid (*i*C6, purity ≥ 98%), hexanoic acid (C6, purity ≥ 98%), octanoic acid (C8, purity ≥ 98%), decanoic acid (C10, purity ≥ 98%), NaH_2PO_4 and $(NH_4)_2SO_4$ were purchased by Sigma Aldrich. Ethyl ether was purchased by J.T. Baked (Phillipsburg-New Jersey – USA), sulfuric acid and NaCl were purchased from Carlo Erba (Milan-Italy). The water used was deionized (resistivity above 18MΩ cm). Standard stock solution for every analyte was prepared at different concentration by dissolving pure analytes standards (200 µL for C2 and 10 µL for C3-C8) in diethyl ether in 10 mL volumetric flask. The mother stock solutions were then used to prepared mix standard solution, by performing proper dilutions. The IS stock solution at a concentration of 170 ppm (µL/L) was prepared by dissolving 170 µL of the 10.000 µL/L mother stock solution in diethyl ether in a 10 mL volumetric flask.

All stock solutions were stored at 4 °C until their use.

2.2.2 Wine model

A wine model was prepared to build calibration curves with the standard additions method and to perform the recovery tests. The model was an hydroalcoholic solution prepared according to a previous work (Lenti *et al.*, 2021). Hence, it was prepared by dissolving absolute ethanol (14.16 mL), glycerol (1 g), D-tartaric acid (0.531 g), D-glucose (0.26 g) and D-fructose (0.26 g) in deionized water in a 100 mL volumetric flask.

2.2.3 Procedure for the extraction of short and medium chain fatty acids

In a 2 mL vial, a wine sample of 0.5 mL and 0.6 g of salt NaH_2PO_4 (enough to reach complete saturation) are added and vigorously mixed with a vortex device for 1 min. The sample is added with 25 µL of internal standard solution (C5 dissolved in ether, at 170 µL/L) and then 0.4 mL of diethyl ether are added for the extraction. The sample is then stirred again with the vortex device for 3 min and the two layers are separated with the help of a centrifuge (5000 rpm) for 5 min. The upper organic phase is collected and injected in the GC-FID with a volume of 1 µL.

Concerning the choice of valeric acid as internal standard, the absence of this analyte in wine samples was supported by the already mentioned study of Perez-Olivèro (2011), since even in this case the analyte was never detected. Also, Selli *et al.* (2004) performed a study for the analysis of volatiles from red wines obtained from a particular variety of *Vitis Vinifera* original from Turkey through the dichloromethane extraction and gas-chromatographic analysis (GC-FID, GC-MS and GC-O) and even in this case valeric acid was not detected et all.

2.2.4 GC-FID analysis

The chromatographic analysis was performed using an Agilent Technologies 6850 GC (Santa Clara, CA, USA) equipped with a split/splitless injector and coupled with a flame ionization detector (FID). The injection was performed in splitless mode (splitless time 3 min) with the injector temperature set at 280 °C. The carrier gas was hydrogen produced by a generator (PGH2-250 from DBS Analytical Instruments, Vigoza, Italy) and the initial hydrogen flow in the column was 2.50 mL/min. The capillary chromatographic column was a ultra-inert polyethylene glycol (PEG) column (DB-WAX UI, length 30 m, 0.25 mm i. d., 0.25 µm film thickness, purchased from Agilent Technologies, Santa Clara, CA, USA). The oven temperature was set at 40°C and maintained for 3 min, then raised at 10 °C/min to 210 °C and then at 40 °C/min to 245 °C and maintained for 0.3 min, for a final run time of about 20 min. The FID temperature was set at 250 °C. The identification of S and MCFAs in real wine sample was assessed by comparison of their retention times with reference standard solution and also confirmed by performing the analysis of standard mixture and a wine sample by GC-MS, using a 6890 N GC coupled with a 5973 N single quadrupole mass spectrometer detector (Agilent Technologies, Santa Clara, CA, USA).

2.2.5 Quantification and method validation

The validation of the method was performed by assessing different parameters, comprising linearity, repeatability, recovery and limits of detection (LOD) and quantification (LOQ). The linearity was assessed by calculating the linear regression coefficients (R^2) obtained from the calibration curves constructed with the method of standard additions in the wine model. Indeed, preliminary experiments suggested that a certain matrix effect was present and able to eventually bias the quantification results. For this reason, the method of standard additions was performed by spiking a wine model (described in Section 2.2.2), with different volumes (10, 20, 30, 40, 50 and 100 µL) of a standard mixture containing the analytes of interest at the appropriate concentrations (12.096 mg/L

for C2, 34 mg/L for C3, 17 mg/L for *i*C4, 106 mg/L for *i*C5, 30 mg/L for C6 and 60 mg/L for C8, in diethyl ether as solvent). Subsequently, repeatability was assessed by preparing and analysing 5 replicates of a wine sample within the same day and 5 replicates prepared in different days and expressed as percentage relative standard deviations (RSD %) obtained for each analyte. LOD and LOQ were calculated by considering the peak areas corresponding to 3 and 10 times the S/N ratio in standard mixtures containing the analytes at known concentrations.

Recovery was assessed by spiking a wine sample with a standard mixture solution containing all the analytes, in order to increase the concentration of each analyte of 100 or 200% of the average concentrations found in the samples. The sample was then extracted following the extraction procedure previously reported in section 2.2.3 and then analysed by GC-FID. Recovery was then calculated for each analyte by applying the equation 3:

$$\boldsymbol{Recovery}\ (\%) = \frac{(\boldsymbol{Css}-\boldsymbol{Cus})}{\boldsymbol{Cps}} \cdot \mathbf{100} \qquad (3)$$

Where:
Css = concentration of the spiked samples
Cus = concentration of the unspiked sample
Cps = concentration spiked to the sample.

2.2.6 Statistical analysis

One-way analysis of variance (ANOVA) followed by Tukey's test for pairwise analysis was used to submit data to assess significant differences ($P < 0.05$) between the different number of extractions and different extraction conditions applied to the samples. It was also applied to the results obtained by the application of the method for the analysis of the 18 DOCG wine samples, to assess significant differences in the volatile acids composition among samples. The software used for this purpose was PAST (Hammer, 2001). The heatmap correlating samples and analytes was obtained by using Rstudio software. The principal component analysis (PCA) was also performed to identify homogenous groups of data, by using R-based software CAT (Leardi *et al.*, 2021).

2.3 Results and discussion

In the present work, a new method for the LLE of short and medium chain free fatty acids, using ethyl ether was developed. Ethyl ether solution of the analytes of interest was analysed by GC-FID with splitless injection mode, to obtain a suitable sensitivity. The chromatographic column used

was a capillary column coated with polyethylene glycol but having high inertness (WAX UI). This column was preferred over the nitro terephthalic acids modified polyethylene glycol (FFAP), with same length, coating thickness and i.d., because the FFAP gave coelution of propionic acid and 2,3-budanediol. Both columns gave coelution problem of butyric acid with an unidentified compound.

The analytes that could be identified and quantified using the WAX UI column were acetic acid (C2), propionic acid (C3), isobutyric acid (*i*C4), isovaleric acid (*i*C5), hexanoic acid (C6) and octanoic acid (C8) (**Figure 4**). Their identity was confirmed also by performing the analysis in the same condition by GC-MS.

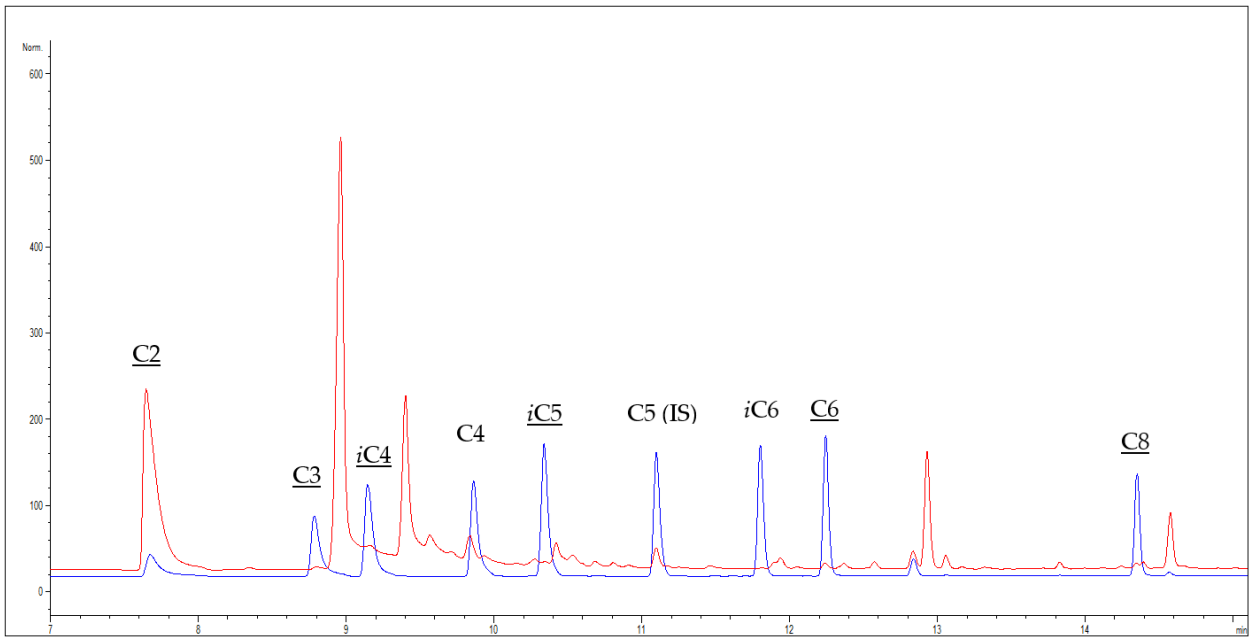


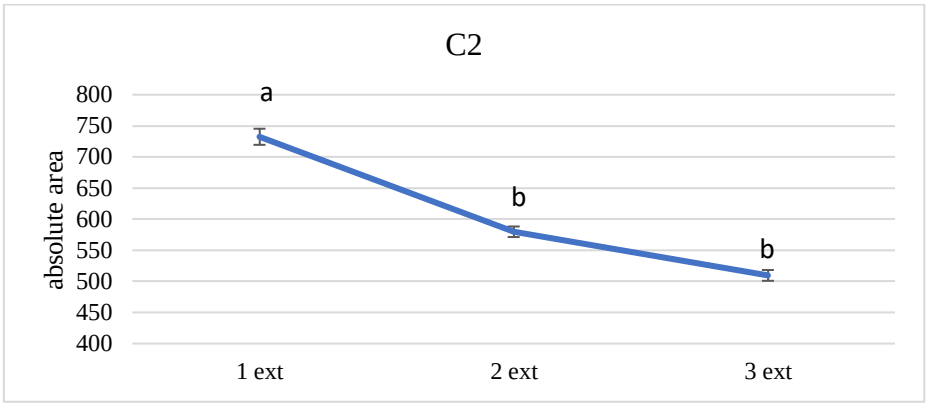
Figure 4. Overlapped chromatograms obtained by the GC-FID analysis of a wine sample (Nero d'Avola) containing the internal standard (IS) (red) and a standard mixture containing analytes C2-C8 at 100 µL/L (blue).

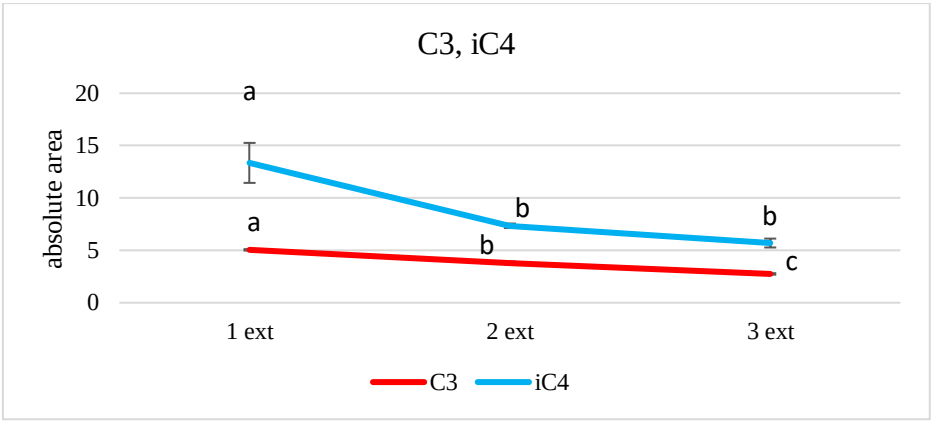
2.3.1 Optimization of the extraction procedure: extractions number evaluation

The number of extractions is an important parameter to be evaluated in LLE. Indeed, if the analytes of interest show great differences each other in terms of polarity, the ideal number of extractions could be different for the different analytes. In the case of short and medium chain free fatty acids, their solubility properties change greatly moving from acetic acid, that is soluble in water, to hexanoic or octanoic acids, that are insoluble in water. This means that their repartition between water and ethyl ether phases changes greatly moving from acetic acid to the longer chain homologous, decreasing significantly with the increase of carbon chain. Moreover, the difference in the repartition between acetic acid and propionic acid, in water or in the organic phase, is much higher than the one

between isovaleric and hexanoic acids, even if the carbon chain length difference is the same. This is due to the different impact of the polar carboxylic end in molecules having a shorter or a longer carbon chain tail. Consequently, a single extraction in diethyl ether could be enough for the less polar analytes (such as hexanoic or decanoic acids) while for the most polar (like acetic acid) it would give a much lower extraction extent and more than one extraction should be performed to enhance its repartition in the organic phase (Scortichini *et al.*, 2020). However, in the specific case under investigation, acetic acid is the most abundant volatile acid, thus it is possible that even with only one or two extractions, a sufficient sensitivity could be obtained for its quantification.

For this reason, the number of extractions to obtain a maximum sensitivity for most of the analytes, was assessed by performing 1, 2, or 3 subsequent extractions and results are shown in **Figure 5** and **Table 5**.





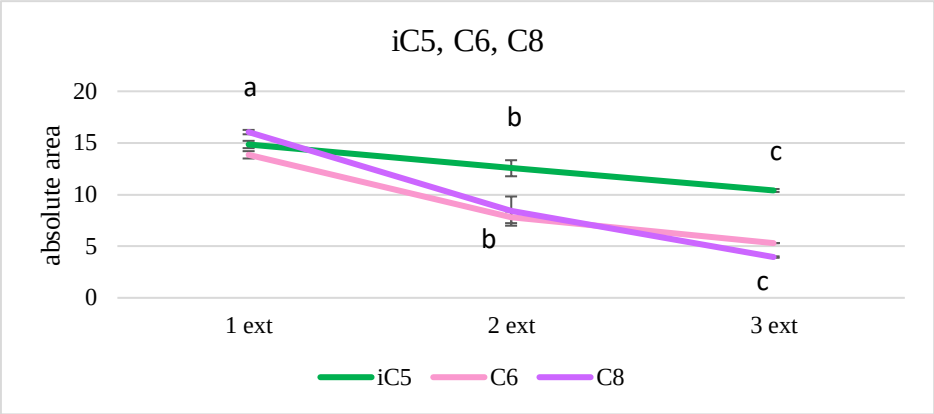


Figure 5. Quantity of short and medium chain free fatty acids extracted from a red wine samples (Nero d'Avola), in terms of peak area units, performing 1,2 or 3 subsequent extractions (ext). Bars indicate. ± standard deviations (*n*=2). Different letters indicate significant differences between the quantity obtained performing a different number of extractions (One-way ANOVA, $P < 0.05$, Tukey's test for pairwise comparison).

Table 5. Mean concentration (mg/L) ± standard deviation (*n*=2) obtained for each analyte from GC-FID analysis performing 1,2 or 3 subsequent ethyl ether extractions from a red wine sample (Nero d'Avola).

Compound	**1st extr**	**2nd extr**	**3rd extr**
acetic acid	493.7 ± 5.4^{a}	429.8 ± 3.6^{b}	400.4 ± 3.4^{c}
propionic acid	0.8 ± 0.0^{a}	0.6 ± 0.0^{b}	0.4 ± 0.0^{c}
isobutyric acid	2.5 ± 0.3^{a}	1.5 ± 0.0^{b}	1.3 ± 0.1^{b}
isovaleric acid	1.2 ± 0.0^{a}	1.1 ± 0.1^{b}	0.9 ± 0.0^{c}
hexanoic acid	2.4 ± 0.1^{a}	1.5 ± 0.1^{b}	1.1 ± 0.1^{c}
octanoic acid	3.3 ± 0.0^{a}	2.0 ± 0.2^{b}	1.3 ± 0.1^{c}

Letters indicate significant differences between the signals (in terms of peak area) obtained performing from 1 to 3 subsequent extractions (extr) (One-way ANOVA, $P < 0.05$, Tukey's test for pairwise comparison).

As expected, a significant decrease of the chromatographic signals was obtained for each analyte increasing the number of extractions from 1 to 2, showing that the second extraction does not provide an important additional recovery of the analyte. Thus, it was decided to proceed with a single extraction.

2.3.2 Optimization of the extraction procedure: the use of salts

To increase the extraction extent of the analytes of interest from the wine matrix, and thus to improve the sensitivity of the method, the addition of different salts was investigated, since their effect on the ionic strength of the solution can greatly affect the repartition of the analytes between the hydro-alcoholic and the organic phase. Indeed, the increase in the ionic strength reduces the solubility of organic compounds in the aqueous matrix, favouring their extraction with the organic solvent. As an example, it was used for the extraction of eight pesticides from water samples and in

the microextraction of volatile compounds from wine, with a significant an increase in the extraction extent (Sáenz-Barrio *et al.* 1996, 2000).

Salts and salts mixtures having high solubility in water and/or producing multiple charge ions were selected: NaH_2PO_4, $(NH_4)_2SO_4$, $(NH_4)_2SO_4/NaH_2PO_4$ (1:7.3) together with the most commonly used NaCl. These salts and salt mixture selection was based on previous study which demonstrated the capability of these salts to improve the headspace SPME of short and medium chain free fatty acids from different food and biological samples, comprising wine (Fiorini *et al.*, 2015). The extraction extent obtained with the mentioned salts with the increase factor compared to the extraction performed without salt, measured in terms of ratio between the signal of the analyte performing the extraction by using a salt, or salt mixture and without using any salt are shown in the **Table 6** and **Figure 6**.

Table 6. Extraction extent of SCFAs and MCFAs by liquid-liquid extraction with ethyl ether from wine (Nero d'Avola) using different salts, salts combination or in absence of salt. Results are reported in terms of concentration (mg/L) ± standard deviation (*n*=2) from GC-FID analysis.

Compound	**No Salt**	**NaCl**	**$(NH_4)_2SO_4$**	**$(NH_4)_2SO_4/NaH_2PO_4$**	**NaH_2PO_4**
acetic acid	490.2 ± 2.6^{a}	633.3 ± 3.7^{a}	742.0 ± 10.5^{b}	775.7 ± 1.3^{b}	777.7 ± 5.8^{b}
propionic acid	0.7 ± 0.1^{a}	1.3 ± 0.1^{a}	1.2 ± 0.1^{a}	1.3 ± 0.2^{a}	2.0 ± 0.3^{b}
isobutyric acid	2.4 ± 0.0^{a}	2.3 ± 0.0^{a}	2.4 ± 0.3^{a}	2.8 ± 0.1^{a,b}	3.5 ± 0.3^{b}
isovaleric acid	1.3 ± 0.1^{a}	1.2 ± 0.0^{a,b}	1.1 ± 0.0^{b}	1.1 ± 0.0^{b}	1.2 ± 0.0^{a,b}
hexanoic acid	2.5 ± 0.2^{a}	2.0 ± 0.2^{a}	2.3 ± 0.0^{a}	2.3 ± 0.1^{a}	2.2 ± 0.1^{a}
octanoic acid	2.9 ± 0.1^{a}	2.5 ± 0.2^{a}	2.8 ± 0.2^{a}	1.8 ± 0.2^{b}	2.7 ± 0.1^{a}

Letters indicate significant differences between the extraction extent for each analyte using different salts (One-way ANOVA, $P < 0.05$, Tukey's test for pairwise comparison).

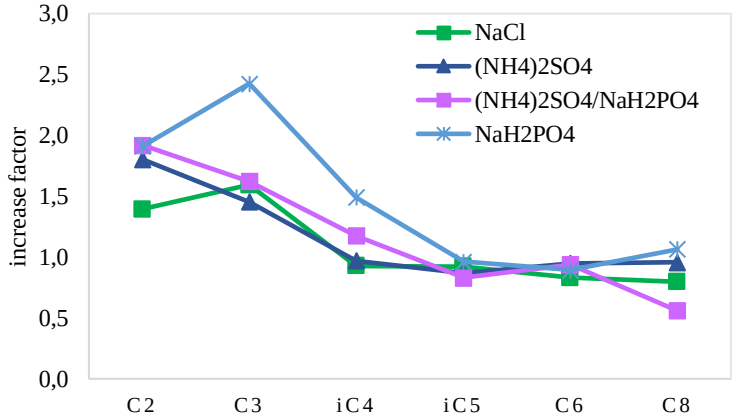


Figure 6. Increase factor calculated as the ratio between the mean peak area obtained from each SCFAs and MCFAs extracted from a wine sample using different salts and the mean peak area obtained without salt.

The addition of salts resulted to produce the maximum increase for two analytes in particular, being acetic and propionic acid, that were extracted 2-2.5 times more when using NaH_2PO_4. This was also the salt that provided the best overall performance with an average increase of 1.5, even if the improvement regarded only the lighter, more polar analytes. This can be explained considering that the lighter, and thus, more polar analytes have a much different repartition between the aqueous and ethyl ether phases, as compared to their homologous that have a longer chain and, consequently, they are more affected by changes in ionic strength of the aqueous phase. The mixture $(NH_4)_2SO_4/NaH_2PO_4$ produced a maximum increase of 1.9 for acetic acid and the average for all the analytes was 1.2, while NaCl produced a maximum increase of 1.7 for propionic acid and with the lower average increase of 1.1. Even if the result is not generalized for all the analytes, it was decided to use NaH_2PO_4, since it greatly improved the extraction of acetic, propionic, isobutyric and octanoic acid in a significant way (**Table 6**). The impact of salts hydrolysis (NaH_2PO_4, $(NH_4)_2SO_4$, $(NH_4)_2SO_4/NaH_2PO_4$) on wine pH was also assessed and monitored since these salts can affect the pH of a solution. Anyway, their effect was negligible in all the cases, allowing to maintain short and medium chain free fatty acids in their undissociated forms, thus extractable with the organic solvent used for the LLE (ethyl ether).

When comparing the results obtained with the LLE of S and MCFAs with the results from DI:HS SPME (**PART 1**) it can be observed how they are not completely in accordance. This is plausible since the two extraction techniques are very different and based on different principles. As a support to this, the comparison between LLE and SPME was recently assessed for the extraction of aroma compounds from rum by Zhang *et al.* (2021). They used three different SPME fibers (50/30 µm DVB/CAR/PDMS, 85 µm CAR/PDMS, 100 µm PDMS and 65 µm PDMS/DVB) to extract volatile for 30 min at 40 °C, while for LLE they performed three subsequent extraction using dichloromethane as extracting solvent, after diluting the sample with distilled water. Statistical analysis confirmed differences among the two methods and underlined how the SPME show lover LODs which implies that the technique is more suitable for qualitative analysis, while, by contrary, LLE is characterized by low RSD which means a good repeatability and accuracy, more suitable for quantitative and semi-quantitative analysis.

2.3.3 Method validation

Linearity was assessed by exploiting the method of standard additions to a wine model to reproduce the matrix effect that otherwise could bias the quantification results. A good linearity was

obtained in the range of concentrations of targeted analytes found in the samples, with R^2 values between 0.994 and 0.997, as reported in **Table 7**.

Table 7. Validation parameters.

Compound	Linearity range (mg L^{-1})	R^2	LOD (mg L^{-1})	LOQ (mg L^{-1})	Recovery (%)	Repeatability (%) (*n*=5)	
						Intraday	Interday
acetic acid	262.5-3024	0.997	0.51	1.70	96.8	2.5	8.5
propionic acid	0.5-5.9	0.995	0.04	0.14	86.4	0.4	2.1
isobutyric acid	0.5-4.3	0.999	0.06	0.19	96.6	1.6	1.6
isovaleric acid	0.5-4.1	0.996	0.04	0.13	81.9	0.0	1.1
hexanoic acid	0.6-7.4	0.997	0.05	0.18	101	2.9	0.5
octanoic acid	1.4-14.6	0.994	0.06	0.21	96	4.9	2.3

Limits of detection (LOD) values were obtained in the range of 0.04-0.51 mg/L, while limits of quantification (LOQ) were in the range 0.13-1.70 mg/L. These values were low enough to permit the quantification of the analytes, reaching a good sensibility of the method. The values of LOD and LOQ obtained were comparable to those reported by Pérez-Olivero (2011) in a study where the analytes were extracted through HS-SPME and analysed by GC coupled to an ion trap mass spectrometer, with LOD values in the range of 0.003-0.275 mg/L. In another study still performed by HS-SPME-GC-MS, LOQ values reported for the quantification of C6 and C8 in Semillon wines, were 8.34 and 0.40 mg/L, respectively, higher, in particular for C6, as compared to the results obtained in the present study which were 0.18 mg/L for C6 and 0.21 mg/L for C8 (Rebière *et al.*, 2010). Instead, significantly lower values of 0.03 mg/L for C6 and 0.02 mg/L for C8 were reported in a work where free fatty acids were determined in musts and wines through LLE with hexane, followed by concentration and derivatization of the analytes with 3% sulfuric acid in methanol and subsequent GC-FID analysis. (Gallart *et al.*, 1997).

The recovery of the method was assessed by spiking wine sample with a solution containing the analytes at different concentrations and for all analytes a good recovery was obtained, in the range 81.9-101%.

Finally, the repeatability was assessed in terms of percentage relative standard deviation obtained for the analysis of a wine sample performed 5 times in a day (intraday repeatability) and 5 times in 5 different days (interday repeatability). The results showed good repeatability values ranging from 0.4 to 4.9% for the intraday and from 0.5 to 8.5% for interday repeatability.

2.4 Application of the method to the DOCG wines of Marche region

2.4.1 Results and discussion

The validated method was then applied for the quantification of short and medium chain free fatty acids in the 18 DOCG wines (listed in **PART 1**, **Table 9**) produced in Marche region and the results are reported in **Table 8**.

Table 8. Mean concentrations (mg/L) ± relative standard deviations (rsd%, *n*=2) of SCFAs and MCFAs in DOCG wine samples.

	Sample	C2		C3		*i*C4		*i*C5		C6		C8	
	Conc. (mg/L)		rsd%		rsd%		rsd%		rsd%		rsd%		rsd%
white wines	VM01	353.4	0.3	0.6	2.7	nd		1.0	0.0	5.0	1.7	7.6	1.2
	VM02	356.5	1,5	0.5	3.0	1.8	3.2	1.2	10.3	5.0	3.5	8.4	3.7
	VJ01	353.6	2.2	0.6	2.8	1.2	5.8	1.0	3.7	4.8	4.6	8.7	8.1
	VJ02	389.5	0.6	0.7	4.6	1.3	2.6	0.8	2.5	5.4	1.9	7.5	1.8
	VJ03	512.2	0.4	0.6	2.2	1.6	7.3	1.1	4.4	3.4	0.4	6	1.3
	PE01	365.9	1.3	1.0	4.3	1.4	1.8	1.0	2.0	4.7	5.8	8.5	1.0
	PE02	381.5	2.2	0.9	0.1	1.8	1.4	1.0	3.4	3.3	1.6	4.4	12.8
	PA01	340.9	0.4	0.7	2.9	1.4	3.6	1.1	4.0	5.6	2.1	10.3	2.9
	PA02	444.4	2.0	0.6	3.5	2.1	2.5	1.0	3.4	5.2	3.4	7.7	4.3
	Mean values	388.7[a]	14.3	0.7	0.0	1.6[a]	19.4	1.0[a]	10.0	4.7[a]	17.3	7.7[a]	21.9
	Range	340.9-512.5		0.5-1.0		1.2-2.1		0.8-1.2		3.3-5.6		4.4-10.3	
red wines	RO01	804.4	1.5	0.9	8.2	3.1	0.4	1.2	11.4	1.83	1.0	1.6	1.8
	RO02	821.6	1.0	0.9	1.1	3	6.5	1.6	7.6	2.3	0.8	2.3	0.0
	CO01	576.6	0.7	0.6	3.5	2.3	0.5	1.1	3.0	1.8	1.0	2.6	2.0
	CO02	797.2	0.8	0.7	5.5	2.9	6.1	1.4	7.7	2.6	10.5	2.3	0.0
	CO03	596.6	0.4	0.9	4.0	2.5	16.7	1.2	1.3	2.5	1.7	3.7	1.5
	VD01	1012.8	0.8	0.9	8.3	2.9	3.9	1.2	13.5	2.6	7.2	2.9	11.0
	VD02	430.8	2.2	0.9	12.4	2.3	2.0	1.2	8.6	2.5	2	3.4	5.6
	VS01	946.6	2.1	0.9	2.9	2.6	6.6	1.2	1.3	2.3	1.8	2.6	0.4
	VS02	978	2.1	0.8	2.3	2.5	5.6	1.3	3.4	2.3	2.8	1.8	1.2
	Mean values	773.8[b]	25.9	1.0	56.3	2.7[b]	11.3	1.3[b]	12.2	2.3[b]	13.1	2.6[b]	26.6
	Range	430.8-1012.8		0.8-2.5		2.3-3.1		1.1-1.6		1.8-2.6		1.6-3.7	

Different letters indicate significant differences between concentrations of analytes in white and red samples (One-way ANOVA, P < 0.05, Tukey's test for pairwise comparison). Nd: < LOD. VM: "Verdicchio di Matelica Riserva"; VJ "Castelli di Jesi Verdicchio Riserva"; PE: "Offida Pecorino"; PA: "Offida Passerina"; RO: "Offida Rosso"; CO: "Cònero"; VD: "Vernaccia di Serrapetrona" sweet; VS: "Vernaccia di Serrapetrona" dry.

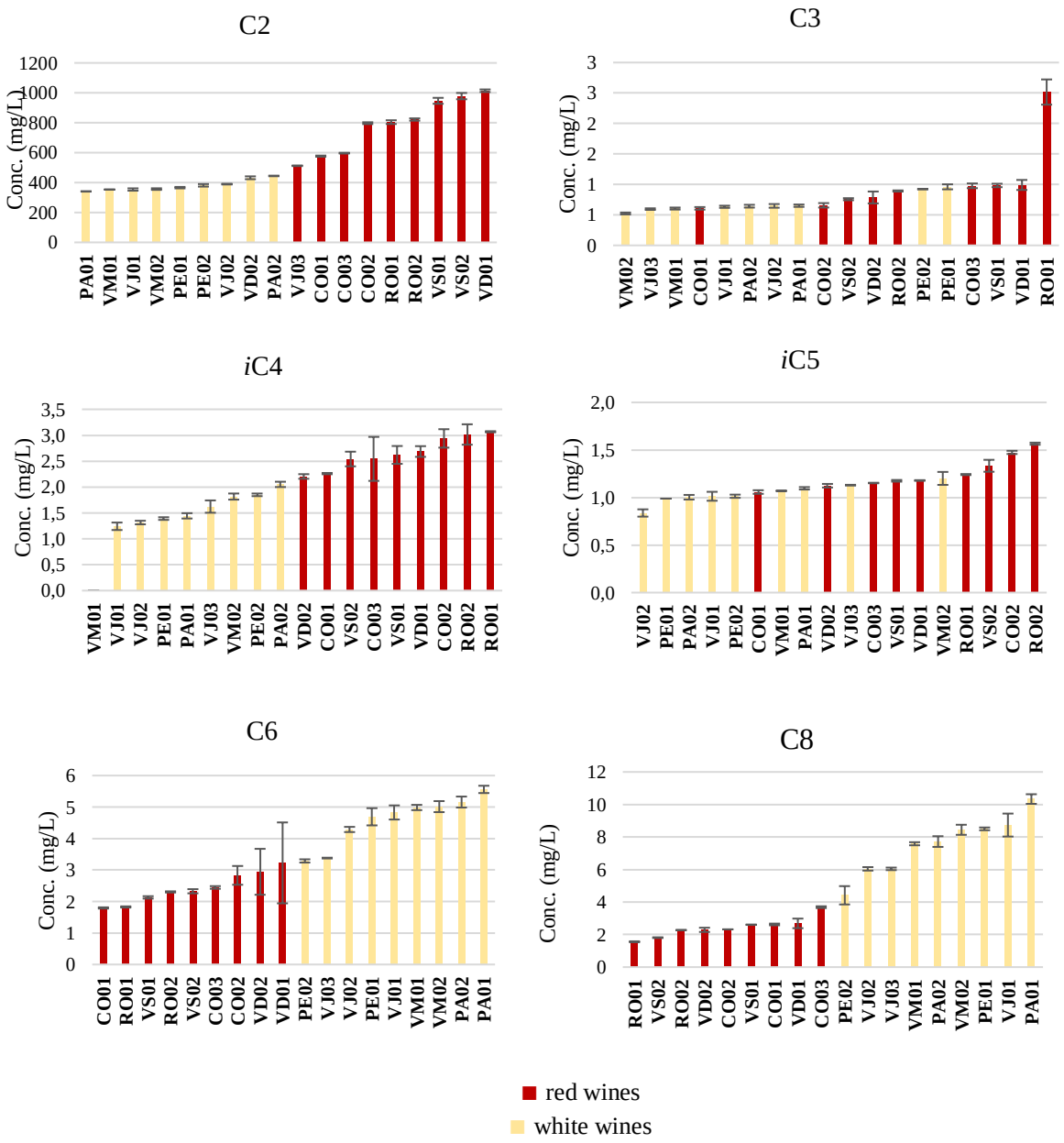


Figure 7. Concentrations of S and MCFAs found in the samples investigated. Bars indicate ± standard deviations.

Acetic acid was obtained in the range 340.9-512.2 mg/L in white wines while in red wines it was obtained in the range 430.8-1012.8 mg/L. However, three red samples had an acetic acid content much lower as compared to the other red wines, whose values were closer to the ones of white wines (VD02 of 430.8, CO01 or 576.6 and CO03 of 596.6 mg/L). Taking into consideration the limits that are imposed by European regulation (CE). N.606/2009 for the content of this acid that expresses the volatile acidity (1080 mg/L for white and rosé wines and 1200 mg/L for red ones), all DOCG wines showed lower concentrations.

The distribution of the analytes was different between red and white wines, especially for the content of acetic acid, hexanoic acid and octanoic acid, with acetic acid being significantly more abundant in red wines and hexanoic and octanoic acid being significantly more abundant in white wines. In fact, the mean quantities of C6 and C8 found in white wines were 4.7 and 7.7 mg/L respectively, while in red wines they were found in average concentrations of 2.3 and 2.6 mg/L, respectively. The same trend was reported in several other studies (Pèrez-Olivero *et al.*, 2011; Shinoara, 1985; Gil *et al.*, 2006).

Propionic, isobutyric and isovaleric acid were found in slightly lower concentrations as compared to the other analytes and they were generally less abundant in white wines as compared to red ones. Concerning propionic acid, in the study of Pérez-Olivero *et al.* (2011) previously mentioned, this analyte was quantified with average quantities of 1.28 mg/L and 1.55 mg/L for white and red wines, respectively. The values were in accordance with our results since we obtained a comparable average value for red wines, being 0.7 mg/L and 1.0 mg/L for white and red wines, respectively. Concerning isobutyric acid, it was quantified with average values of 1 mg/L and 1.76 mg/L for white and red wines. In our case this analyte was found with an average value of 1.6 mg/L for white wines and 2.7 mg/L for red wines, hence showing slightly higher quantities. The values obtained, instead, for isovaleric acids were 1 mg/L and 1.25 mg/L for white and red wines respectively, while the ones from the mentioned work showed values of 0.7 and 1.0 mg/L. Anyway, little discrepancies between results can be explained by the different techniques used. In fact, the method proposed by Pèrez-Olivero *et al.* (2011) involved the extraction through HS-SPME followed by GC-ITMS analysis.

Louw *at el.* (2009), performed the characterization of South African young red and white wines by analysing the volatile profile after the LLE with ether, followed by GC-FID analysis in two types of white young wines (Sauvignon Blanc and Chardonnay) and four of red young wines (Pinotage, Cabernet Sauvignon, Merlot and Shiraz). Taking into consideration the range of mean values obtained for white and red wines, even in this case acetic acid was obtained with higher concentrations in red wine samples (491.3-597.5 mg/L) compared to white ones (395.4-408.1 mg/L), while hexanoic and octanoic acid showed lower concentrations for red wines and with values comparable to our results (being the ranges for hexanoic 1.4-1.9 mg/L and 5.2-5.8 and for octanoic acid 1.3-1.8 and 4.7-6.2 mg/L, in red and white wines, respectively). For the other analytes Louw *et al.* obtained propionic acid in the range 8.8-14.9 mg/L in white wines and 2.8-41.0 mg/L in red wines, while in the DOCG wine samples this compound was found in lower concentrations, given that the mean values for white wines was 0.7 mg/L and for red wines 1.0 mg/L. Conversely, the values

obtained for isobutyric acid were more in accordance with our results, being the range for white wines 1.0-1.02 mg/L (1.2-2.1 mg/L for DOCG wines) and for red wines 1.4-2.2 mg/L (2.3-3.1 mg/L for DOCG wines). Finally, isovaleric acid was obtained with very low concentrations by Louw *et al.*, in the range 0.02-0.04 mg/L for white wines and 1.4-2.8 mg/L for red wines. DOCG wines showed slightly higher values for white wines, being in the range 0.8-1.2 mg/L and more in accordance for red wines, with a range 1.1-1.8 mg/L.

Another study that reported the quantification of short and medium chain free fatty acids from wines was carried out by Calleja & Falqué (2005) who quantified these analytes in two samples of red Mencìa wines. The samples were previously adjusted to a pH 7, the analytes were then extracted three times with diethyl ether-pentane (1:1, v/v), concentrated under nitrogen flux and finally analysed with GC-MS. The average values obtained were of 1.29 mg/L for isobutyric, 0.72 mg/L for isovaleric, 1.05 mg/L for hexanoic and 0.94 mg/L for octanoic acid. Differently, the relative average concentrations obtained for the DOCG wine red samples were higher, being 2.7, 1.3, 2.3 and 2.6 mg/L, respectively.

Finally, the OAV values were also calculated by dividing the concentration of each analyte by its olfactory threshold (OTH). It was done to assess which analytes could play a role in the overall aroma of the wines under investigation (**Table 9**).

Table 9. Olfactory threshold (OTH) and odor active values (OAV) calculated for the DOCG wine samples.

		C2	C3	*i*C4	*i*C5	C6	C8
	OTH (mg/L)	200[a]	20[b]	0.23[c]	0.03[c]	0.42[c]	0.50[c]
white wines	VM01	1.8	0.0	0.0	33.3	11.9	15.2
	VM02	1.8	0.0	0.8	36.4	11.9	16.8
	VJ01	1.8	0.0	0.5	30.6	11.4	17.4
	VJ02	1.9	0.0	0.6	25.5	12.9	15.0
	VJ03	2.6	0.0	0.7	34.2	8.1	12.0
	PE01	1.8	0.0	0.6	30.0	11.2	17.0
	PE02	1.9	0.0	0.8	30.3	7.9	8.8
	PA01	1.7	0.0	0.6	33.3	13.2	20.6
	PA02	2.2	0.0	1.0	30.3	12.4	15.4
	Mean value	**1.9**	**0.0**	**0.7**	**31.5**	**11.2**	**15.4**
red wines	RO01	4.0	0.1	1.4	36.4	4.4	3.2
	RO02	4.1	0.0	1.3	48.5	5.5	4.6
	CO01	2.9	0.0	1.0	33.3	4.3	5.2
	CO02	4.0	0.0	1.3	42.4	6.2	4.6
	CO03	3.0	0.0	1.1	34.8	6.0	7.4
	VD01	5.1	0.0	1.3	36.4	6.2	5.8

VD02	2.2	0.0	1.0	36.4	6.0	6.8
VS01	4.7	0.0	1.1	36.4	5.5	5.2
VS02	4.9	0.0	1.1	39.4	5.5	3.6
Mean value	**3.9**	**0.1**	**1.2**	**38.2**	**5.5**	**5.2**

[a]Guth, 1997; [b]Lambrechts & Pretorius, 2000; [c]Ferreira *et al.*, 2000. VM: "Verdicchio di Matelica Riserva"; VJ: samples of "Castelli di Jesi Verdicchio Riserva"; PE: "Offida Pecorino"; PA: "Offida Passerina"; RO: "Offida Rosso"; CO: "Cònero"; VD: "Vernaccia di Serrapetrona" sweet; VS: "Vernaccia di Serrapetrona" dry.

For propionic acid in all samples and isobutyric acid in white wines, the OAVs were obtained with values < 1 meaning that these compounds are not relevant in the aroma of samples under investigation. Differently isobutyric acid in red wine samples was obtained with a mean value of 1.2, hence, in these samples, it might actively contribute to the aroma. Acetic acid OAVs were obtained with a range of 1.7-5.1, where the mean value for red wines (3.9) was obviously higher than the one of white wines (1.9). Hence, acetic acid may play an active role on the aroma of these wine samples.

Concerning the other compounds, very high values were obtained, always being much higher than 1, for all wine samples. The OAV mean value obtained for isobutyric was indeed 6.8 for white wines and 11.6 for red wines, while isovaleric acid was much higher with mean values of 31.5 and 38.2, respectively. Very high values for isovaleric acid were also obtained by Gil *et al.* (2006) where it was 51.8 for white samples and 58.1 for red wines. Hexanoic and octanoic acids showed lower values, but still higher than one, hence even these analytes should be actively involved in the aroma of all DOCG wines under investigation.

2.4.2 Statistical analysis

2.4.2.1 One-way ANOVA

To characterize the profile of the DOCG wines of Marche region, the one-way analysis of variance (ANOVA) coupled with Tukey's test for pairwise comparison, between wine samples divided into DOCGs typology, was performed. Hence, the eighteen samples were divided into eight groups and the ANOVA was performed to point out, if present, statistically significant differences ($P < 0.05$) in the short and medium chain free fatty acids composition of the investigated DOCGs and the results are reported in **Table 10**.

Table 10. Average concentration (mg/L) of the SCFAs and MCFAs in the different DOCG samples, divided into eight groups (distinguishing Vernaccia DOCG wines into two different groups: dry and sweet; and Offida DOCG into three groups: Pecorino, Passerina and Offida Rosso).

	Verdicchio di Matelica Riserva	**RSD %**	**Castelli di Jesi Verdicchio Riserva**	**RSD %**	**Offida Pecorino**	**RSD %**	**Offida Passerina**	**RSD %**	**Offida Rosso**	**RSD %**	**Cònero**	**RSD %**	**Vernaccia di Serrapetrona sweet**	**RSD %**	**Vernaccia di Serrapetrona dry**	**RSD %**
C2	355.0^{a}	0.6	418.4^{a}	19.9	373.7^{a}	3.0	392.7^{a}	18.6	813.0^{b}	1.5	656.8^{b}	18.6	721.8^{b}	57.0	962.3^{c}	2.3
C3	0.6^{a}	12.9	0.6^{a}	25.0	0.9^{b}	7.4	0.7^{a}	10.90	0.9^{b}	0.0	0.7^{a}	20.8	0.9^{b}	0.0	0.9^{b}	8.3
*i*C4	1.8		1.4^{a}	15.2	1.6^{a}	17.7	1.8^{a,c}	28.3	3.1^{b}	2.3	2.6^{b}	11.9	2.6^{c}	16.3	2.6^{c}	2.8
*i*C5	1.1^{a}	12.9	1.0^{a}	15.8	1.0^{a}	0.0	1.1^{a}	6.7	1.4^{b}	20.2	1.2^{b}	12.4	1.2^{b}	0.0	1.3^{b}	5.7
C6	5.0^{a}	0.0	4.5^{a}	22.6	4.0^{a}	24.7	5.4^{a}	5.2	2.1^{b}	16.1	2.3^{b}	19.0	2.6^{b}	2.8	2.3^{b}	0.0
C8	8.0^{a}	7.1	7.4^{a}	18.3	6.5^{a}	44.9	9.0^{a}	20.4	2.0^{b}	25.4	2.9^{b}	25.7	3.2^{b}	11.2	2.2^{b}	25.7

RSD% is the percentage standard deviation obtained among samples belonging to the same DOCG class. Letters indicate significant differences between the concentrations (mg/L) in the eight different DOCG wines groups (One-way ANOVA, $P < 0.05$, Tukey's test for pairwise comparison). When no letters and RSD % are reported, no replicates were available to performed ANOVA.

The ANOVA underlined the differences previously discussed. Indeed, differences raised mainly between red and white DOCGs wines in the amount of the analytes. The former showed higher concentrations in terms of acetic, propionic, isobutyric and isovaleric acids, while the latte in hexanoic and octanoic acids. At the same time, within the red and white DOCGs some differences were pointed out by the analysis of variance. In fact, "Vernaccia di Serrapetrona" dry was the one showing highest abundance of acetic acid. Also, "Offida Rosso" seemed to be enriched in isobutyric acid.

The differences in five grape varieties used to produce the different DOCGs (Verdicchio, Pecorino, Passerina, Montepulciano and Vernaccia Nera) or the different geographical production areas seemed to play an irrelevant role in the final short and medium chain free fatty acids profile of the wines. Indeed, the most important discrimation parameter was the difference in white or red wines.

2.4.2.2 Principal component analysis

The principal component analysis (PCA) was performed to identify homogenous groups of data by considering the samples divided into the eight DOCG groups and the six analysed volatile acids. The first two principal components (PC1 and PC2) were able to explain most of the variance, with a high sum of 93.3 %. The biplot correlating scores (DOCG wines) and loadings (the analysed volatile acids) calculated on the first two principal components, is shown in **Figure 8.**

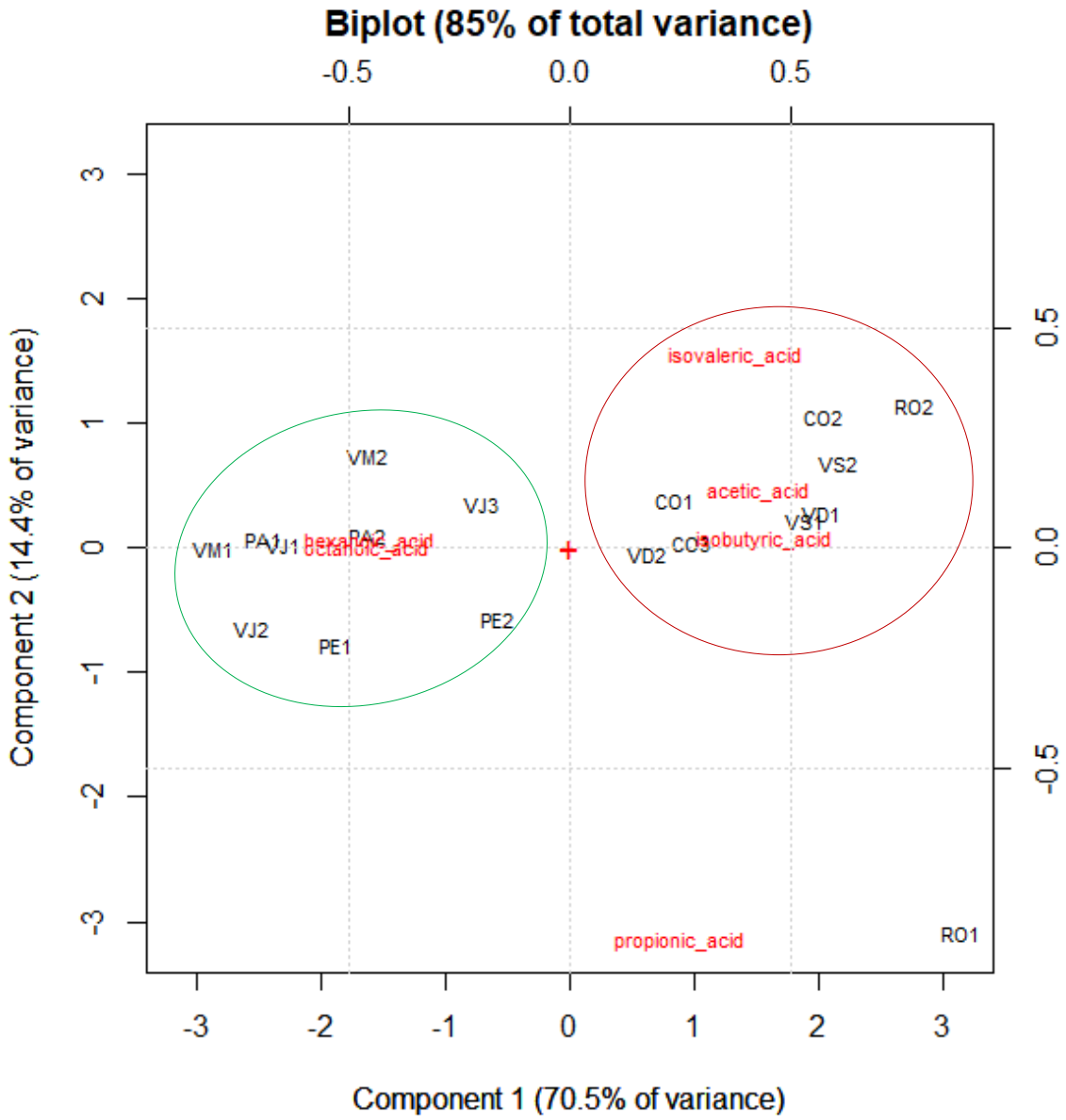


Figure 8. Principal component analysis and related biplot, obtained using the concentrations of all SCFAs and MCFAs analysed as variables (in red), and the DOCG wine samples investigated (in black) as scores. VM: "Verdicchio di Matelica Riserva"; VJ "Castelli di Jesi Verdicchio Riserva"; PE: "Offida Pecorino"; PA: "Offida Passerina"; RO: "Offida Rosso"; CO: "Còmero"; VD: "Vernaccia di Serrapetrona" sweet; VS: "Vernaccia di Serrapetrona" dry.

The PC1 was able to explain most of the variance (82.3%) and, along this component, white DOCGs s and red DOCGs were clearly separated. Furthermore, some clusters of data were pointed out. Indeed, three white DOCG wines were clustered and positioned close to hexanoic and octanoic acids (green circle), indicating that these DOCGs were characterized by a high content of the mentioned acids. In the same way, all red DOCGs wines were grouped in a cluster comprising acetic,

isobutyric and isovaleric acids (red circle). One sample of "Offida Rosso" (RO01) was the one showing the most peculiar behaviour, not being positioned close to other red DOCG wines.

It can be explained by the fact that this wine sample contained higher concentration of propionic acid when compared to the other white DOCGs (P <0.05) as also reported in **Table 10**. Indeed, also propionic acid was positioned far from the other analytes (and close to RO01 wine sample), being not able to clearly discriminate between white or red DOCGs.

The heatmap confirmed the clustering of samples in the two main groups, almost perfectly dividing red and white DOCG wines (**Figure 9**).

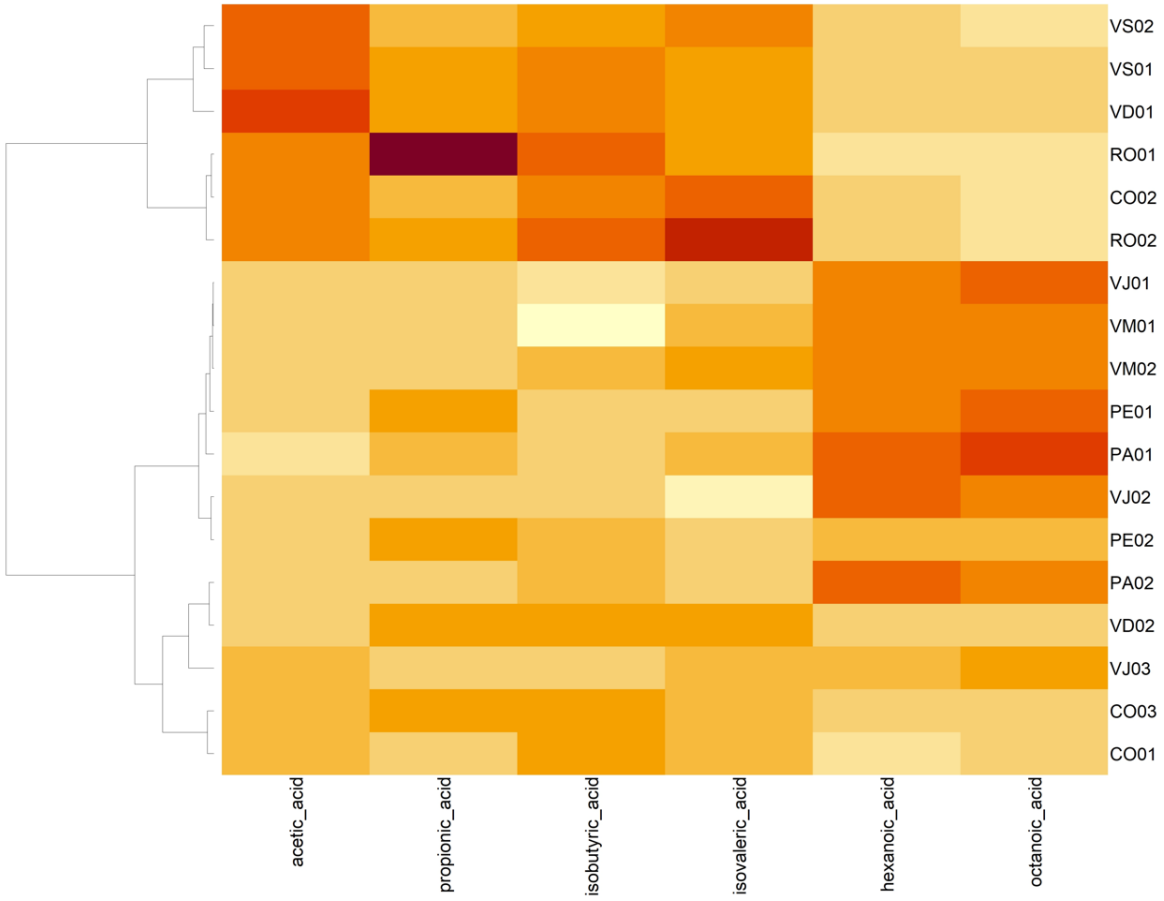


Figure 9. Heatmap of the short and medium chain free fatty acids in DOCG wine samples investigated (variables clustered on the vertical axes). VM: "Verdicchio di Matelica Riserva"; VJ: samples of "Castelli di Jesi Verdicchio Riserva"; PE: "Offida Pecorino"; PA: "Offida Passerina"; RO: "Offida Rosso"; CO: "Cònero"; VD: "Vernaccia di Serrapetrona" sweet; VS: "Vernaccia di Serrapetrona" dry.

The heatmap also pointed out the higher concentration of propionic acid for the "Offida Pecorino" DOCG, comparable to those obtained for red DOCG wines.

2.5 Conclusions

A new method for the determination of short and medium chain free fatty acids from wine samples was developed and validated, being these analytes important molecules related to wine quality.

The proposed method allows to perform the extraction of the analytes by using small amounts of sample and solvent (0.5 and 0.4 mL, respectively), few and unexpensive reagents and the use of an analytical instrument which is commonly available in most of laboratories (GC-FID). The method is also rapid, requiring a relatively short time of about 30 min for the complete extraction and analysis. The number of extractions was initially investigated together with the use of salts able to increase the extraction extent of analytes. A single extraction and the use of NaH_2PO_4 allowed to greatly increase method sensitivity.

Good linearity, repeatability, recovery and sensitivity were obtained, making the procedure a suitable tool for the quantification of acetic, propionic, isobutyric, isovaleric, hexanoic and octanoic acids from wine.

The method was then applied for the quantification of the analytes in 18 DOCG wine samples of Marche region. Different statistical analyses (ANOVA coupled to Tukey's test for pairwise comparison and PCA) were performed in order to characterize the profile of the difference DOCGs investigated and this pointed out that the most relevant differences raised between white and red DOCGs being the former enriched in hexanoic and octanoic acids and the latter in the other analytes (acetic, propionic, isobutyric and isovaleric acids). Hence, the profile of short and medium chain free fatty acids seemed not to be strictly dependent on the grape variety used to produce a determine DOCG of the geographical production area. Only one sample of "Offida Rosso" showed the most peculiar behaviour when considering the PCA, being the white DOCG more enriched in propionic acid.

On the other hand, every DOCG seemed to be characterized by a peculiar profile in the short and medium chain free fatty acids composition, which should be better investigated and eventually confirmed by analysing samples from different wineries and, also, different vintages. This would, indeed, help to verify if the profile of the volatile acids could be used to assess DOCG wines of Marche region authenticity.

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3: Investigation of polyphenols

3.1 The role of polyphenols in wine

Polyphenols are a large family of organic compounds, naturally occurring as plant secondary metabolites. They are present also in grapes and, consequently, in wine where they play essential roles from the organoleptic point of view. Indeed, they contribute to colour, astringency, bitterness and also to the stability, by interacting with proteins and being involved in oxidation reactions and in the aging behaviour of wine (Carando *et al.*, 1999).

Besides, phenolic compounds are widely known and studied for their health benefits (Rasouli *et al.*, 2017). Polyphenols are in fact strong antioxidants and epidemiological studies showed that, due to their presence, a moderate consumption of wine may protect against cardiovascular diseases (Lippi *et al.*, 2010; Arts, 2001; Renaud & de Lorgeril, 1992). Several works have been focused on the beneficial effects of moderate wine consumption and the most representative ones were recently overviewed by Lucarini *et al.* (2021).

Both white and red wines contain a high number of polyphenols, even if it the content of phenolic substances in red wines can be more than 10-fold higher than the content of white wines (Fuhrmann *et al.*, 2001). This difference arises from the distinct vinification pathways between the two wine typologies. In fact, the vinification for red wines is performed with the presence of the mash during fermentation, leading to an abundant extraction of these compounds from berries.

The final phenolic content, and thus, the qualitative and quantitative profile is very variable, being influenced by a huge number of factors. In fact, during ripening, grapes undergo several chemical changes that can influence the final non-volatile polyphenolic content together with climatic and geographical vineyard conditions (*terroir*) and the technological practise to which grapes undergo (Fang *et al.*, 2008; de Freitas, 2000; Reynolds, 2010; van Leeuwen, 2010). The ranges in which the major polyphenols have been found in red wines, collecting data from different studies, are reported in **Table 1**.

Table 1. Concentration ranges of the major polyphenols in red wines (Li & Duan, 2018).

Phenolic compounds	Concentration range (mg/L)
Hydroxybenzoic acids[a]	8-54
Hydroxycinnamic acids[a]	11-164
Flavonols[a]	4-114

Flavanols[b]	436-3600
Anthocyanins[c]	185-895

[a] Bautista-Ortín *et al.*, 2007; Garcia-Falcon *et al.*, 2007¸Gutiérrez *et al.*, 2005; Kallithraka *et al.*, 2006. [b] Bautista-Ortín *et al.*, 2007; González-Centero *et al.*, 2016; Koundouras *et al.*, 2013; Pérez-Lamela *et al.*, 2007. [c] Bautista-Ortín *et al.*, 2007; González-Centero *et al.*, 2016; Gutiérrez et al., 2005; Kallithraka *et al.*, 2006; Pérez-Lamela *et al.*, 2007.

From the sensorial point of view, polyphenols are known to be responsible for the colour of wines, but they are also involved in gustative attributes such as bitterness, sourness and astringency, even if in some cases a direct correlation between mouth perceptions and polyphenolic composition has not been well-established.

Astringency is defined as drying, roughing and puckering of the oral cavity that, beyond polyphenols, can be elicited by different substances such as multivalent metallic cations, organic acids and minerals (Pires *et al.*, 2020). It accounts for more than a half of the total terms of wine mouth-feel characteristics. The perception of astringency is the result of a highly complex process that depends on the presence of individual wine components that may exert both synergic or antagonistic effect on compounds and thus affecting the overall perceived intensity (Riou *et al.*, 2002; Sowalsky & Noble, 1998). Polyphenolic compounds that are believed to largely be responsible for astringency are the polymeric flavan-3-ols, occurring both in free from and conjugated to anthocyanins (Vidal *et al.*, 2004).

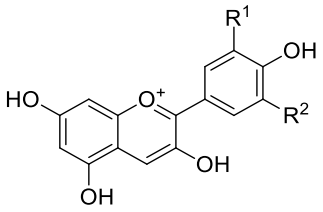
Bitterness is one of the five basic tastes. Even though the mechanisms of its origin and perception are well understood, there is still a limited available data regarding the relationship between structure and sensorial perception. Bitterness is usually regarded as an unpleasant taste, even if in the case of some beverages it is a necessary attribute, when present in proper moderate amounts and intensities (Soares *et al.*, 2015).

From the point of view of the chemical structure polyphenols are a very heterogenous family of compounds which includes several and different components. Generally, they can be divided into two main families: flavonoid and non-flavonoid. The class of non-flavonoids comprises mainly hydroxybenzoic acids and hydroxycinnamic acids, together with derivates such as stilbenes. Flavonoids include flavonols, flavan-3-ols, polymer of flavan-3-ols also called procyanidins and anthocyanins (Minussi *et al.*, 2003). The main difference between the two groups stands in the basic structures. While non-flavonoids contain one phenol ring, flavonoids contain two phenol rings which are connected by an oxygen-containing central pyran ring.

3.1.1 Flavonoids

Flavonoids comprise in turn several classes, where anthocyanins (**Figure 1**) and flavonols (**Figure 2**) play the most important roles in wine colour.

Anthocyanins

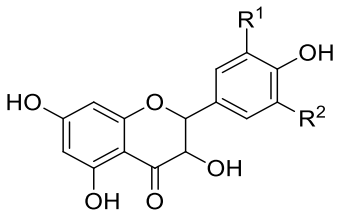


	R^1	R^2
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Malvidin	OCH_3	OCH_3
Peonidin	OCH_3	OCH_3
Petunidin	OH	OCH_3

Figure 1. Most abundant anthocyanins in wine.

Anthocyanins are polyphenols that are present only in grape skins and they are the major responsible for the colours of red fruits and vegetables and of wine. They are responsible not only for the initial red colour of wine, but also for the colour changes that may occur progressively during ageing, being a result of their condensation with flavanols and other compounds (pyruvic acid, vinylphenol, glyoxylic acid, etc.) and the red or blue shadows that they can show is strictly connected to the pH. Every anthocyanin has been associated to different colours; in particular, cyanidin to orange red, peonidin to garnet red, delphinidin, petunidin and malvidin to ruby red and pelargonidin to orange.

Flavonols



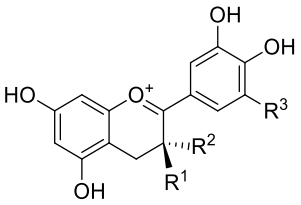
	R^1	R^2
Kaempferol	H	H
Myrcetin	OH	OH
Quercitin	OH	H
Isorhamnetin	H	OCH_3
Laricitin	OH	OCH_3
Syringetin	CH_3	OCH_3

Figure 2. Major flavonols in wine.

Flavonols, structurally very similar to flavones, are mainly represented by kaempferol, quercetin and myricetin (Makris *et al.*, 2006). These compounds are white to yellow pigments found in grapes skin, thought to be important co-pigments for anthocyanins. The proportion of flavonols that affects the colours in white wines is extremally small, while in red wines these yellow pigments

are masked by the purplish red of anthocyanidins. These compounds are associated to astringency sensations (Ferrer-Gallego *et al.*, 2016).

Flavan-3-ols

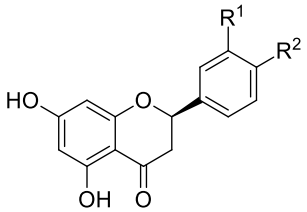


	R[1]	**R[2]**	**R[3]**
(+)-Catechin	H	H	OH
(-)-Epicatechin	H	OH	H
Gallocatechin	H	OH	OH
Epigallocatechin	OH	H	OH

Figure 3. Major flavan-3-ols in wine.

Flavan-3-ols are a very important class of polyphenols, being one of the most abundant in nature. The difference among flavan-3-ols consist of the stereochemistry of carbon 3 and on the hydroxylation degree (**Figure 3**). The most important flavan-3-ols found in wine are (+)-catechin or (-)-epicatechin, which are mainly present in seeds and skin of grapes. The colour browning of white wine during aging has been attributed to the reaction of (+)-catechin with tartaric acid, which provoke the accumulation of xanthylium cation pigments (Yuan *et al.*, 2022).

Flavanones



	R[1]	**R[2]**
Naringenin	H	OH
Hesperetin	OH	CH_3

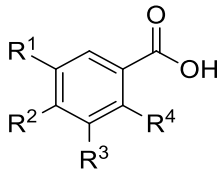
Figure 4. Major flavanones in wine.

Naringenin is the most abundant flavanone in wine and it can reach concentrations up to 25 and 7.7 mg/Kg in red and white wines, respectively (Nemzer *et al.*, 2022).

3.1.2 Non-flavonoids

Concerning non-flavonoids, phenolic acids are colourless compounds mainly present in fresh grapes, but they can also derive from the breakdown of anthocyanins or released from barrels woods. The main difference between hydroxybenzoic and hydroxycinnamic acids lies in the number of carbons which compose the basic chemical structure, which is 7 and 9 carbon atoms, respectively.

Hydroxybenzoic acids

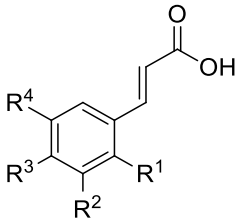


	R^1	R^2	R^3	R^4
Gallic acid	OH	OH	OH	H
Gentisic acid	OH	H	H	OH
Syringic acid	OCH_3	OH	OCH_3	H
Protocatechuic acid	H	OH	OH	H
Vanillic acid	H	OH	OCH_3	H

Figure 5. Major hydroxybenzoic acids in wine.

For hydroxybenzoic acids, the five compounds listed in **Figure 5** are the ones more commonly found in wines (Baderschneider & Winterhalter, 2001). Gallic acid is the most abundant in red wines, found in free form in grapes, and it is the precursor of hydrolysable tannins.

Hydroxycinnamic acids

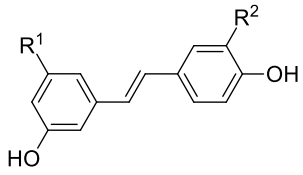


	R^1	R^2	R^3	R^4
Caffeic acid	H	OH	OH	H
Ferulic acid	H	OCH_3	OH	H
p-Coumaric acid	H	H	OH	H
o-Counaric acid	OH	H	H	H
Sinapic acid	H	OCH_3	OH	OCH_3

Figure 6. Major hydroxycinnamic acids in wine.

Hydroxycinnamic acids (some examples reported in **Figure 6**) are the most representative phenolic acids in grapes and wine (Baderschneider & Winterhalter, 2001). These compounds are associated to wine browning processes, and they are also the precursors of volatile phenolic compounds (Kallithraka *et al.*, 2009). They exist as tartaric acid esters and are hydrolysed during winemaking processes and ageing (Buiarelli, 2010).

Stilbenes



	R^1	R^2
trans-Resveratrol	OH	H
trans-Piceid	*O*-glc	H
Piceatannol	OH	OH

Figure 7. Major stilbenes in wine.

Among the major stilbenes (**Figure 7**), resveratrol in the most studied, due to the beneficial effects associated to its consumption. It is mainly concentrated in grapes skin, but it is usually found

in very tiny concentrations, hence its determination in wines has always been quite challenging (Fabjanowicz *et al.*, 2018).

Co-pigmentation is a process that brings to the formation of very complex polymers, which are referred to as tannins. The major sensorial characteristic associated to tannins is the astringency and, in some cases, bitterness. In addition to mouthfeel, tannins play also an important role in the stabilization of wine colour by reacting with anthocyanins and producing polymeric pigments (du Toit, 2010).

Tannins are generally divided into two groups: condensed tannins and hydrolysable tannins. Condensed tannins, are oligomers of flavon-3-ols, comprising catechin, epicatechin, gallocatechin, and in red wine they can reach concentrations up to 4 g/L. They are extracted from skin, flesh and seeds of grapes during the winemaking processes. Hydrolysable tannins, being composed by non-flavonoids polyphenols, derive from oak barrels and occur only in low concentrations in oak aged wines. They are normally glucose molecule acylated with galloyl groups, whose variable amount and composition depends on the aging time in barrels (Smith *et al.*, 2015). From the organoleptic point of view, hydrolysable tannins are able to greatly impact the mouthfeel with the so-called "structure" and with the characteristic astringency sensations.

Since polyphenols are known to be of paramount importance in wines, being the responsible of key sensorial characteristics such as colour, flavour and taste, and since they are also positively correlated to some health-promoting properties, the study of phenolic composition in grapes and wines is gaining more and more importance. Moreover, it may also allow to identify markers of a particular grape variety or wine and indeed, this has already found applications for authentication purposes and for protecting consumers from frauds (Anastasiadi *et al.*, 2009).

Beyond the few works that have been published, dealing with the characterization of phenolic profile of wines produce in Marche region, there is still a lack on those with controlled and guaranteed designation of origin (Boselli *et al.*, 2006; Sagratini *et al.*, 2012; Fiorini *et al.*, 2014).

Starting from these premises, the analysis of the polyphenolic profile of the DOCG wine samples under investigation (listed in **PART 1**, **Table 9**) was performed with the aim to characterize the five different DOCG wines produced in Marche region and to find, if present, those phenolic compounds able to discriminate one DOCG over the other, thus that can be possibly used as authenticity markers.

3.2 Materials and Method

3.2.1 Reagents and standards

PhytoLab provided analytical standards for kaempferol-3-glucoside and quercetin-3-glucoside (Vestenbergsgreuth, Germany). Sigma-Aldrich provided the reference materials for the remaining 35 of the 37 phenolics (Milan, Italy). Stock solutions of each analyte (1000 mg L^{-1}) were made by dissolving pure reference materials in methanol (HPLC-grade) and then keeping them at 5 °C in glass stoppered bottles until analysis. Working solutions of standards at different concentrations were made fresh by diluting stock solutions with methanol (HPLC-grade). Merck provided formic acid at a concentration of 99% (Darmstadt, Germany). Methanol of HPLC quality was acquired from Sigma-Aldrich, located in Milan, Italy. A Milli-Q SP Reagent Water System filtered deionized water to give ultrapure water with a resistivity of >18 M cm (Millipore, Bedford, MA, USA). Sartorius Stedim provided 0.2 μm polyamide filters which were used to filter all liquids (Goettingen, Germany). Phenex™ RC 4 mm 0.2 μm syringeless filters purchased from the company of Phenomenex, located in Castel Maggiore, BO, Italy, were used to filter all samples before injection into the HPLC instrument.

3.2.2 Sample preparation

Wine samples were prepared by dilution (1:5) with water and were filtered through 0.2 μm syringeless filters before HPLC-MS/MS analysis.

3.2.3 HPLC-MS/MS analysis

The quantification of 37 phenolic analytes was carried out, by using a modified version of a previously described method (Mustafa *et al.*, 2022). The HPLC-MS/MS investigations were carried out with an Agilent 1290 Infinity series and a Triple Quadrupole 6420 (Agilent Technology, California, USA), and linked to an electrospray ionization (ESI) source that operated in negative and positive ionization modes. Using Optimizer Software, the MS/MS parameters of each standard were optimized by using flow injection analysis (FIA). The separation of phenolic compounds was obtained by direct injection of diluted wine (1:5) using gradient elution mode on a Phenomenex Synergi Polar–-reversed phase (RP) C18 column (250 mm x 4.6 mm, 4 μm) using a mixture of water and methanol as solvents A and B, respectively, both with 0.1% formic acid. For column protection, a polar RP security guard cartridge preceded the column (4 mm x 3 mm ID). The mobile phase

composition was made up of the following components: 0–1 min, isocratic condition, 20% B; 1–25 min, 20–85% B; 25–26 min, isocratic condition, 85% B; 26–32 min, 85–20% B. A 0.2 μm polyamide filter was used to filter all solutions and solvents. The injection volume was 2 μL, and the flow rate was kept at 0.8 mL/min. The temperature of the column was set to 30 °C, and the drying gas temperature in the ionization source was set to 350 °C. The flow rate of the gas was set to 12 L/min, the capillary voltage was 4000 V and the nebulizer pressure was 55 psi. The peak areas were integrated for quantitation after detection in the dynamic-multiple reaction monitoring (dynamic-MRM) mode. Each analyte's most abundant product ion was employed for quantification, while the other ions were used for qualitative analysis. Each compound's unique time window (Δ retention time) was set at 2 min. The HPLC-MS/MS acquisition parameters (dynamic-MRM mode) used for the analysis of the marker compounds are reported in **Table 2**.

Table 2. HPLC-MS/MS acquisition parameters.

No.	Compounds	Precursor ion, *m/z*	Product ion, *m/z*	Fragmentor, V	Collision energy, V	Polarity	Retention time (Rt, min)
1	gallic acid	169	125.2*	97	12	Negative	6.96
2	neochlorogenic acid	353	191.2*, 179	82	12, 12	Negative	9.52
3	catechin	289	245.2*,109.2	131	8, 20	Negative	11.44
4	procyanidin B2	576.99	576.99*, 321.2	160	0, 32	Negative	12.41
5	chlorogenic acid	353	191.2*, 127.5	82	12, 20	Negative	12.42
6	*p*-hydroxybenzoic acid	137	93.2*	92	16	Negative	12.86
7	epicatechin	289	245.1*, 109.1	126	8, 20	Negative	13.03
8	3-hydroxybenzoic acid	137	93.2*	88	8	Negative	13.59
9	caffeic acid	179	135.2*, 134.1	92	12, 24	Negative	13.65
10	vanillic acid	167	152.4*, 108.1	88	12, 20	Negative	14.32
11	resveratrol	227	185*	131	12	Negative	14.40
12	syringic acid	196.9	182.2*, 121.2	93	8, 12	Negative	15.28
13	procyanidin A2	575	575*, 285	170	0, 20	Negative	16.18
14	*p*-coumaric acid	163	119.2*, 93.2	83	12, 36	Negative	16.70
15	ferulic acid	193	134.2*, 131.6	83	12, 8	Negative	17.10
16	3,5-dicaffeoylquinic acid	514.9	353.1*, 191	117	8, 28	Negative	17.61
17	rutin	609	300.2*, 271.2	170	32, 50	Negative	17.73
18	isoquercitrin	463	271.2*, 300.2	155	44, 24	Negative	18.36
19	delphindin-3,5-diglucoside	462.9	300.1*	165	24	Negative	18.38
20	phloridzin	435.39	273*, 167	155	8, 28	Negative	18.83
21	quercitrin	446.99	300.2*, 301.2	160	24, 16	Negative	19.61
22	myricetin	316.99	179.1*, 182	150	16, 24	Negative	19.61
23	naringin	578.99	271.3*, 151.3	170	32, 44	Negative	19.62
24	kaempferol-3-glucoside	447	284.2*, 255.2	170	24, 40	Negative	19.77
25	ellagic acid	301	301*, 229	170	0, 24	Negative	21.41
26	quercetin	300.99	151.2*, 179.2	145	16, 12	Negative	21.87
27	phloretin	272.99	167*, 123	116	8, 20	Negative	22.30
28	isorhamnetin	314.99	300.2*, 196.1	145	16, 4	Negative	24.57
1	delphindin-3-galactoside	465.01	303*	121	20	Positive	11.36
2	cyanidin-3-glucoside	449	287.3*, 255.6	121	20, 20	Positive	13.14
3	petunidin-3-glucoside	479.01	317*, 302	121	20, 44	Positive	13.26
4	pelagonidin-3-rutinoside	579.01	271*	145	32	Positive	14.56

5	pelargonidin-3-glucoside	433.01	271*, 121	116	24, 50	Positive	14.52
6	malvidin-3-galactoside	493.01	331*, 315.1	121	20, 50	Positive	14.64
7	hyperoside	465.01	303*, 61.1	97	8, 50	Positive	18.33
8	hesperidin	611.01	303*, 334.8	112	20, 12	Positive	20.19
9	kaempferol	287.01	153*, 69.1	60	36, 50	Positive	23.84

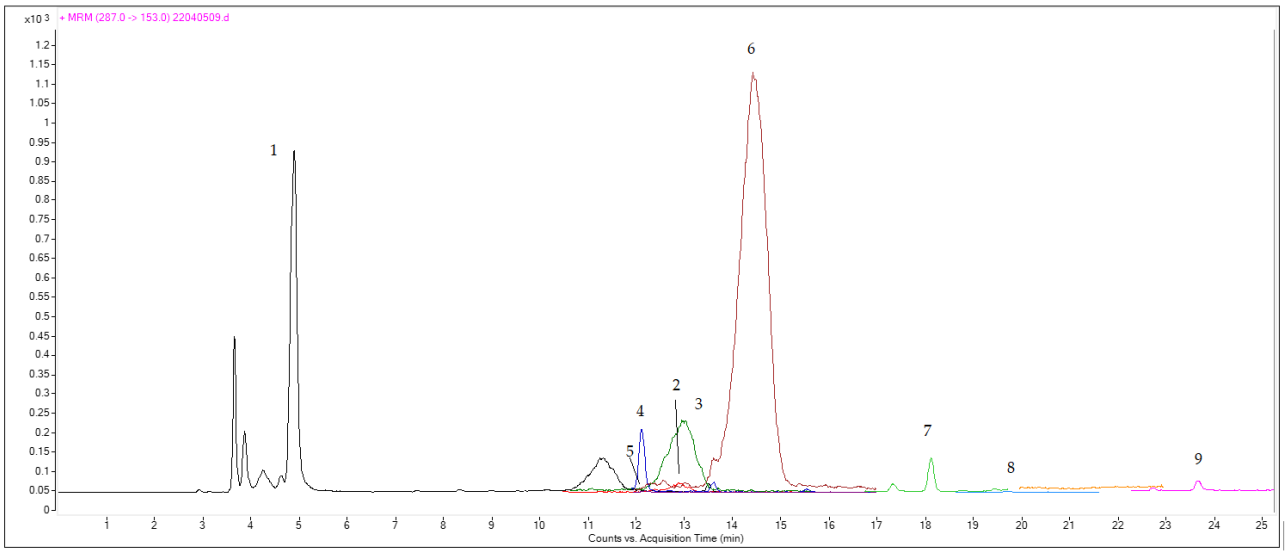
3.2.4 Statistical analysis

One-way analysis of variance (ANOVA) followed by Tukey's test for pairwise analysis was used to assess significant differences (P <0.05) in the phenolic content among the 18 DOCG wine samples. The software used for this purpose was PAST (Hammer, 2001). The heatmap correlating wine samples and phenolic substances was obtained by using Rstudio software. The principal component analysis (PCA) was also performed to identify homogenous groups of data, by using R-based software CAT (Leardi *et al., 2021).*

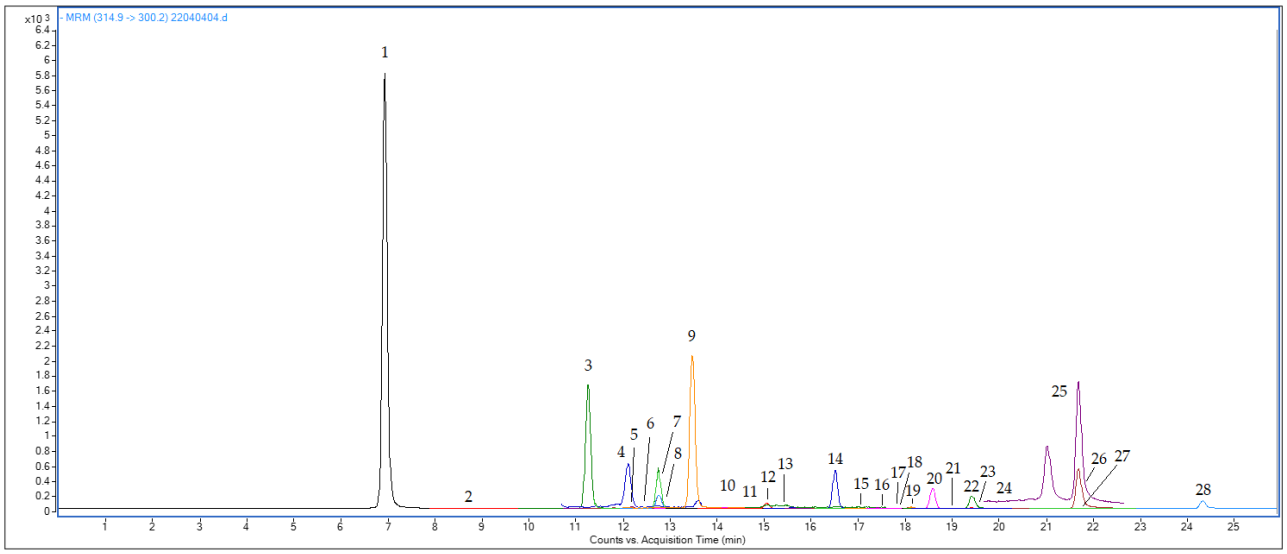
3.3 Polyphenol composition in DOCG wines from Marche region

3.3.1 Results and discussion

By the analysis of polyphenols substances, a total of 37 compounds were detected and quantified. An example of the chromatograms obtained by the HPLC analysis is shown in **Figure 8**, while the results obtained by the analysis of the phenolic substances in the samples under investigation are reported in and **Table 3**.



(a)



(b)

Figure 8. (a) Example of a chromatogram obtained by the HPLC analysis of the 9 positive ions acquisition used for quantification. Different colours indicate different ions: (1) delphinidin-3-galactoside, (2) cyanidin-3-glucoside; (3) petunidin-3-glucoside; (4) pelagonidin-3-rutinoside; (5) pelargonidin-3-glucoside; (6) malvidin-3-galactoside; (7) hyperoside; (8) hesperidin; (9) kampferol. (b) Example of a chromatogram obtained by the HPLC analysis of 28 negative ions acquisition used for quantification. Different colours indicate different ions: (1) gallic acid; (2) neochlorogenic acid; (3) catechin; (4) procyanidin B; (5) chlorogenic acid; (6) *p*-hydroxy benzoic acid; (7) epicatechin; (8) 3-hydroxybenzoic acid; (9) caffeic acid; (10) vanillic acid; (11) resveratrol; (12) syringic acid; (13) procyanidin A2; (14) *p*-coumaric acid; (15) ferulic acid; (16) 3,5-dicaffeoylquinic acid; (17) rutin; (18) isoquercitrin; (19) delphinidin 3,5-diglucoside; (20) phloridzin; (21) quercitrin; (22) myricetin; (23) naringin; (24) kaempferol-3-glucoside; (25) ellagic acid; (26) quercetin; (27) phloretin; (28) isorhamnetin.

Table 3. Concentrations (mg/L) of phenolic compounds in wine samples analysed by HPLC-MS/MS.

		White wines									red wines								
No.	**Compound**	**VM01**	**VM02**	**VJ01**	**VJ02**	**VJ03**	**PE01**	**PE02**	**PA01**	**PA02**	**R001**	**R002**	**C001**	**C002**	**C003**	**VD01**	**VD02**	**VS01**	**VS02**
	phenolic acids																		
1	gallic acid	4.13	5.43	14.94	3.21	9.26	12.17	36.05	29.61	62.92	155.96	162.02	353.01	239.26	167.98	102.74	116.49	105.11	167.23
2	neochlorogenic acid	0.02	0.04	0.03	0.02	0.02	0.09	0.08	0.06	0.16	0.16	0.11	0.02	0.02	0.06	0.06	0.01	0.02	0.00
3	chlorogenic acid	0.12	0.30	0.34	0.23	0.23	0.26	0.08	0.32	0.09	0.22	0.18	0.13	0.11	0.12	0.20	0.12	0.07	0.04
4	*p*-hydroxybenzoic acid	1.11	1.48	1.06	0.93	0.97	0.68	0.73	0.72	1.81	1.74	3.54	1.77	1.32	3.84	3.80	1.67	3.60	3.24
5	3-hydroxy benzoic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
6	caffeic acid	1.45	7.21	7.41	9.78	17.97	6.18	4.22	2.32	3.49	35.66	33.98	22.43	20.34	12.84	17.02	21.07	15.36	55.84
7	vanillic acid	23.52	24.05	24.05	20.31	17.64	31.00	11.76	26.19	20.85	147.52	296.11	95.68	59.33	112.78	75.36	91.40	54.52	81.78
8	syringic acid	0.44	0.51	0.47	0.37	0.44	0.56	0.51	0.60	0.44	14.67	12.49	10.28	8.08	11.65	10.63	8.96	9.82	6.48
9	*p*-coumaric acid	1.58	6.12	4.16	7.62	14.57	5.30	2.06	2.11	1.64	11.87	12.39	4.83	7.16	8.89	4.52	6.19	2.96	13.45
10	ferulic acid	0.40	2.26	2.62	2.12	2.41	5.85	0.73	2.61	1.52	0.10	0.05	0.01	0.03	0.20	0.04	0.13	0.03	0.04
11	3,5-dicaffeoylquinic acid	0.013	0.014	0.021	0.013	0.013	0.015	0.008	0.022	0.015	0.026	0.047	0.010	0.010	0.014	0.006	0.004	0.006	0.010
12	ellagic acid	1.51	0.04	0.60	0.14	0.07	0.04	0.86	1.00	1.39	13.14	18.91	15.72	16.43	8.09	5.68	2.16	3.24	5.11
	flavonoids																		
	A) anthocyanins																		
13	delphinidin 3,5 diglucoside	0.02	0.01	0.30	0.01	0.01	0.01	0.08	0.01	0.01	0.26	0.22	5.57	1.02	0.35	0.19	0.88	0.15	0.04
14	delphinidin-3-galactoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5.79	7.22	4.11	12.04	4.77	0.57	0.69	0.18	1.38
15	cyanidin-3-glucoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.63	4.87	2.83	4.05	2.67	0.19	0.31	0.10	0.97
16	petunidin-3-glucoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5.05	6.53	3.91	9.62	4.66	0.59	0.92	0.21	1.20
17	pelargonidin-3-rutinoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
18	pelargonidin-3-glucoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.01	0.07	0.01	0.01	0.01	n.d.	n.d.	n.d.	n.d.
19	malvidin-3-galactoside	n.d.	0.030	0.015	0.003	n.d.	0.041	0.062	0.029	0.056	28.687	54.007	16.295	40.472	27.588	3.743	7.747	1.658	5.733
	B) flavonols																		
20	rutin	0.007	0.004	0.004	0.003	0.003	0.004	0.009	0.004	0.006	0.006	0.009	0.009	0.007	0.010	0.004	0.007	0.004	0.003
21	isoquercitrin	0.03	0.00	0.37	0.02	0.02	0.01	0.08	0.01	0.02	0.33	0.26	6.79	1.15	0.42	0.21	1.09	0.15	0.04
22	quercitrin	0.02	0.03	0.02	0.01	0.01	0.00	0.02	0.02	0.01	3.73	1.48	9.44	4.82	4.72	0.11	0.06	0.10	0.73
23	myricetin	0.02	0.02	0.01	0.03	0.01	0.01	0.01	n.d.	n.d.	4.96	8.74	17.84	19.90	5.00	5.77	3.71	1.84	4.02

24	kaempferol-3-glucoside	0.01	0.01	0.07	0.02	n.d.	0.01	0.01	0.01	n.d.	0.03	0.03	0.81	0.12	0.05	0.01	0.10	0.03	0.02
25	quercetin	2.03	0.03	0.02	0.22	n.d.	n.d.	0.01	0.01	0.01	9.84	17.78	35.88	21.66	4.28	11.76	14.17	4.36	12.84
26	isorhamnetin	0.028	0.002	0.002	0.008	0.002	0.001	0.001	0.002	0.002	0.406	1.730	0.897	0.702	0.196	0.339	0.316	0.135	0.262
27	hyperoside	0.04	0.01	0.74	0.02	0.01	0.01	0.15	0.01	0.03	0.51	0.28	10.20	1.80	0.51	0.36	1.61	0.23	0.05
28	kaempferol	0.04	0.01	n.d.	n.d.	n.d.	0.03	0.02	0.03	n.d.	0.92	0.58	9.01	3.63	0.03	0.87	0.90	0.16	0.70
	C) flavan-3-ols																		
29	catechin	7.53	9.60	15.65	6.18	10.56	6.20	16.27	7.42	11.07	36.55	63.82	52.95	69.12	51.45	60.04	41.18	47.41	74.39
30	epicatechin	0.87	2.39	4.90	0.76	1.23	1.49	5.07	0.97	2.04	8.77	17.69	7.52	16.68	11.02	9.85	5.41	7.17	13.62
31	procyanidin B2	0.68	1.79	7.76	0.75	1.36	1.45	8.09	2.53	3.31	32.08	63.60	31.51	69.10	34.09	34.94	18.02	16.45	36.44
32	procyanidin A2	0.09	0.06	0.06	0.09	0.07	0.04	0.03	0.05	0.06	0.17	0.07	0.03	0.02	0.05	0.07	0.07	0.03	0.08
	D) dihydrochalcones																		
33	phloridzin	0.22	0.19	0.28	0.24	0.22	0.08	0.15	0.07	0.10	0.85	0.71	1.52	1.38	0.82	1.01	0.85	0.76	0.81
34	phloretin	0.036	0.007	0.003	0.003	0.016	0.002	0.002	0.002	0.002	0.016	0.048	0.009	0.017	0.007	0.010	0.008	0.003	0.004
	E) flavanones																		
35	hesperidin	0.33	0.31	0.25	0.21	0.23	0.30	0.40	0.35	0.73	0.22	0.27	0.22	0.21	0.30	0.25	0.31	0.27	0.28
36	naringin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.71	n.d.	2.09	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	F) Stilbene																		
37	resveratrol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total phenolic content		46.27	61.95	86.16	53.30	77.34	71.85	91.25	77.08	113.88	522.89	789.84	721.23	629.63	479.46	350.94	346.33	276.14	486.81

VM: “Verdicchio di Matelica Riserva”; VJ “Castelli di Jesi Verdicchio Riserva”; PE: “Offida Pecorino”; PA: “Offida Passerina”; RO: “Offida Rosso”; CO: “Cònero”; VD: “Vernaccia di Serrapetrona” sweet; VS: “Vernaccia di Serrapetrona” dry.

The overall phenolic profile obtained among the 18 wine samples was rather variable, even between samples belonging the same DOCG. At first, what can be observed is the expected difference in the total amount of polyphenols between white and red wines, related to the two different vinification techniques (**Figure 9**).

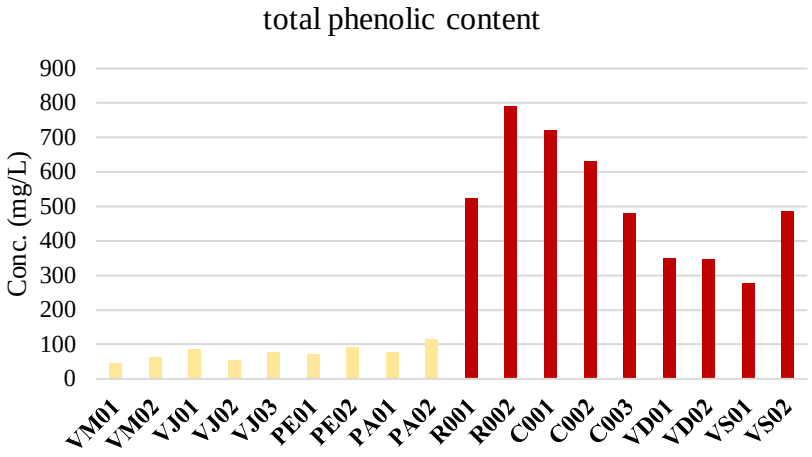


Figure 9. Total phenolic content in the 18 DOCG wine samples.

Indeed, while the average of phenolic content for white wines was 75.5 mg/L, for red wine samples it was 509.3 mg/L.

Regarding phenolic acids, eleven different compounds were detected and identified. In almost all the samples (expect for RO02), gallic acid was the most abundant phenolic acid, ranging from 3.3 to 62.9 mg/L for white wines and from 102.7 to 353.0 mg/L in red ones. Grape seeds and skin are rich in gallic acid and this explains the big differences between white and red wines since the second ones are produced with the maceration step, so that these analytes can be extracted from the skins in a higher extent with respect to the vinification of white grapes.

Gallic acid was detected in all samples, in the range 3.21-62.92 mg/L for white wines and 102.74-353.01 mg/L for red wine samples. White wines produced with Verdicchio grape variety were characterized by the lowest content of this acid. In fact, in "Verdicchio di Matelica Riserva" and "Castelli di Jesi Verdicchio Riserva" the compound was obtained with a mean concentration of 7.4±4.8 mg/L, while for the "Offida" white DOCGs the mean value was much higher (35.2±21.1 mg/L).

Salucci *et al*. (2017) studied the polyphenol profile of some Italian wines produced from grape varieties grown in the Marche region, including a sample of "Verdicchio dei Castelli di Jesi Doc", Classico Riserva and, in this case, gallic acid was found in a mean quantity of 42 mg/L. In the case

of DOCG "Castelli di Jesi Verdicchio Riserva" (VJ 01, VJ02 and VJ03) the values ranged from 3.3 to 14.9 mg/L, while for the two samples of "Verdicchio di Matelica Riserva" (VM01 and VM02, made with the same grape variety) it showed concentrations of 4.1±0.0 and 5.4±0.0 mg/L. Hence, in our case, the values found were much lower. The same can be observed for most of the other polyphenols that were identified and quantified. Caffeic acid was indeed found by Salucci *et al*. at a concentration of 69 mg/L, while in DOCG Verdicchio samples it ranged from 7.2 to 17.9 mg/L. Epicatechin, which showed the highest content of 139 mg/L, in the samples under investigation was obtained in a range of 0.76-5.07 mg/L. These results underline how the difference in *terroir* and vinification can greatly influence the final profile of a wine, even if produced with the same grape variety.

Concerning "Offida Passerina" white wines, the phenolic content was studied, together with "Verdicchio dei Castelli di Jesi", by Boselli *et al*. (2006). They performed the analysis in DOC wine samples through HPLC-DAD and HPLC-ESI-tandem MS. The comparison between the two wines made with the two homonymous grape varieties showed different and peculiar phenolic compositions. Boselli *et al.* reported that the Verdicchio was richer in phenolic acids compared to Passerina, while in our case, apart for gallic acid, the two wine typologies showed comparable content of this class of compounds.

Wines made with Passerina grape variety were also the subject of the study of Carbone & Fiordiponti (2016). In this case, the wine investigated was "Passerina del Frusinate", a wine with recognised geographical indication produced in Lazio region, but with the same grape variety of our samples "Offida Passerina" DOCG wines (PA01 and PA02). In Frosinone province, this wine can be produced with or without maceration and in the mentioned study the phenolic profile was assessed by HPLC-DAD in three different maceration times (0, 18 and 24 h). The values obtained for gallic, vanillic and syringic acids were not comparable to the ones obtained for DOCG "Offida Passerina" wine samples. Indeed, for "Passerina del Frusinate" samples the compounds were obtained in the ranges of 3.42-10.6 mg/L for gallic acid, 5.23-16.2 mg/L for vanillic acid and 3.3-8.5 mg/L for syringic acid. Differently, in the two samples of "Offida Passerina" under investigation, they showed 29.6±0.0 and 62.9±0.0 mg/L for gallic acid, 26.2±0.0 and 20.8±0.0 mg/L for vanillic acid and 0.6±0.0 and 0.44±0.0 mg/L for syringic acid. On the other hand, other phenolic acids were obtained with values more in accordance with our results. In fact, *p*-coumaric acid showed an average of 1.7 mg/L in the reported study and a concentration of 0.7±0.0 and 1.8±0.0 mg/L for the two DOCG wines. Finally, ferulic acid was found by Carbone & Fiordiponti with an average concentration of 1.5 mg/L, while in our two samples with concentrations of 2.6±0.0 and 1.5±0.0 mg/L.

The sample PA02 showed a much higher quantity of gallic acid as compared to all other white DOCG wine samples, suggesting that this phenolic compound may be used as a marker for the identification of this wine, since its quantity is twice compared to that found in the other sample "Offida Passerina" (PA01).

Concerning Pecorino grape variety and wine, no information is reported in literature regarding phenolic contents in "Offida Pecorino" wines, so that a comparison with literature and with wines produced with the same grape variety is not possible. However, referring to the previous cited works reporting results for white wines, it can be noted that PE01 showed a quantity of gallic acid in the range obtained by Barbone & Fiordiponti (2016). Differently, the content of gallic acid in the other "Offida Pecorino" wine sample (PE02) was 62.9±0.0 mg/L, relatively close to the value of 42 mg/L, found Salucci *et al.* (2017). On the other hand, vanillic acid for the same PE02 was obtained with values more in accordance with the study of Barbone & Fiordiponti.

Moreover, the phenolic acids content between the two samples of "Offida Pecorino" was very diverse. In fact, while PE01 is rich in vanillic acid with 31±0.0 mg/L and has a lower content of gallic acid of 12.2±0.0 mg/L, for PE02 the trend was the opposite, with quantities of 11.7±0.0 and 36.0±0.0 mg/L, respectively. Furthermore, PE01 is the white wine with the highest amount of vanillic acid (31±0.0 mg/L) and ferulic acid (5.8±0.0 mg/L), which could be markers for the identification of this wine.

Among the flavonoids, flavan-3-ols were the class detected in higher amount in white wines. Catechin for example, showed an average quantity of 10.0 mg/L and, as expected it was the most abundant flavan-3-ol, responsible for the characteristic taste of wine (Lunte *et al.*, 1988). Two samples in particular, the "Castelli di Jesi Verdicchio Riserva" (VJ01) and the sample "Offida Pecorino" (PE02), showed the highest concentrations, that were 15.6±0.0 and 16.3±0.0 mg/L, respectively, while other samples, showed lower concentrations, in the range 6.2-11.1 mg/L. The two samples showed also the highest amounts of epicatechin (4.9±0.0 and 5.1±0.0 mg/L, respectively) and procyanidin B2 (7.7±0.0 and 8.1±0.0 mg/L).

Interestingly, in the class of flavanones, naringin was only detected in "Offida Pecorino" (PE01) and "Offida Passerina" (PA02) wine, with low values of 3.7±0.0 and 2.1±0.0 mg/L, respectively. The two wines are made with different grape variety, but the values were quite similar. Anyway, the two samples greatly differ for the amounts of other compounds, such as gallic (36.0±0.0 and 62.9±0.0 mg/L, respectively) and vanillic acid (11.7±0.0 and 80.8±0.0 mg/L, respectively) so that, even in this case, a peculiar polyphenol profile can be highlighted and eventually used to discriminate the different wines. Moreover, the sample of "Offida Passerina" PA02 was the one with

the highest amount of gallic acid among all white wines, even if not comparable with the quantity present in red samples. For this reason, these compounds could be eventually considered as a marker of this wine in particular.

Concerning red wines, among phenolic acids, even in this case gallic and vanillic acids showed the highest concentrations. The first one ranged from 102.7 to 353.0 mg/L, while the second one from a lower value of 54.5 mg/L to 296.1 mg/L.

Caffeic acid showed a more peculiar behaviour. Indeed, in one sample of "Cònero" (CO03) it was obtained with a value of 167.9 mg/L, relatively close to the "Offida Rosso" DOCG (RO01, 155.9 and RO02, 162.0 mg/L) and to one sample of "Vernaccia di Serrapetrona" dry (VS02, 167.2 mg/L). Lower values were obtained by the other three samples of "Vernaccia di Serrapetrona" sweet and dry, being 102.7 mg/L for VD01, 116.5 mg/L for VD02 and 105.1 mg/L for VS01. Differently, very high values were obtained for two samples of "Cònero", being 353.0 mg/L for CO01 and 239.3 mg/L for CO02. Hence, caffeic acid did not show a characteristic behaviour that could be possibly link to the grape variety.

Vanillic acid, which was obtained as the second most abundant phenolic acid, with an average quantity of 112.7±74.3 mg/L, showed a great variability among samples. Even in this case, there was not a clear division between wine typology and, as an example, for the two samples of "Offida Rosso" (RO01 and RO02), this compound was obtained in two very different concentrations of 147.7 and 296.1 mg/L. Moreover, the RO02 was the sample with the highest amount of vanillic acid while for the other compounds it showed comparable amounts.

Salucci *et al*. (2017), reported the phenolic profile of a sample of "Cònero DOC Riserva", a wine produced with Montepulciano grape variety, the same used for "Offida Rosso" and "Cònero" wines in a minimum amount of 85%. Anyway, the results were not in agreement with the ones obtained for DOCG wines, which resulted much higher for all compounds. Gallic acid, in fact, was found in a concentration of 1898 mg/L, while in our samples it was found in the range 167.9-353 mg/L in "Cònero" wines and 155.9-162.0 mg/L in "Offida Rosso" wines.

Concerning the other phenolic acids, caffeic acid was found in the range of 12.8-55.8 mg/L. and, even in this case, the great variability among samples showed some peculiarities. The highest quantity was found in the sample of "Vernaccia di Serrapetrona" dry (VS02), while the other sample (VS01) showed one of the lowest quantities (15.4 mg/L). By contrary, syringic acid was obtained with an average value of 10.3 mg/L but it did not show a great variability among the samples.

Boselli *et al.* (2004) reported a study on the chemical and sensorial characterization of DOCG red wines from Marche region. In particular, the study regarded five different wines, including a

sample of “Rosso Cònero” (made with the minimum amount of 85% of Montepulciano grapes, as the “Rosso Cònero” DOCG) and “Vernaccia di Serrapetrona” sweet. They reported the absolute area values obtained by the HPLC analysis of phenolic substances finding that gallic acid, catechin and quercitin in the two wine typologies showed appreciable differences. Interestingly, in our case a clear distinction between wine typologies was not obtained, rather, same wine typologies showed variable amounts of these compounds. The three samples of “Cònero” wines, for example, showed different amounts of catechin (36.5, 63.8 and 52.9 mg/L), making the compound eventually suitable to discriminate the three different wines, while in the study of Boselli *et al.*, the “Cònero” wine samples did not show a great variability.

On the other hand, for caffeic acid, Boselli *et al.* obtained again a certain variability among the samples, in accordance with our results.

Sagratini *et al.* (2012) studied the volatile and polyphenolic content of Montepulciano monovarietal wines from Marche and Abruzzo regions, to see if the profile of both volatile and phenolic substances was able to discriminate the two different production regions. For Marche region wines, gallic acid was found in the range of 10.2-18.9 mg/L, much lower than the result obtained in the DOCG wine samples (105.1-353.1 mg/L). The same trend can be highlighted also for caffeic acid and *p*-coumaric acid, which in the case of DOCG wines showed higher concentrations. Ferulic acid was not detected at all by Sagratini *et al.*, while the samples under investigation showed a low range of concentrations (0.01-0.2 mg/L). On the other hand, resveratrol was found in mean concentration of 2.2 mg/L, while in the DOCG wine samples here investigated, it was always found below the LOD, and thus not quantified.

In the class of flavonoids, malvidin-3-galactoside was also found with a great variability. In this case the wines of “Vernaccia di Serrapetrona” (VS01, VS02, VD01 and VD02) were obtained with the lowest amount of this compound (2-8 mg/L), while the other samples showed much higher concentrations, but without a clear distinction between samples. In fact, the highest amounts of malvidin-3-galactoside were obtained for the sample “Offida Rosso” (RO02, 54 mg/L) and the “Cònero” wine samples (CO02 ,40 mg/L), followed by RO01 (29 mg/L) and CO03 (28 mg/L).

In the class of anthocyanins, delphinidin-3-galactoside, showed an interesting trend among samples. In “Vernaccia di Serrapetrona” it was obtained with very low values, in the range 0.2-1.4 mg/L. In the other samples the quantities were much higher and, in the sample, “Cònero” (CO02) it reached a concentration of 12.0 mg/L, which is also the highest as compared to all the other samples.

Quercetin was the most abundant flavonol and the highest quantity of 35.8 mg/L was found in a sample of “Cònero” wine. Anyway, a diverse trend between wine typologies was not evidenced

by quercetin amounts, since this compound showed very variable quantities among the same wine DOCG.

Flavan-3-ols were also obtained with very high quantities, but also high variability among samples. Catechin, for example, was found in the range of 36.5-74.4 mg/L being the lowest value obtained in the "Offida Rosso" sample (RO01) and the highest in a "Vernaccia di Serrapetrona" dry (VS02). This compound is also the most abundant in sample VS02, together with caffeic acid (55.8 mg/L), showing the highest concentration with respect to all the other samples.

In agreement with our results, also in the study reported by Boselli *et al.* (2004), catechin showed a modest variability in the "Vernaccia di Serrapetrona" wine samples.

Fiorini *et al.* (2013) reported the only study performed to assess the polyphenolic profile of "Vernaccia di Serrapetrona" with DOCG designation, investigating different vintages, and performing the analysis through HPLC-MS. The concentration of epicatechin reported in that study (0.8-13.9 mg/L) are similar to those found in the present investigation (5.4-13.6 mg/L), while gallic acid, catechin, caffeic acid and *p*-coumaric acid were found in much lower amount.

Environmental factors such as geographical production area, soil composition, climatic conditions and also viticultural practises play an essential role in the biosynthesis and accumulation of phenolic compounds in grape berries and thus, in the final phenolic composition in wine. Hence, the phenolic composition has been recognised as an important indicator for the differentiation of wine according to origin (Geana *et al.*; 2014; Jaits *et al.*, 2010). Sagratini *et al.* (2012), for example, reported the determination of phenolic substances, together with the volatile fraction, in Montepulciano monovarietal wines produced in Abruzzo and Marche region. The multivariate chemometric analyses underlined some significant differences in the phenolic and volatile profile of the wines produced in the two different Italian regions, suggesting that the different *terroir* was able to greatly influence the phenolic and volatile profiles of the final product.

3.3.2 Statistical analysis

To characterize the phenolic profile of the five DOCGs of Marche region, different statistical analyses were carried out. It was decided to divide the eighteen wine samples into eight groups, by considering also some DOCG "subclasses". Hence, the DOCG "Vernaccia di Serrapetrona" was divided into sweet and dry typologies and, the "Offida" DOCG was divided into three groups: "Offida Pecorino", "Offida Passerina" and "Offida Rosso". For "Offida" in particular, two wines are white

and the other is red, produced with three different grape varieties (Montepulciano, Pecorino and Passerina) so they cannot be considered under the same more general “Offida” DOCG, especially when comparing the phenolic content.

3.3.2.1 One-way ANOVA

One-way analysis of variance (ANOVA) coupled to Tukey’s test for pairwise comparison was thus performed on samples divided into the eight DOCG groups to assess possible significant differences (P <0.05) in their phenolic profile and the results are reported in **Table 4**.

Table 4. Average concentrations (mg/L) of the phenolic compounds in the different DOCG samples, divided into eight groups (distinguishing "Vernaccia di Serrapetrona" DOCG wines into two different groups: dry and sweet, VD and VS, respectively; and Offida DOCG into three groups: Pecorino, Passerina and Offida Rosso, PE, PA and RO, respectively).

	VM	RSD %	VJ	RSD %	PE	RSD %	PA	RSD %	RO	RSD %	CO	RSD %	VD	RSD %	VS	RSD %
Total phenolic content	46.27^{a,c}	20.5	86.16^{a}	23.5	71.85^{a,c}	16.8	77.08^{a,d}	27.3	522.89^{b,c}	28.8	721.23^{b,d}	9.6	350.94^{d}	0.9	276.14^{c,d}	39.1
phenolic acids																
gallic acid	4.78^{a}	19.2	9.14^{a}	63.4	24.11^{a}	70.0	46.27^{a}	50.9	158.99^{b}	2.7	253.42^{b,c}	26.8	109.6^{b,c}	8.9	136.17^{b}	32.3
neochlorogenic acid	0.03^{a}	47.1	0.02^{a}	24.7	0.09^{a}	8.3	011^{a}	64.3	0.14^{a}	26.2	0.03^{a}	69.3	0.08^{a}	101.0	0.01^{a}	141.4
chlorogenic acid	0.21^{a}	60.6	0.29^{a}	23.8	0.17^{a}	74.9	0.21^{a}	79.3	0.2^{a}	14.1	0.12^{a}	8.3	0.16^{a}	35.4	0.06^{a}	38.6
p-hydroxybenzoic acid	1.30^{a,b}	20.2	0.99^{b}	6.7	0.71^{a}	5.0	1.27^{b}	60.9	2.64^{b,c}	48.2	2.31^{b,c}	58.2	2.74^{b,c}	55.1	3.42^{c}	7.4
3-hydroxybenzoic acid	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
caffeic acid	4.33^{a}	94.1	11.72^{a,c}	47.3	5.2^{a}	26.7	2.9^{a}	28.5	34.82^{b,d}	3.4	18.54^{c,d}	27.2	19.05^{c,d}	15.0	35.6^{a,d}	80.4
vanillic acid	23.78^{a,c}	1.6	20.7^{b}	15.6	21.38^{a,b}	63.6	23.52^{a,b}	16.1	221.82^{a,c}	47.4	89.26^{c}	30.6	83.38^{c}	13.6	68.15^{a,c}	28.3
syringic acid	0.48^{a}	10.4	0.43^{a}	12.0	0.54^{a}	6.6	0.52^{a}	21.8	13.58^{b}	11.4	10.0^{b}	18.0	9.8^{b}	12.1	8.15^{b}	29.0
p-coumaric acid	3.85^{a}	83.4	8.78^{a}	60.4	3.68^{a}	62.3	1.88^{a}	17.7	12.13^{a}	3.0	6.96^{a}	29.3	5.36^{a}	22.1	8.21^{a}	90.4
ferulic acid	1.33^{a}	98.9	2.38^{a}	10.5	3.29^{a}	110.0	2.07^{a}	37.3	0.08^{a}	47.1	0.08^{a}	130.5	0.09^{a}	74.9	0.04^{a}	20.2
3,5-dicaffeoylquinic acid	0.014^{a}	5.2	0.02^{a}	29.5	0.01^{a}	43.0	002^{a}	26.8	0.04^{a}	40.7	0.01^{a}	20.4	0.01^{a}	28.3	0.01^{a}	35.4
ellagic acid	0.78^{a,b}	134.1	0.27^{a}	106.6	0.45^{a,b}	128.9	1.2^{b,c}	23.1	16.03^{d}	25.5	13.4^{d}	34.5	3.92^{a,c,d}	63.5	4.18^{a,c,d}	31.7
flavonoids																
A) anthocyanins																
delphinidin 3,5 diglucoside	0.02^{a}	47.1	0.11^{a,b}	157.0	0.05^{a}	110.0	0.01^{a}	0.0	0.24^{a,b}	11.8	2.31^{a}	122.8	0.54^{a}	91.2	0.1^{a}	81.9
delphinidin-3-galactoside	n.d.		n.d.		n.d.		n.d.		6.51^{a}	15.5	6.97^{a}	63.1	0.63^{a}	13.5	0.78^{a}	108.8
cyanidin-3-glucoside	n.d.		n.d.		n.d.		n.d.		3.75^{a}	42.2	3.18^{a,b}	23.7	0.25^{b}	33.9	0.54^{a}	115.0
petunidin-3-glucoside	n.d.		n.d.		n.d.		n.d.		5.79^{a}	18.1	6.06^{a}	51.2	0.76^{a}	30.9	0.71^{a}	99.3
pelargonidin-3-rutinoside	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
pelargonidin-3-glucoside	n.d.		n.d.		n.d.		n.d.		0.04^{a}	106.1	0.01^{a}	0.0	n.d.		n.d.	
malvidin-3-galactoside	0.03		0.01^{a}	94.3	0.05^{a}	28.8	0.04^{a}	44.9	41.35^{b}	43.3	28.12^{b}	43.0	5.75^{a}	49.3	3.70^{a}	78.0
B) flavonols																
rutin	0.006^{a}	38.6	0.003^{a}	17.3	0.01^{a}	54.4	0.005^{a}	28.3	0.008^{a}	28.3	0.009^{a}	17.6	0.006^{a}	38.6	0.004^{a}	20.2
isoquercitrin	0.015^{a}	141.4	0.14^{a}	147.9	0.05^{a}	110.0	0.015^{a}	47.1	0.29^{a}	16.8	2.79^{a}	125.1	0.65^{a}	95.7	0.095^{a}	81.9
quercitrin	0.025^{a}	28.3	0.013^{a}	43.3	0.01^{a}	141.4	0.015^{a}	47.1	2.61^{a,b}	61.1	6.33^{b}	42.6	0.08^{a}	41.6	0.42^{a}	107.3

myricetin	0.02[a]	0.0	0.017[a]	69.3	0.01[a]	0.0	n.d.		6.85[a,b]	39.0	14.25[b]	56.7	4.74[a,b]	30.7	2.93[a,b]	52.6
kaempferol-3-glucoside	0.01[a]	0.0	0.05[a]	78.6	0.01[a]	0.0	0.01		0.03[a]	0.0	0.33[a]	128.6	0.06[a]	115.7	0.03[a]	28.3
quercetin	1.03[a]	137.3	0.12[a]	117.9	0.01		0.01[a]	0.0	13.81[a,b]	40.7	20.6[a]	76.8	12.97[b]	13.1	8.6[a]	69.7
isorhamnetin	0.02[a]	122.6	0.0[a]	86.6	0.00[a]	0.0	0.002[a]	0.0	1.07[a]	87.7	0.60[a]	60.5	0.33[a]	5.0	0.20[a]	45.2
hyperoside	0.03[a]	84.9	0.26[a]	163.1	0.08[a]	123.7	0.02[a]	70.7	0.40[a]	41.2	4.17[a]	126.2	0.95[a]	89.7	0.14[a]	90.9
kaempferol	0.025[a]	84.9	n.d.		0.025[a]	28.3	0.03[a]		0.75[a]	32.1	4.22[a]	107.0	0.89[a]	2.4	0.43[a]	88.8
C) flavan-3-ols																
catechin	8.57[a]	17.1	10.80[a]	43.9	11.24[a,b]	63.4	9.25[a,b]	27.9	50.20[b,c]	38.4	57.84[c]	16.9	50.61[c]	26.4	60.9[c]	31.3
epicatechin	1.63[a]	65.9	2.30[a]	98.7	3.28[a]	77.2	1.5[a]	50.3	13.23[a]	47.7	11.74[a]	39.4	7.63[a]	41.1	10.40[a]	43.9
procyanidin B2	1.24[a,b]	63.6	3.29[a]	118.0	4.77[a,b]	98.4	2.92[a,b]	18.9	47.84[a,b]	46.6	44.9[b]	46.8	26.48[a,b]	45.2	26.45[a,b]	53.5
procyanidin A2	0.08[a]	28.3	0.07[a]	20.8	0.04[a]	20.2	0.06[a]	12.9	0.12[a]	58.9	0.03[a]	45.8	0.07[a]	0.0	0.06[a]	64.3
D) dihydrochalcones																
phlorizin	0.21[a,c]	10.3	0.25[a,c]	12.4	0.12[a,c]	43.0	0.09[a,c]	25.0	0.78[a]	12.7	1.24[b]	29.9	0.82[b,c]	32.84	0.79[a,b]	4.5
phloretin	0.01[a]	68.1	0.01[a]	102.3	0.00[a]	0.0	0.00[a]	0.0	0.03[a]	70.7	0.01[a]	48.1	0.01[a]	15.7	0.00[a]	20.2
E) flavanones																
hesperidin	0.32[a]	4.4	0.23[a]	8.7	0.35[a]	20.2	0.54[a]	49.8	0.25[a]	14.4	0.24[a]	20.3	0.28[a]	15.2	0.28	
naringin	n.d.		n.d.		3.71		2.09		n.d.		n.d.		n.d.		n.d.	
F) stilbenes																
resveratrol	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	

RSD% is the percentage standard deviation obtained among samples belonging to the same DOCG class. VM: “Verdicchio di Matelica Riserva”; VJ “Castelli di Jesi Verdicchio Riserva”; PE: “Offida Pecorino”; PA: “Offida Passerina”; RO: “Offida Rosso”; CO: “Cònero”; VD: “Vernaccia di Serrapetrona” sweet; VS: “Vernaccia di Serrapetrona” dry. Letters indicate significant differences between the concentrations (mg/L) in the 8 different DOCGs (One-way ANOVA, $P < 0.05$, Tukey’s test for pairwise comparison). When no letters and RSD % are reported, no replicates were available to performed ANOVA.

For most of the phenolic substances, significant differences (P <0.05) able to stand out one DOCG over the other did not arise. Anyway, some interesting peculiarities can be highlighted. Regarding phenolic acids, for example, the content of vanillic acid in the "Offida Rosso" was higher when compared to all other DOCGs (even if with P >0.05) even if this DOCG is produced with the same grape variety of "Cònero" wines.

In the class of flavan-3-ols procyanidin B2 seemed to be more characteristic of wines produced with Montepulciano grape variety, since it was present in higher amount in "Offida Rosso" and "Cònero" wines, compared to the "Vernaccia di Serrapetrona". The same trend for wines produced with Montepulciano grape variety was also observed for malvidin-3-galactoside in the class of anthocyanins and quercitrin among flavonols.

Hence, while red wines showed a certain discrimination among DOCG, the phenolic profiles of white DOCG wines were more comparable, even though made with different grape varieties (Verdicchio, Pecorino and Passerina).

3.3.2.2 Principal components analysis

The PCA was then performed on the results to identify homogenous groups of data, taking into consideration the 34 phenolic compounds as variables and the 18 wine samples. The first two principal components (PC1 and PC2) were able to explain the 66.7% of the total variance and the score plot of the samples obtained highlighted the presence of some clusters of data (**Figure 10**).

Figure 10. Principal component analysis and related score plot, obtained using all phenolic substances in the DOCG wine samples investigated. VM: "Verdicchio di Matelica Riserva"; VJ "Castelli di Jesi Verdicchio Riserva"; PE: "Offida Pecorino"; PA: "Offida Passerina"; RO: "Offida Rosso"; CO: "Cònero"; VD: "Vernaccia di Serrapetrona" sweet; VS: "Vernaccia di Serrapetrona" dry.

The first component (PC1) was the one explaining most of the variability among samples (48.6%) and a clear division of samples was obtained for white wine samples (in the left part of the score plot, in the green circle) from the other red wine samples, due to their comparable phenolic profiles. Indeed, as previously discussed, all white wine samples contained small concentrations of the phenolic substances, and the statistical analysis did not point out any significant difference in their composition (**Table 4**).

By contrary, red wines did not show a circumscribed cluster and they were more homogeneously distributed along the two components. The samples "Vernaccia di Serrapetrona", sweet and dry, were close, thus exhibiting a similar phenolic content (red circle). Moreover, the cluster including "Vernaccia di Serrapetrona" wine samples was positioned between the grouping of white DOCGs (green circle) and the more distributed samples "Cònero" (CO01, CO01 and CO03) and "Offida Rosso" (RO01 and RO02) samples, meaning that this DOCG exhibits an intermediate

behaviour when considering the phenolic substances composition. “Vernaccia di Serrapetrona” is a red wine, but, at the same time, it is characterized by a lower abundance of substances such as anthocyanins. These are pigments able to contribute to the colour and their lower concentrations when compared to the other red DOCGs (**Table 4**) explains the less intense colour that characterizes the DOCG and thus also the proximity of the wine samples to the white DOCGs in the score plot.

Finally, while red wine samples RO01 and CO03 were positioned relatively close to the “Vernaccia di Serrapetrona” red wines, the remaining wine samples (CO01,CO02 and RO02) showed a more pronounced peculiar behaviour. This was also highlighted by the heatmap in **Figure 11** where these three samples (CO01, CO02 and RO02) were positioned in a distinct cluster (bottom part).

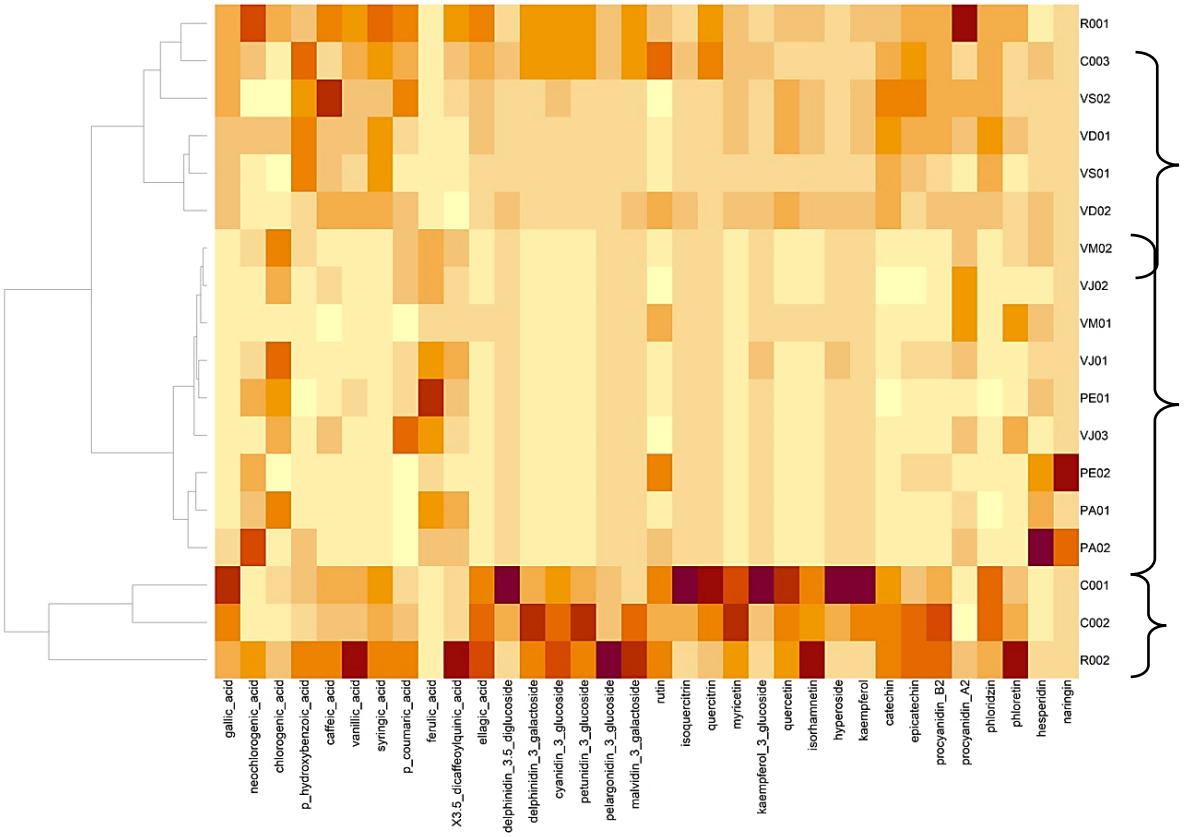


Figure 11. Heatmap of the phenolic substances concentration in DOCG wine samples investigated (variables clustered on the vertical axes). VM: “Verdicchio di Matelica Riserva”; VJ: samples of “Castelli di Jesi Verdicchio Riserva”; PE: “Offida Pecorino”; PA: “Offida Passerina”; RO: “Offida Rosso”; CO: “Cònero”; VD: “Vernaccia di Serrapetrona” sweet; VS: “Vernaccia di Serrapetrona” dry.

The other upper cluster, comprising all the other wine samples, was in turn divided into two clusters, one grouping white wine samples (bottom cluster) and the other red wine samples (upper cluster).

3.4 Conclusions

The results obtained for the study of the phenolic profile in the DOCG wines and the statistical analyses performed, highlight that, beyond the main differences between red and white wines, every wine sample was characterised by a peculiar phenolic content profile. When considering the five DOCGs, significant differences raised mainly between wines produced with diverse grape varieties. The DOCG wines produced with Montepulciano grape variety ("Offida Rosso" and "Cònero"), for example, were comparable and were also the ones showing generally highest concentrations of phenolic substances such as procyanidin B2. By contrary, other compounds, such as catechin, were found in comparable concentrations in wines made with Montepulciano and Vernaccia Nera grape varieties. Interestingly, the "Offida Rosso" DOCG seemed to be particularly rich of vanillic acid when compared to all other DOCGs, making this compound a possible authenticity marker of this wine typology. Finally, for white DOCG wines the overall phenolic profile did not show significant differences, even if produced with three different grape varieties and other classes of compounds could be probably more useful to be exploited to identify authenticity markers.

The analysis of the same wines produced in other years could help to confirm if the profile is specific to the wine or depends on the pedoclimatic conditions of the specific year. Furthermore, other DOCGs produced in Italy should also be taken into consideration in order to better investigate the presence of peculiarities which may be attributed to a characteristic DOCG wine of Marche region.

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4: Investigation of elemental composition

4.1 Introduction

Traceability aims to link food with the soil from which its raw material is originated. Traceability in wine industry has a very important role since it ensures the registration on specific documents of all manipulations of raw materials, ingredients and the final products. Moreover, wine is an easily adulterated product due to its chemical composition. Wine supply chain requires traceability from grape production to processing and distribution and, in this context, minerals can have an important role (Palade & Popa, 2014). In fact, in literature some works reported that the lanthanides are able to maintain their distribution passing from soil to plants, suggesting that they may be used as geographical markers to find the provenance of a particular foodstuff (Tyler, 2004). This linkage between soil and food has been reported by authors for different food products such as tomatoes or hazelnuts from Piemonte region (Bettinelli *et al.*, 2005; Oddone *et al.*, 2009), suggesting that this relationship may be used also for wine traceability.

However, the situation is more complex for wine since the production chain involves several steps. In fact, besides the natural mineral content of the soil and the grape vine's capacity to uptake and accumulate minerals in grapes, there are also a huge number of factors which can alter the final composition. In fact, the winegrower is also an important variable in shaping a wine's sensorial attributes by choosing vine variety and vineyard management practices depending on local soil and climatic conditions (Ferretti & Febbroni, 2022).

At the same time, the investigation of wine mineral profile has been already used as an interesting tool to discriminate the geographical origin, because it reflected the vineyard soil mineral composition from which it derives (Aceto *et al.*, 2013; Almeida & Vasconcelos, 2003; Geana *et al.*, 2013; Pérez-Álzarez *et al*., 2019; Galgano *et al.*, 2008). Some works reported also that the distribution of lanthanides is maintained unaltered between soil and must and thus, the elemental analysis was exploited to assess wine origin (Aceto *et al.*, 2009).

Shimizu *et al.* (2018) reported the study of elemental composition that was used to discriminate Japanese wines produced in different areas. This approach was used to study the provenience of Italian wines, such as Nebbiolo or Moscato, as reported by some authors (Marengo & Aceto, 2003; Aceto *et al.*, 2013). More recently Grainger *et al.* (2021) analysed the elemental composition of 14 wines made with the same clone of *Vitis vinifera* cv. Pinot noir. They analysed 47 different minerals and they observed how the wine composition was affected by soil and microclimate conditions; indeed, in some cases their amounts varied by 10-fold among samples. Pepi & Vaccaro

(2018) reported the study of major and trace elements to assess the geochemical fingerprint of Prosecco wines from four distinct wineries located in the Veneto alluvial plain.

Minerals of primary and natural origin, which came from soil, comprise the largest total metal content in wine; anyway, the overall metal composition can be considered as having a two-fold origin, divided into endogenous and exogenous sources (**Figure 1**).

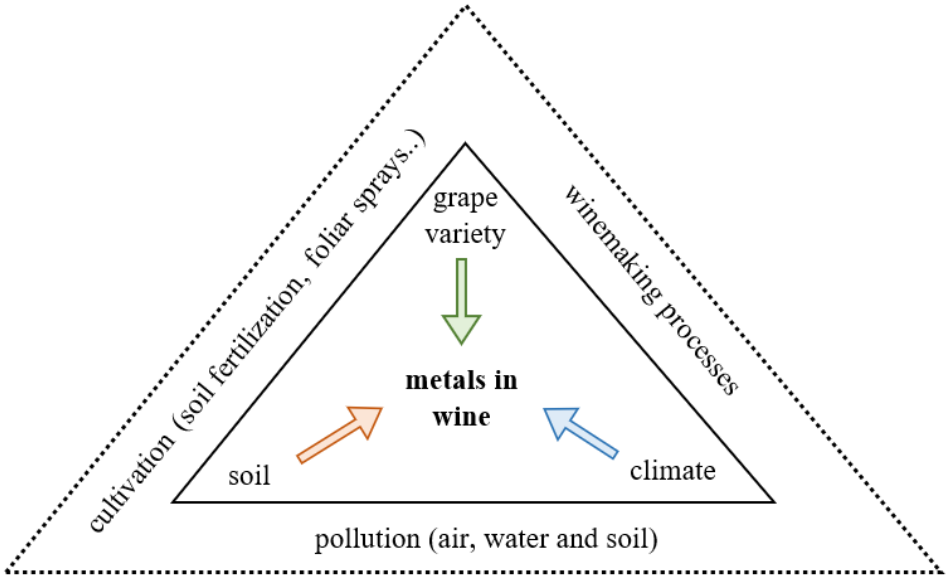

Figure 1. Endogenous (solid line) and exogenous (dotted line) sources of metals.

The endogenous sources can be considered those natural aspects related to the environment in which grapes are cultivated such as the soil composition, the climatic conditions, but also the selected grape variety. By contrary, exogenous factors can be considered as those derived from human impact, hence including environmental pollution, the use of fertilizers and/or insecticides and winemaking techniques which are also aspects able to affect the final minerals concentrations in wine (Hopfer *et al.*, 2015). Concerning winemaking processes, for example, the fining treatments used to remove proteins and to reduce the risk of protein haze in wine involves the addition of clay material (bentonite) whose main constituent is montmorillonite, that contains sodium and calcium (Catarino *et al.*, 2008). This means that the addition of bentonite increases the final content of Na and Ca, but it can affect also the content of other minerals such as Li, Be, Mg, Al, Sc, V, Mn, Fe, Co, Ni, Ga, Ge, As, Sr, Y, Zr, Nb, Mo, Cd, Sn, Sb, Ba, W, Tl and Bi (Catarino *et al.*, 2008). For red wines in particular, the length and intensity of the maceration steps, which are adjusted and modified depending on the desired characteristics of the final product, can also have an important influence on the wine mineral composition. In fact, it has been observed that when the maceration is prolonged, the concentration of minerals such as Cr, Cu, Fe and Zn increase (Pohl, 2007). Moreover, the final wine ageing step can impact the mineral composition. Indeed, it has been observed that the concentrations of some

minerals such as Al, Fe and V increase during oak barrel ageing (Almeida & Vasconcelos, 2003; Hopfer *et al.*, 2015). By contrary, other minerals like Mg, V, Co, Ni and Sr, increase during ageing in oak staves (Kaya *et al.*, 2017).

The final elemental composition in wine is, therefore, the result of different factors and some of them are out of control or not-discriminating at all. Depending on the discriminating power, three different groups of elements can be pointed out as reported by some authors (Marengo *et al.*, 2003; Volpe *et al*., 2009; Pohl, 2007):

- Elements whose concentrations are not influenced by the production cycle, but by the mineral composition of the soil or the capacity of the vine to uptake them, which is influenced by the rootstock of the vine. The elements that belong to this class are usually Al, B, Ba, Li, Mn, Mo, Rb, Si, Sr and Ti (Geana *et al.*, 2013).
- Elements that are present as the sum of natural and production cycle sources, which are Ca, Fe, Mg, Cu, Zn, F, K, Na, P and that can consequently vary greatly during winemaking.
- Elements whose concentrations can be totally attributed to artificial factors, as in the case of Pb that is due to fungicidal treatments. This class comprises also elements such as Co, Hg, Cr, Ni and V.

Anyway, also the elements that do not belong to the first group can be used to discriminate different wines, even if not from a geographical point of view, since also winemaking techniques can afford peculiarities which can help wine authentication.

Elements can be also classified in terms of their abundances in grapes. The main elements present are Al, Fe, Cu, Mn, Rb, Sr and Zn which can range from 0.1 to 10 mg/L, while the trace elements are normally Ba, Cd, Co, Cr, Li, Ni, Pb and V and they can be normally found in the range 0.1-1 mg/L (Pérez-Álvarez *et al.*, 2019).

Beyond the importance of minerals for the authenticity and traceability, they play also important roles in other contexts. Fe, Zn, Cu, Cr and Se, for example, are essential for human's organism because they form an integral part of some enzymes which are involved in the metabolism and other biochemical processes (Pan *et al*., 2013).

On the other hand, several metals and metalloids, as in the case of Pb and Cd, are known for their toxicity (Galani-Nikolakaki *et al*., 2002). For this reason, the OIV (Office International de la Vigne et du Vin) has determined the acceptable levels of some metals in wine, whose concentrations are listed in **Table 1**.

Table 1. Maximum acceptance limits for metals (OIV).

Metal	Concentration (mg/L)
Arsenic	0.2
Boron	80
Bromine	1
Cadmium	0.01
Silver	< 0.1
Fluorine	1
Zinc	5

(OIV Official Website. Available online: https://www.oiv.int/public/medias/3741/e-code-annex-maximum-acceptable-limits.pdf)

Minerals are also important for the efficiency of the alcoholic fermentation and for the prosthetic metallo-enzyme activation of the yeasts (Rodriguez Mozaz *et al.*, 1999).

Even if they constitute a relatively low portion in wine, they can have a relevant impact on it, by contributing to stability, clarity and conservation, i.e. precipitation of K and Ca under the form of tartrate salts (McKinnon & Scollary, 1997). Potassium, indeed, is an important mineral, but whose accumulation brings to a not negligible pH increase. This is the reason why its concentration is usually mitigated by the addition of tartaric acid during winemaking.

Concerning the sensorial characteristics, minerals are able to affect flavour, freshness, aroma, colour and taste, even if the excessive presence of elements such as Al, Fe, Zn and Cu can give negative effects on the organoleptic properties of the wine (Gennaro *et al.*, 1986).

Starting from these premises the analysis of minerals in wine is an important tool to trace and authenticate the provenience. For this reason, the elemental analysis of the 18 DOCG wine samples produced in Marche region (listed in **PART 1**, **Table 9**) was performed to identify their mineral content and to observe if differences raised between samples produced in different areas of the region, due to the different soil composition. A comparison was performed between all samples, but also among samples divided according to the DOCGs to point out, if present, significant differences and peculiarities which can differentiate and characterize the five DOCGs of Marche region.

4.1.1 Soil characteristics of Marche Region DOCG wines

Marche region is characterized by a great *terroir* diversification due to both the influence of the Apennine Mountains to the west and the Adriatic Sea to the east (Magnani *et al.*, 2019). Consequently, even if produced in a relatively small geographical area, the mineral profile of the

DOCG wines produced in Marche region can be very different, due to the different soil composition and climatic conditions.

The characteristics of the soils of provenience for the five wine typologies under investigation are listed:

1) “Verdicchio di Matelica Riserva”: The soil is mostly characterized by limestone-pelitic rocks (32%, thus) and marly and calcareous rocks (26%). Conglomerate and arenite substrates are present together with deposits belonging to Pleistocene terraces.

2) “Castelli di Jesi Verdicchio Riserva”: The hilly areas are characterized by a high presence of clays, calcium carbonate, poor permeability, erodibility, diverse pelitic and calcarenitic fraction.

3) “Offida Pecorino”, “Offida Passerina” and “Offida Rosso”: The soils are cambisols and they are characterized by a good aggregation and porosity. In these areas a homogenous distribution of carbonates is present.

4) “Cònero”: The geographical area is characterized mainly by milestone and cretaceous rocks.

5) “Vernaccia di Serrapetrona” sweet and dry: Cultivation soils are thin and stony, directly developed on the limestock-marl rock. The ground is mostly calcareous, stony and rarely fluvic. They are arenaceous-clayey with the presence of sands due to marine sedimentation.

Therefore, the geographical areas of grape cultivation used for production the DOCGs wines in Marche region are very diverse, hence a certain degree of differentiation should be transferred in the final wine mineral content. Important variances in the content of some minerals can arise from parameters, such as vinification processes, as previously discussed, which can modify the real mineral content present in the soils of origin and in grapes. At the same time, some minerals remain unaltered during wine production processes, so that these should be the ones investigated more in detail in order to obtain, if present, important information about the geographical area of origin.

4.2 Materials and methods

4.2.1 Sample preparation

Previous assays suggested that preparation of wine samples by microwave digestion was not appropriate due to a significant loss of elements. For this reason, wine samples were simply diluted and ready for direct analysis by ICP-MS. The dilutions (1:20) were performed in 500 µL of wine

samples in both acid and basic forms, using HNO_3 or NH_3, respectively.

4.2.2 Calibration and quantification

Quantification of the 40 elements was performed by constructing calibration curves using four different standard mixtures:

1. Minor elements quantification was performed with different standards mixture. Multi elements calibration standard containing Ag, Al, As, Ba, Be, Ca, Cd, Co, Cr, Cs, Cu, Fe, Ga, K, Li, Mg, Mn, Na, Ni, Pb, Rb, Se, Sr, Ti, U, V, and Z at a concentration of 10 mg/L. Multi elements calibration standard containing Au, HF in traces, Ir, Pd, Pt, Rh, Ru, Te and S at a concentration of 10 mg/L in 5% HNO_3. Appropriate dilutions with 1% HNO_3 were performed to obtain standard mixtures at eight different concentrations (0.01, 0.1, 1, 5, 10, 50, 100 and 500 µg/L).
2. Major elements calibration was performed by using Na, K, Ca, Mg, P and S standards at 1000 mg/L in 1% HNO_3, by preparing mixture standards at different concentrations (0.5, 1, 2.5, 5, 10, 25, 50 and 100 µg/L) by appropriate dilutions with 1% HNO_3.
3. Mercury calibration curve was prepared starting from a 10 mg/L Hg primary standard (in 1% HNO_3) to obtain five different concentrations (0.1, 0.5, 1, 5 and 10 µg/L). Au was added (2 mg/mL) to prevent Hg reduction.
4. Basic calibration for S, Cl, S, Br, I was performed by diluting with NH_3 a standard mixture containing the elements (at 10 mg/L in H_2O) to obtain eight different concentrations (0.1, 1, 5, 10, 50, 100, 500 µg/L).

4.2.3 ICP-MS analysis

The analysis for multi-element characterization was performed using 7500cx Series ICP-MS (Agilent Technologies, Santa Clara, USA) equipped with an autosampler. The operating conditions of the instrument are shown in **Table 2**.

Table 2. ICP-MS operating conditions.

Power	1500 W
Carrier gas (He)	1.11 L/min
Make-up gas	0.00 L/min
Sample depth	7.5 mm
Nebulizer pump	0.1 rps

Spray chamber Temperature	2 °C

4.2.4 Statistical analysis

One-way analysis of variance (ANOVA) followed by Tukey's test for pairwise analysis was used to submit data to assess significant differences (P <0.05) between the concentrations of the elements among the different DOCGs or to compare amounts between white and red wine DOCGs. The principal component analysis (PCA) was also performed to identify homogenous groups of data (cluster), by using R-based software CAT (Leardi *et al.*, 2021).

4.3 Elemental composition of the DOCG wines from Marche region

4.3.1 Results and discussion

The elemental composition of the 18 DOCG wine samples under investigation (listed in **PART 1**, **Table 9**), determined by means of ICP-MS analysis, is reported in **Table 3**.

Table 3. Major and trace elements obtained by the ICP-MS analysis of DOCG wine samples, expressed in mg/L.

	VM01	VM02	VJ01	VJ02	VJ03	PE01	PE02	PA01	PA02	RO01	RO02	CO01	CO02	CO03	VD01	VD02	VS01	VS02	RSD% Range
Li	0.02	0.01	0.02	0.02	0.01	0.02	0.02	0.01	0.03	0.03	0.02	0.03	0.02	0.02	0.02	0.01	0.02	0.01	0.07-0.28
Be	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.002.16
B	8.16	5.53	4.65	5.90	5.42	6.55	7.12	6.27	6.86	7.46	8.47	7.01	7.22	7.50	8.08	5.37	7.20	5.88	0.05-0.25
Na	16.40	14.39	23.53	13.70	18.45	18.93	25.70	15.86	23.83	23.42	20.47	15.64	19.06	21.11	11.54	6.80	7.38	13.07	0.06-0.6
Mg	88.85	86.15	91.15	89.35	83.15	99.90	107.55	93.85	112.05	129.90	132.95	119.45	118.05	195.05	107.10	88.75	102.65	117.70	0.08-0.22
Al	0.42	0.50	0.74	0.32	0.45	1.71	0.95	0.54	0.97	1.83	0.43	0.23	0.44	0.65	0.29	0.68	0.38	0.33	0.17-0.45
P	297.65	248.25	279.15	207.60	223.65	196.90	349.70	244.40	312.25	366.80	297.95	437.10	337,55	303.80	232.15	261.25	212.60	289.85	0.07-0.33
K	592.00	646.50	726.50	671.50	711.00	885.50	959.50	861.00	827.00	1140.0	1269.50	820.50	942.00	1028.0	1052.5	717.50	1019.50	1091.0	0.08-0.20
Ca	43.32	77.15	57.25	55.45	56.75	93.90	107.00	71.55	107.65	68.10	68.10	52.45	46.26	72.90	53.25	56.50	58.30	93.40	0.08-0.29
Si	26.31	28.19	36.25	39.74	38,59	34.29	34.41	35.26	31.39	37.65	40.74	32.80	33.52	34.31	29,26	31.89	32.87	41.47	0.10-0.50
S	75.70	140.15	99.65	159.80	149.35	217.35	169.40	176.25	223.05	233.7	184.15	145.3	228.15	275.65	147.10	96.00	244.15	190.15	0.14-0.99
Cl	10.44	10.73	22.03	17.74	13.54	29.76	17.54	23.57	19.80	25.02	22.96	20.52	18.61	22.41	21.75	10.55	17.91	19.71	0.14-0.98
V	0.00	0.00	0.00	0.02	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0,00	0.00	0.00	0.00	0.01	0.00	0.20	0.08-0.87
Cr	0.01	0.02	0.01	0.01	0.01	0.03	0.02	0.02	0.02	0.04	0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.04	0.09-0.33
Mn	0.42	0.55	0.45	0.46	0.30	0.67	0.78	0.75	0.78	1.06	0.93	0.62	0.92	1.64	0.34	0.33	0.36	1.00	0.10-0.25
Fe	0.75	0.34	0.35	0.44	0.27	1.00	1.80	0.54	1.80	1.68	1.26	1.01	1.63	1.57	0.92	1.12	1.18	2.86	0.07-0.28
Co	0,00	0.00	0,00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.20-0.61
Ni	0.00	0.01	0.02	0.01	0.00	0.26	0.01	0.02	0.02	0.57	0.01	0.01	0.01	0.03	0.01	0.08	0.01	0.02	0.10-1.06
Cu	0.02	0.64	0.09	0.02	0.04	0.05	0.04	0.29	0.02	0.13	0.02	0.06	0.07	0.39	0.18	0.06	0.22	0.04	0.06-0.27
Zn	0.59	0.45	0.39	0.27	0.26	2.37	0.44	0.48	0.51	3.59	0.60	0.28	0.64	0,68	0.46	0.48	0.45	0.75	0.09-0.33
Ga	0.02	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.04	0.02	0.02	0.03	0.04	0.03	0.03	0.03	0.06	0.27-0.46
As	0.01	0.01	0.01	0.02	0.02	0.02	0.01	0.01	0.01	0.02	0.02	0.01	0.02	0.02	0.02	0.02	0.01	0.03	0.53-1.24
Se	0.01	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.00	0.01	0.01	0.00	0.01	0.01	0.00	0.00	0.01	0.01	0.00-3.82

Rb	1.22	1.32	1.43	1.14	1.23	1.19	1.33	1.19	1.18	1.61	1.20	1.94	1.88	1.68	1.20	1.09	1.11	1.34	0.04-0.22
Sr	0.00	0.42	0.49	0.49	0.34	0.53	0.70	0.42	0.75	0.78	0.76	0.74	0.67	0.97	0.79	1.16	0.70	1.04	0.08-0.21
Ru	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00-3.65
Pd	0.01	0.00	0.01	0.01	0.14	0.01	0.01	0.01	0.01	0.02	0.01	0.07	0.02	0.09	0.03	0.07	0.00	0.01	0.17-1.13
Ag	0.02	0.00	0.00	0.03	0.02	0.02	0.00	0.02	0.00	0.02	0.00	0.02	0.02	0.02	0.03	0.03	0.02	0.00	0.00-4.03
Cd	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00-3.53
Sn	0.01	0.00	0.00	0.00	0.00	0.01	0.00	0.05	0.00	0.02	0.00	0.01	0.02	0.02	0.00	0.01	0.01	0.00	0.15-1.93
Sb	0.01	0.00	0.00	0,00	0.00	0.00	0.00	0.01	0,00	0,01	0.00	0.01	0.01	0.00	0.00	0.01	0.00	0.00	0.35-3.83
Cs	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.01	0,00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03-0.26
Ba	0.09	0.06	0.06	0.05	0.06	0.07	0.09	0.06	0.10	0.16	0.11	0.07	0.13	0.17	0.15	0.12	0.14	0.29	0.04-0.24
Au	0.01	0.00	0.00	0.01	0.08	0.00	0.01	0.01	0.00	0.02	0.01	0.16	0.02	0.18	0.02	0.14	0.00	0.01	0.16-2.16
Hg	0.01	0.06	0.00	0.01	0.00	0.00	0.08	0.00	0.06	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.00-4.98
Tl	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.23-1.67
Pb	0.01	0.01	0.01	0.01	0.01	0.03	0.02	0.02	0.03	0.02	0,00	0.01	0.01	0.04	0.00	0.01	0.01	0.01	0.08-0.31
U	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00-2.22
Br	0.03	0.03	0.04	0.03	0.03	0.04	0.04	0.04	0.04	0.04	0.16	0.06	0.04	0.05	0.05	0.03	0.04	0.06	0.34-1.29
I	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.01	0.01	0.10-0.52

RSD% range is referred to the relative standard deviation range obtained between wine samples, for each element. VM: “Verdicchio di Matelica Riserva”; VJ “Castelli di Jesi Verdicchio Riserva”; PE: “Offida Pecorino”; PA: “Offida Passerina”; RO: “Offida Rosso”; CO: “Cònero”; VD: “Vernaccia di Serrapetrona” sweet; VS: “Vernaccia di Serrapetrona” dry.

The results show that the levels for the selected hazardous metals never exceed the maximum acceptance values dictated by OIV, reported in **Table 1**.

There is not a clear difference between the average values obtained for white wines and for red samples. In fact, considering the most abundant element, which is Na, it was obtained with an average value of 19.0±4.4 mg/L for white wines and 15.4±6.1 mg/L for red ones (P >0.05). The elements Si, S and Cl showed similar concentrations (33.8±4.5 and 34.9±4.1 mg/L, 156.7±48.5 and 193.8±57.2 mg/L and 18.4±6.3 and 19.9±4.2 mg/L, respectively, P >0.05). Differently, K showed a lower concentration for white wines, being 764.5±123.8 mg/L and a higher value of 1008.9±165.5 mg/L for red wines (P <0.05).

A more detailed analysis of the results can highlight interesting observations. Some elements showed a relatively high variability among samples, even between samples of wines whose grapes are cultivated in the same area. For example, Ca was obtained in concentrations of 43.3 and 77.1 mg/L in the two samples of "Verdicchio di Matelica Riserva" (VM01 and VM02), but the variable amounts of the element can be linked also to winemaking practices. Also, S showed very diverse values, being one 75.7 mg/L and the other 140.1 mg/L in the two samples. S also varied greatly among the samples "Castelli di Jesi Verdicchio Riserva" VJ01, VJ02 and VJ03, ranging from 99.6 to 238.4 mg/L. Anyway, like Ca, also this element should not be considered when searching for elements able to discriminate geographical origin of wines. Indeed, sulphur dioxide derivatives can be added in several chemical forms in grape musts and juice as antioxidant and anti-microbial agents and they are subsequently converted partially into sulphide by the action of yeast. Thus, sulphur content does not reflect soil composition.

Concerning the other elements, the five Verdicchio wine samples showed some similarities, that can be possibly attributed to the close geographical production area and grape variety. Mg, for example, is a metal considered to arise only from soil, without important modifications due to winemaking processes. In fact, the five samples of Verdicchio show comparable values of this elements (with mean areas of 87.5±2.0 mg/L for "Verdicchio di Matelica Riserva" wines and 87.8±4.2 mg/L for "Castelli di Jesi Verdicchio Riserva", P >0.05). The same can be highlighted also for Mn (mean values being 0.49±0.1 and 0.40±0.1 mg/L, respectively).

The samples "Offida Pecorino", "Offida Passerina" and "Offida Rosso", even if produced with the relative homonymous grape varieties, are produced in the same geographical area, and this is in accordance with their mineral composition, since for almost all elements the samples showed comparable results. In particular, the two samples "Offida Rosso" (RO01 and RO02) were characterized by a very similar elemental profile and the most variable elements between all samples

of Offida wines was Ca, ranging from 71.5 to 107.6 mg/L (but with no significant differences when comparing the mean values for the three DOCGs). At the same time, Mg showed a significant difference (P <0.05) between white (mean value of 94.7±9.8 mg/L) and red samples (mean value of 123.5±30.1 mg/L).

"Cònero" DOCG is produced in an area which is relatively close to the one of "Castelli di Jesi Verdicchio Riserva", but for some elements, such as Mg, K, Fe, the samples of "Cònero" wines (CO1, CO02 and CO03) showed slightly higher results, statistically significant for K and Fe (P <0.05). The other elements were obtained with similar quantities.

Finally, "Vernaccia di Serrapetrona", is a wine produced in a geographical area near the production area of Verdicchio. Apart for elements such as Mg and Fe, the other elements were obtained with quantities relatively close to the ones obtained for the Verdicchio wines. Mg was obtained with a mean concentration of 104.1±12.0 mg/L while in Verdicchio wines it was obtained in the range in concentration 87.7±3.1 mg/L; while Fe was obtained in a concentration of 1.52±0.9 mg/L in the Vernaccia samples and 0.43±0.2 mg/L in the Verdicchio ones (and in both cases P <0.05).

Concerning "Offida Pecorino" and "Offida Passerina" white wines, Biancolillo *et al.* (2022) recently performed a varietal discrimination between these two wines made with the same grape variety, together with Trebbiano, since the three varieties belong to the same Trebbiano family. This study was performed by assessing volatile, phenolic and mineral profile and submitting results to several statistical analysis, coupled also to sensorial evaluation.

Among the most abundant minerals, Na was obtained with an average value of 17.8 mg/L in the "Offida Passerina" wine samples, while in "Offida Pecorino" wines it was found in a mean concentration of 22.5 mg/L. The results are completely in accordance with our values, since for "Offida Passerina" the mean between the two samples was 19.8±5.6 mg/L, while for "Offida Pecorino" 22.3±4.8 mg/L. Mg in the study of Biancolillo *et al.* was obtained in very similar concentrations when comparing Passerina and Pecorino wines (79 and 77 mg/L, respectively). Even if obtained with higher values, also the "Offida Passerina" and "Offida Pecorino" DOCGs showed comparable mean values (102.9±12.9 and 103.7±5.4 mg/L) and this difference may be attributed to the different geographical areas. The samples of Pecorino were slightly richer in terms of K, and this trend was also found in our samples when comparing the two different wine typologies (922.5 mg/L in Pecorino samples and 844.0 mg/L in Passerina ones). In general, also the composition for other minerals, such as Ca, Mn, Fe and Sr, was comparable in the two wines typologies, while when comparing the mean values obtained for the Marche region samples to the ones obtained in the Abruzzo wine samples, the results are slightly diverse maybe due to the different geographical areas.

Concerning the other wine typologies investigated in the present work, there is a lack in the literature for the study of the mineral composition of wines produced with Verdicchio, Montepulciano and Vernaccia Nera grape varieties in the Marche region, thus, comparison can be only performed with wines produced in other Italian regions. Ferretti & Febbroni (2022), for example, investigated the *terroir* influence on traceability in grapes, must and Gewürztraminer wines, from the South Tyrol region. The study was conducted on wines produced in seven vineyards located in a small geographic area with a high *terroir* variability and the elemental composition was assessed through ICP-MS. Results showed how this variability was able to influence and impact wines sensorial characteristics. Mn was found in the range 0.45-1.33 mg/L, values comparable to the ones obtained in the 18 DOCG wine samples (0.30-1.64 mg/L). On the other hand, Fe was obtained with a slight different results, since in the South Tyrol wines it was in the range 0.1-0.2 mg/L, while in our case in the range 0.27-2.86 mg/L. Sr is a metal which is strongly dependent on the soil where the grapes are cultivated (Geana *et al.*, 2013). For the Gewürztraminer wines Sr was obtained in the range 0.08-0.16 µg/L, while in Marche region DOCG wines investigated it showed much higher quantities of 0.4-1.16 mg/L. The same order of magnitude was obtained for another important geographical marker, Rb. Indeed, it was obtained in the range 0.42-1.16 µg/L in South Tyrol wines and in the magnitude of mg/L in our samples, probably due to the different and distant Italian regions where they are produced.

4.3.2 Statistical analysis

4.3.2.1 Principal component analysis

The principal component analysis (PCA) was performed to identify homogeneous groups of data by considering all the analysed elements as variables and all wine samples. The total variance explained by the first five principal components (PCs), was of 70.1%, with the first PC (PC1) explaining the 27.58% and the second (PC2) the 14.71%. The score plot obtained for the wine samples investigated is shown in **Figure 2**.

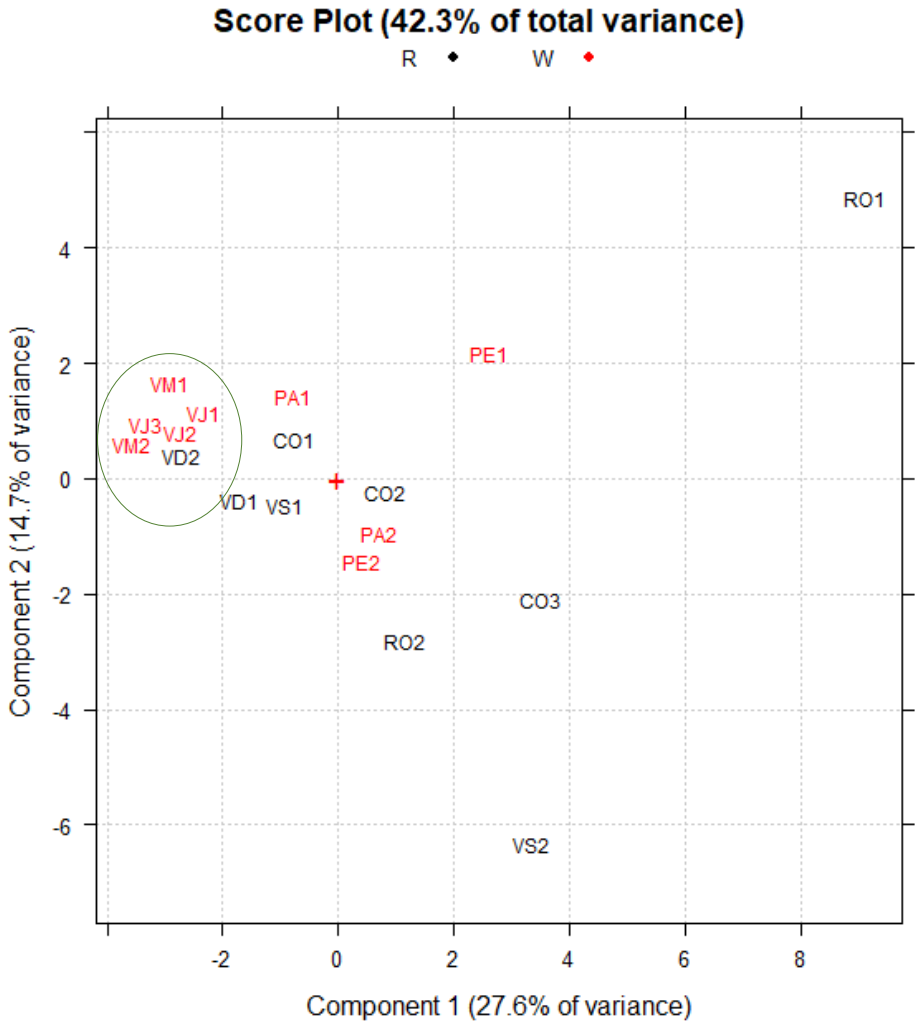

Figure 2. Principal component analysis and related score plot, obtained using all the DOCG wine samples investigated. VM: "Verdicchio di Matelica Riserva"; VJ "Castelli di Jesi Verdicchio Riserva"; PE: "Offida Pecorino"; PA: "Offida Passerina"; RO: "Offida Rosso"; CO: "Cònero"; VD: "Vernaccia di Serrapetrona" sweet; VS: "Vernaccia di Serrapetrona" dry. Different colours indicated white (in red) or red (in black) wine samples. Green circle indicates clustering of wine samples.

Despite the poor variance degree explained by the first two principal components, a certain clustering was observed for Verdicchio wines, which includes "Verdicchio di Matelica Riserva" and "Castelli di Jesi Verdicchio Riserva" and that are produced in relatively close geographical areas (green circle). At the same time, also "Offida Pecorino" white wines (PA01, PA02, PE01 and PE02) were relatively close, even if distributed close to some red wine samples (CO01 and CO02) and thus not exhibiting a characteristic elemental composition.

Given that, as already said, some minerals seemed not to be strictly associated to the soil and thus to geographical area, but their presence probably derives from the treatments (such as the use of fertilizers or addition of some agents during winemaking steps), a selection of those elements probably associated only to a natural origin, was performed. This selection comprised B, Li, Mn, Sr

and Ba and the PCA was performed again taking into consideration the five minerals. In this case the variability explained by the first two principal components was 72.2 %. The biplot is reported in **Figure 3**.

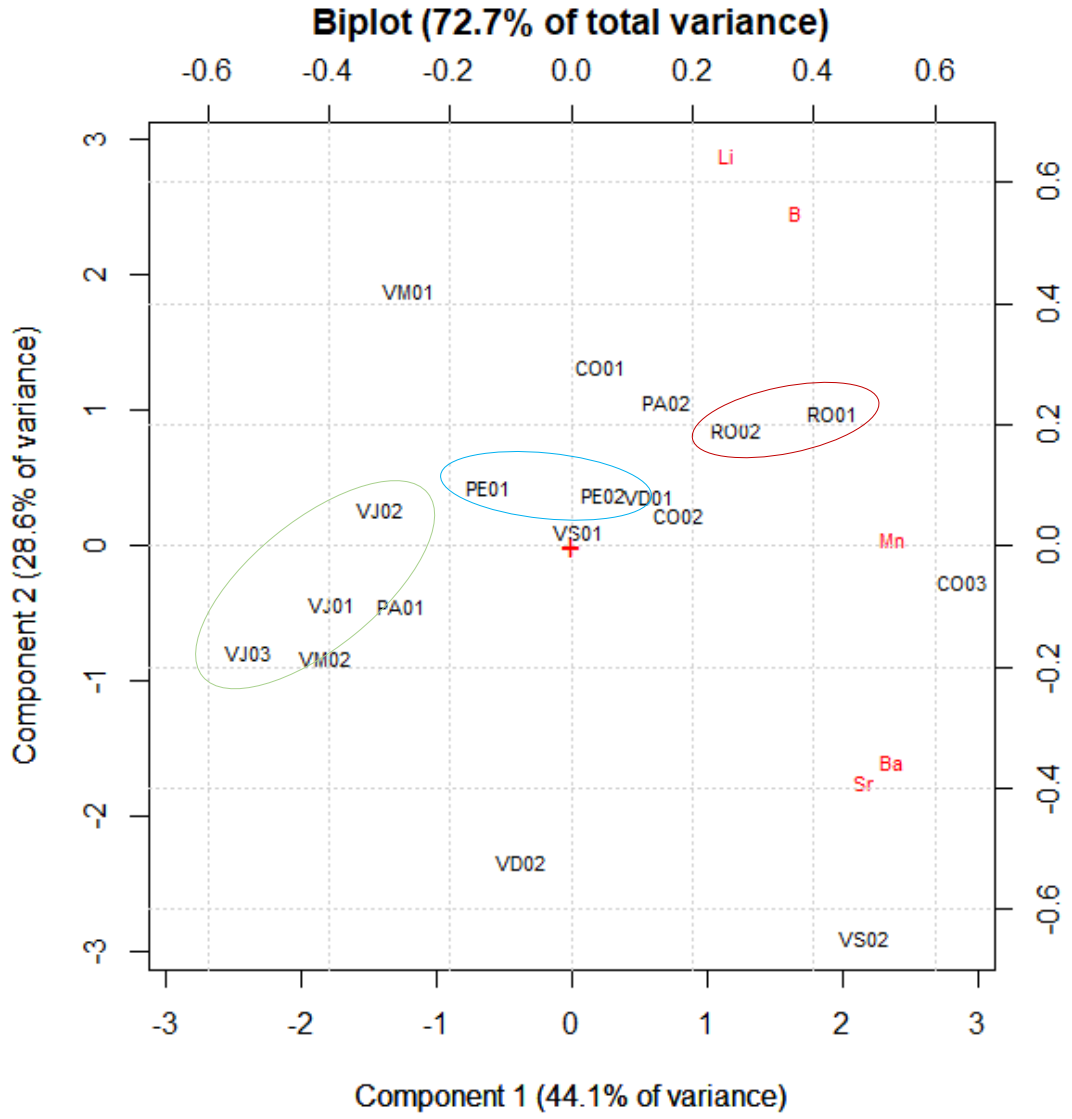

Figure 3. Principal component analysis and related biplot, obtained the five selected elements as variables (in red) to optimize separation according to soil composition and the DOCG wine samples (in black) as scores. VM: "Verdicchio di Matelica Riserva"; VJ "Castelli di Jesi Verdicchio Riserva"; PE: "Offida Pecorino"; PA: "Offida Passerina"; RO: "Offida Rosso"; CO: "Cònero"; VD: "Vernaccia di Serrapetrona" sweet; VS: "Vernaccia di Serrapetrona" dry. Green, blue and red clusters indicate different groupings of wine samples.

The biplot obtained by considering only those elements attributed to natural sources did not point out clear clustering of samples according to DOCGs typology and thus, clusters able to discriminate the geographical area of origin. However, a certain degree of grouping can be highlighted for "Castelli di Jesi Verdicchio Riserva" (green circle), "Offida Pecorino" (blue circle) and "Offida Rosso" (red circle), even if samples of other DOCGs are also distributed close to these

clusters. Anyway, the fact that these samples, that belong to the same DOCG, are situated close each other suggest that the DOCG may be characterized by a peculiar elemental composition which should be better investigating. By contrary, the other DOCGs samples did not show the same behaviour, as in the case of "Vernaccia di Serrapetrona" DOCG samples (VD01, VD02, VS01 and VS02) which are more homogenously distributed in the biplot.

4.3.2.2 One-way ANOVA

To better investigate the results obtained by the ICP-MS elemental analysis of the samples and by the PCA, the analysis of variance (ANOVA) coupled with Tukey's test for pairwise comparison was carried out by dividing the 18 DOCG wine samples into the eight DOCGs groups and the five selected elements. The results obtained by the ANOVA are reported in **Table 4**.

Table 4. ANOVA performed among the average concentrations (mg/L) of the five selected minerals in the different DOCG samples, divided into eight groups (distinguishing "Vernaccia di Serrapetrona" into two different groups: dry and sweet, and "Offida" DOCG into three groups: Pecorino, Passerina and Offida Rosso).

	Verdicchio di Matelica Riserva	**RSD %**	**Castelli di Jesi Verdicchio Riserva**	**RSD %**	**Offida Pecorino**	**RSD %**	**Offida Passerina**	**RSD %**	**Offida Rosso**	**RSD %**	**Cònero**	**RSD %**	**Vernaccia di Serrapetrona sweet**	**RSD %**	**Vernaccia di Serrapetrona dry**	**RSD %**
B	6.80^{a}	27.2	5.30^{a}	11.8	6.84^{a}	5.9	6.57^{a}	6.4	7.90^{a}	9.0	7.24^{a}	3.4	6.73^{a}	28.5	6.54^{a}	14.3
Li	0.012^{a}	47.1	0.02^{a}	34.6	0.02^{a}	0.0	0.02^{a}	70.7	0.02^{a}	28.3	0.02^{a}	24.7	0.02^{a}	47.1	0.02^{a}	47.1
Sr	0.52^{a}	26.1	0.44^{a}	19.7	0.62^{a}	19.5	0.59^{a}	40.0	0.77^{a}	1.8	0.79^{a}	19.8	0.98^{a}	26.8	0.87^{a}	27.6
Mn	0.46^{a}	19.0	0.40^{a}	22.2	$0.73^{a,b}$	10.7	$0.77^{a,b}$	2.8	1.0^{b}	9.2	$1.06^{a,b}$	49.5	0.34^{a}	2.1	0.68^{a}	66.6
Ba	$0.07^{a,b}$	28.3	0.06^{a}	10.2	$0.08^{a,b}$	17.7	$0.08^{a,b}$	35.4	$0.14^{a,b}$	26.2	$0.12^{a,b}$	40.8	$0.14^{a,b}$	15.7	0.20^{b}	49.3

RSD% is the percentage relative standard deviation obtained between wine samples belonging to the same DOCG class. Letters indicate significant differences between the concentrations (mg/L) in the eight different DOCG wines groups (One-way ANOVA, $P < 0.05$, Tukey's test for pairwise comparison).

In most of the cases significant differences did not arise in the amount of the selected minerals. Elements such as Li, B and Sr for example, did not show significant differences among DOCGs, but neither among samples (**Figure 3**). By contrary, taking into consideration the biplot, Mn showed higher abundances in red samples, in particular in samples RO01, RO02 and CO03. These can be considered as peculiarities of the single samples since these elements did not show significant differences when takin into consideration the DOCG class, as reported in **Table 4**. Ba was obtained with very low concentrations (in the range 0.05-0.29 mg/L) with the highest amount in a "Vernaccia di Serrapetrona" dry sample (VS02). Anyway, even in this case this can be considered as a peculiarity of the single wine sample. Indeed, Ba was obtained in comparable amounts along the different DOCGs, with the exception for "Castelli di Jesi Verdicchio Riserva" where it showed the lowest concentration.

The lack in a clear discrimination of DOCGs in terms of elemental composition can be attributed to the relatively small area of Marche region. Indeed, even if these wines are produced in circumscribed areas (described by the relative production disciplinaries), these areas are close each other and, in some cases, adjoined, thus this result was expected.

4.4 Conclusions

The ICP-MS analysis of 40 elements was performed to characterize the DOCG wines under investigation. While some differences raised between samples produced in close geographical area, important differences able to discriminate the mineral composition between the five DOCG did not arise. Among the high number of elements quantified in the samples, a selection considering only those elements that are attributed to natural sources and whose quantities in wine are not influenced or modified during winemaking processes, was performed. Among the selected elements the principal component analysis (PCA) and the analysis of variance (ANOVA) were performed.

Li, B, Sr and Mn were obtained in comparable amounts in the different DOCGs (P <0.05), hence a peculiar profile depending on the specific production geographical area was not observed. Concerning Ba, it was generally obtained in higher amount in red wine samples, even if without statistically significant differences with respect to white wines.

The lack of a clear discrimination between DOCGs elemental composition can be attributed to the relatively small wines production geographical area. For this reason, further studies to compare the obtained elemental profiles of DOCGs wines from Marche region to those of wines produced with the same grape varieties but in other Italian regions, could clarify if the more diverse geographical areas would result in a more diverse elemental composition.

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5: Organic acids in wine

5.1 Introduction

Organic acids are known to play important roles contributing to maintain the nutritional values of some foods, due to their antioxidant, antimicrobial and anti-inflammatory properties.

They can be exploited in different contexts and some of them are directly added to foods as acidulants or stabilizers. For example, they are used in the control of sugars fermentation performed by yeasts and bacteria, which bring to the formation of volatile acids that can negatively affect the quality of some food products (Jurado-Sánchez *et al.*, 2011).

Organic acids are also able to contribute to the organoleptic properties, being responsible for the flavour, colour and aroma of some foods. They are naturally presents in many different products, from fruits to vegetable and beverages and thus their quantitative determination is of great importance and interest, being strictly correlated to food quality. By now several studies have been published focused on their determination in different matrices, from mushrooms to coffee (Galli & Barbas, 2004; Valentão *et al.*, 2005; Sandín-España *et al.*, 2016).

Concerning wine, their presence is very important for its stability and the analysis of organic acids is required as quality control during the different steps of winemaking. In fact, important and uncontrolled changes due to wine spoilage could be associated also to changes of the organic acids content. For this reason, they are monitored starting from the grapes before the harvest, continuing to the alcoholic fermentation and in wine stabilization processes.

Wine acidity is given by volatile (expressed in terms of acetic acid g/L) and fixed acidity, whose sum is the wine total acidity. Organic acids that compose fixed acidity can be distinguished between the ones which are naturally present in grapes such as malic, tartaric and citric acids and those that are formed during fermentation processes, such as succinic, acetic and lactic acids. Anyway, there are also acids present in minor quantities, which are shikimic, galacturonic, glucuronic, citramyl, ketoglutaric acids, etc.

Some organic acids can be added in wine or must, to adjust the acidity with the aim to produce more balanced wines or to repair to a natural insufficient acidity which may arise from both climatic reasons or oenological practises. Tartaric, citric, malic and lactic acids are used for this purpose and the quantities which can be used are strictly dictated by legislation (CE n.187/01 2022). Indeed, the net cumulative increase of acidity should never exceed 4 g/L (expressed as tartaric acid).

The major organic acids present in wine, with their concentration ranges and sensorial attributes are represented **Table 1**, while their chemical structures are shown in **Figure 1**.

Table 1. Concentration ranges and sensorial attributes of major organic acids in wine.

Acid	Concentration range (g/L)	Sensorial attribute
tartaric acid	4.5-15	tart[a]
malic acid	2-6.5	sour[b]
citric acid	0.5-1.0	fresh[c]
lactic acid	1.2-7.4	smooth
shikimic acid	0.01-0.15	-
pyruvic acid	0.01-0.5	slightly sour[d]
succinic acid	0.5-1.5	salty bitter[e]

[a]Ribéreau *et al.*, 2006; [b]Ribéreau *et al.*, 2000; [c] Bely *et al.*, 2005; [d]Margalit, 1997; Chidi *et al.*, 2018; [e]Baroň & Fiala., 2012.

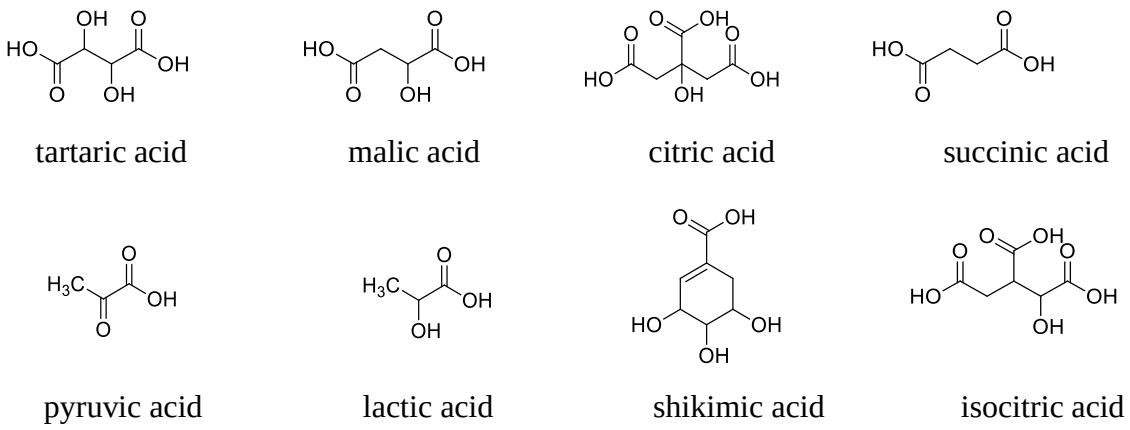

Figure 1. Chemical structures of the main organic acids.

Generally, these acids can be found in wine in both free form or combined with cations to form the relative salts and the sum of all the free organic acids is defined as the wine total acidity. This parameter is essential from an oenological point of view, for both the protection against microbial attacks and for the organoleptic characteristics which are typical of wines to submit to prolonged aging. Moreover, a strong technological and economic interest has prompted numerous researchers to investigate the ability of organic acids, tartaric in particular, to interact with proteins or other molecules present in the hydroalcoholic matrix and, indeed, studies confirmed also the role of tartaric acid in the astringency perception (Zhao *et al.*, 2022).

This acid, together with malic, citric and succinic are the most abundant in wine, whose concentrations depend on the grape variety and climatic conditions during the years (Peres *et al.*, 2009).

Tartaric acid plays an important role in the maintenance of chemical stability, colour and taste of wine. The acid taste is, in fact, attributed to the presence of this compound and its presence in fermenting must is essential since it lowers the pH at a value where many undesirable spoilage bacteria cannot live. During the fermentation step its content tends to slightly decrease, as a result of its precipitation under the form of potassium salts. Furthermore, wine can be added with tartaric acid to remove excess.

The acid taste of wine is also influenced by citric acid, which is important in biochemical and metabolic processes. This acid is able to inhibit the growth of yeasts and for this reason it is frequently used as acidifying agent in food and beverages, but in wine, when it is present in excessive amounts, it can indicate adulteration (Silva *et al.*, 2015). Oenological practises allow the addition of citric acid to a final concentration in the wine that should not exceed 1 g/L.

Isocitric acid differs from citric acid for the position of the hydroxy group, and it is commonly used as a marker to detect the authenticity and quality of fruit products.

Pyruvic and succinic acids are by-products of nitrogen yeast metabolism hence they are produced during fermentation; but, while pyruvic acid content in wine is limited, succinic acid is usually present at higher concentrations and it can contribute with bitter and salty tastes (Lelova *et al.*, 2018). The particular taste is also the reason why it is not commonly used as acidulant agent in foods (Ribereau-Gayon, 2006).

Malic acid is a compound naturally present in many fruits such ad green apples and grapes, where it can impart the characteristic sour taste. It can reach very high concentrations, up to 16 g/L as in the case of grapes harvested in cool climatic regions (Krueger, 2012; Gallander, 1977). Together with tartaric acid it contributes to the 70-90 % of total grape juice acidity and during fermentation processes it tends to decrease due to the action of yeasts (Gao & Fleet, 2004).

Lactic acid is produced during malolactic fermentation by the decarboxylation of malic acid performed by lactic acid bacteria (**Figure 2**).

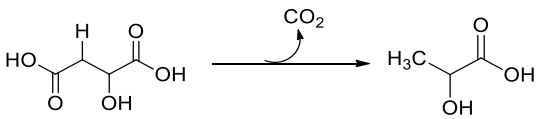

Figure 2. Summarized scheme of malolactic fermentation.

Malolactic fermentation is always sought for red wines, while in white wines it depends on the wine and production regions. From the sensorial point of view this fermentation step provokes a diminishing of fruity characters, followed by a smoother and a more harmonious taste. At the same time, even if it can be beneficial to some wines, it may bring to unpleasant off-flavours, for example to the production of diacetyl, in some wines such as Chenin blanc, where it is not desired (Bartowsky & Henschke, 2004).

Finally, shikimic acid in wine plays an essential role, being the precursors of benzoic and cinnamic acid, aromatic amino acids and flavonoids. It is considered a marker of varietal origin among *Vitis vinifera*, since its concentrations is very variable (Mardones *et al.*, 2005), according to the variety. Indeed, it is of a few mg/L in Pinot and reaches concentrations up to 50 mg/L in Chardonnay and Trebbiano. Its content decreases considerably during the ripening of the grapes and therefore it depends on the harvest period (Tessini *et al.*, 2009). From the sensorial point of view, it has not relevant roles.

Generally, organic acids can be viewed as indicators for the deterioration due to storage or aging of wine, since the class and content of these compounds is able to give characteristics taste to wine. Moreover, they can be used also to assess authenticity since the acidic profile has been correlated with the region of winemaking, to processing techniques (alcoholic and malolactic fermentation) and to wine maturation (Jurado-Sánchez *et al.*, 2011; Zheng *et al.*, 2009).

In particular, tartaric and malic acids have been used to discriminate between wines produced in regions with different climate temperatures. Indeed, the classic fermentation is characteristic for the south regions in Europe, i.e., Italy, French, Spain and Portugal and this results in a higher amount of tartaric acid, while malolactic fermentation is very common in regions of north Europe (Austria, Germany, Czech Republic and UK). In these regions the climate is colder and, the intensity of sunshine is lower and this leads to higher levels of carboxylic acids, particularly malic acid (Lasik, 2013). Furthermore, the type of fermentation is determined by the chemical composition of grapes, and this means that the major differences of wines produced in the north or south regions derives from the different ratios among carboxylic acids (malate, tartrate, lactate and citrate) (Abrahamse & Bartowsky, 2011).

Due to the importance of organic acids in wine, different studies have been reported aimed to characterize wines considering their organic acids composition (Geana *et al.*, 2016; Coelho *et al.*, 2018; Ivanova-Petropulos *et al.* 2020).

At the same time, there is still a lack in the characterization of the organic acid content in wines with DOCG designation produced in Marche region, or produced with the corresponding grape varieties (Verdicchio, Pecorino, Passerina, Montepulciano and Vernaccia Nera), given that only few works have been reported by now (Boselli *et al.*, 2004). Biancolillo *et al.* (2022), for example, performed the characterization of Trebbiano d'Abruzzo, Pecorino and Passerina white wines produced in Abruzzo, thus taking into considering wines produced with the same grape varieties of "Offida Pecorino" and "Offida Passerina" Marche DOCG wines under investigation. A varietal discrimination was pointed out by performing the sensorial analysis and a multi-block classification based on volatiles, polyphenols, major components and organic acids, the last analysed by UHPLC.

Starting from these premises, the analysis of seven organic acids, namely tartaric, succinic, citric, isocitric, shikimic, malic and lactic acids was performed by HPLC analysis, to characterize the organic acids profile of the 18 DOCG wine samples under investigation (listed in **PART 1, Table 9**). The results were then submitted to different statistical analyses with the aim to characterize the organic acids profile of the five DOCGs produced in Marche region.

5.2 Materials and method

5.2.1 Reagents and standards

1000 mg/L standard solutions for the organic acids were purchased by Sigma Aldrich. Stock solutions for identification of organic acids in wine samples were prepared by diluting the 1000 mg/L solution 1:10. The quantification of organic acids has been performed through calibration curves by choosing the appropriate range of concentrations (for shikimic acid 5, 12.5, 25, 37.5 and 50 mg/L, while for the others 100, 250, 500, 750 and 1000 mg/L).

5.2.2 Sample preparation

Samples were directly analysed, after been diluted (1:10, 1:5 and 1:2) with ultrapure water (Milli-Q) and injected, by using a 1 mL syringe equipped with regenerated cellulose filter (RC, 0.20 μm).

5.2.3 HPLC-DAD analysis

The instrument used for the determination of organic acids was a 1260 Infinity Quaternary

LC coupled with a Diode Array Detector (HPLC-DAD, Agilent Technologies, Santa Clara, USA). The HPLC column was a LiChroCART 250x4.6mm, Purospher RP-18e with a particle size of 5µm. The detector used was a Variable Wavelength Detector (VWD). The light source for the UV wavelength range was a deuterium lamp that emits light over the 190 – 600 nm wavelength range and 210 nm was used for the quantification of organic acids. As solvent, a gradient mixture of sulphuric acid and acetonitrile was used.

Table 2. Gradient program set for HPLC-DAD analysis.

Time (min)	MeCN [%]	Sulphuric acid 10mM [%]
0	5.0	95.0
3	5.0	95.0
4	10.0	90.0
10	10.0	90.0
12	70.0	30.0
16	70.0	30.0
18	5.0	95.0

The identification of compounds was performed by comparing retention times to those of pure standards, injected with the same instrumental conditions and method and at a concentration of 100 ppm.

5.2.4 Statistical analysis

The principal component analysis (PCA) was performed in order to identify homogenous groups of data (cluster). The PCA was performed using R-based software CAT (Leardi *et al.*, 2021). The heatmap was also designed for an additional graphical representation of results, by using RStudio software.

5.3 Organic acids profile of the DOCG wines from Marche region

5.3.1 Results and discussion

A total of seven organic acids were detected and quantified. An example of a chromatogram obtained by the HPLC-DAD analysis is shown in **Figure 3** while the results obtained by the analysis of the organic acids in the samples under investigation are reported in **Table 3**.

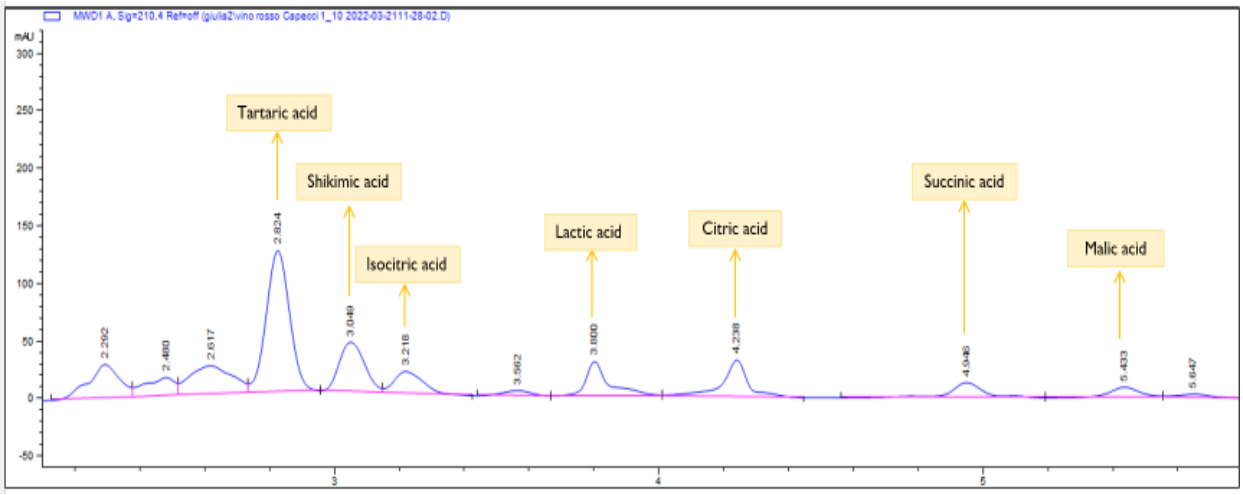

Figure 3. Example of a chromatogram obtained by the HPLC-DAD analysis of a wine sample.

Table 3. Concentrations (mg/L) of the organic acids in wine samples analysed by HPLC-DAD.

		tartaric acid	shikimic acid	isocitric acid	lactic acid	succinic acid	malic acid	citric acid
					(mg/L)			
white wines	VM01	2707.9	16.9	652.11	1115.8	934.58	106.5	1935.3
	VM02	2474.9	23.8	3117.18	623.5	1129.06	156.7	3212.3
	VJ01	2626.7	19.3	1844.43	1095.2	973.20	108.0	1875.4
	VJ02	2267.1	15.3	2163.25	672.9	875.17	94.8	1495.9
	VJ03	2412.5	18.2	2087.31	283.2	1014.53	193.8	1919.3
	PE01	2440.4	41.0	2588.52	1030.6	936.39	474.7	2708.2
	PE02	2664.0	31.1	3512.80	777.5	1113.79	470.2	2725.8
	PA01	1806.0	36.1	3805.78	728.9	1070.47	280.2	1999.5
	PA02	2987.8	29.5	2652.19	917.5	901.88	182.1	2764.6
	range	1806.1 - 2987.8	16.9-41.0	652.1-3805.8	283.2-1115.8	875.2-1129.1	106.5-474.7	1495.9-3212.3
red wines	RO01	1875.7	45.2	279.3	3895.35	1560.01	631.5	1815.8
	RO02	1686.7	38.5	210.2	4153.57	1773.60	619.8	2045.4
	CO01	2528.5	57.5	572.0	2516.45	1440.88	518.6	2623.3
	CO02	2624.7	50.3	145.8	3133.72	1752.57	902.5	3057.6
	CO03	2743.0	37.1	522.7	4770.32	1432.40	1210.2	2322.9
	VD01	1485.0	62.3	310.5	4904.92	1948.52	676.9	2258.8
	VD02	1658.6	44.8	926.2	2706.21	1301.50	312.9	2359.5
	VS01	1291.9	55.5	216.1	5696.90	1749.78	668.1	2533.3
	VS02	1799.8	53.2	323.1	2822.85	1975.05	363.3	2165.3
	range	1291.9-2743.0	37.1-62.3	145.8-926.2	2516.5-2904.9	1301.5-1975.1	312.9-1210.2	1815.8-3057.6

VM: "Verdicchio di Matelica Riserva"; VJ "Castelli di Jesi Verdicchio Riserva"; PE: "Offida Pecorino"; PA: "Offida Passerina"; RO: "Offida Rosso"; CO: "Cònero"; VD: "Vernaccia di Serrapetrona" sweet; VS: "Vernaccia di Serrapetrona" dry.

White and red wines showed different organic acids profiles. White wines were more enriched in tartaric and isocitric acids, while red wines showed higher abundances of shikimic, lactic, succinic and malic acids. Citric acid could not be quantified due to coelution problems. The method used for the determination of organic acids is still under optimization, and for this reason, citric acid is not going to be considered in the further discussion and elaboration of results .

Tartaric acid is the most abundant organic acid and it is the main responsible of wine acid taste. White wines are usually more acid than red wines and this is in accordance with the results, since this acid was obtained in a mean value of 2487.5±329.9 mg/mL in white wines and a lower mean value of 1966.0±530.1 mg/L in red wines. At the same time, in "Cònero" wine samples (CO01, CO02 and CO03) tartaric acid was obtained with values more in accordance with those of white wines (being 2528.5, 2624.7 and 2743.0 mg/mL, respectively).

Shikimic acid is considered a variety marker since its abundance is supposed to be strictly dependent on the grape variety. In red wines, where it showed an overall higher abundance, there was not a clear difference between wines produced with Montepulciano (whose minimal amount in the investigated samples is 85%) and Vernaccia Nera grape varieties. On the other hand, for white wines produced with Verdicchio grape variety, this compound showed very low concentrations (in the range 15.3-23.8 mg/L) when compared to the other white wines produced with Pecorino or Passerina grape varieties where it was obtained in the range 29.5-40.9 mg/L. Shikimic acid plays also an important role as precursor of benzoic and cinnamic acid, aromatic amino acids and flavonoids (flavones, antocyanidins, flavonols, tannins, etc.) transferred to wine during winemaking processes and this explains its higher abundances in red wines (Ribereau-Gayon *et al.*, 2001).

Isocitric acid was obtained in very different amounts between white and red wine sample. Indeed, in white wines it was approximately 6-fold more abundant, ranging from 652.1 to 3805.8 mg/L, compared to red samples where it ranged between 145.7 and 926.2 mg/L. Within white wines, it showed some differences depending on grape variety. In Verdicchio wine samples (VM01, VM02, VJ01, VJ02 and VJ03) isocitric acid showed lower concentrations (in a mean of 1972.8 mg/L) compared to wines produced with Pecorino or Passerina vines (3050.6 and 3229.0 mg/L, respectively).

Concerning malic acid, it was more abundant in red wine samples when compared to white ones. Furthermore, the concentrations of malic acid showed a precise profile depending on grape variety. For example, in white wine samples the mean concentration in Verdicchio wines was 131.9±41.9 mg/L, in "Offida Pecorino" wines 472.4±3.1 mg/L and in "Offida Passerina" wines 231.2±69.4 mg/L; while its concentration was more comparable in red wine samples.

Lactic acid was more abundant in red samples (in the range 2516.5-2904.9 mg/L), with respect to white wines (283.2-1115.8 mg/L) and this trend was expected, due to malolactic fermentation. Finally, succinic acid, which is another organic acid that originates during fermentation, was obtained in a slightly lower range of concentrations in white wines (875.2-1129.1 mg/L) when compared to red wine samples (1301.5-1975.1 mg/L).

In the past, some authors performed the characterization of wines by evaluating organic acids as possible markers. Geana *et al.* (2016) for example, performed the classification of Romanian red wines made with different varieties (Cabernet Sauvignon, Merlot, Pinot Noir, Feteasca Neagra and Mamaia). The results showed that all wines were characterized by a precise organic acids profile, being their content very variable among the samples investigated. Shikimic acid, for example, ranged from 9.1 mg/L in Mamaia wines to 63.2 mg/L in Cabernet Sauvignon confirming its high variability depending on grape variety. In the same way, in the DOCG wine samples investigated, this compound showed a great variability, in the range 16.9-62.3 mg/L.

Coelho *et al.* (2018) performed a study for the simultaneous determination of sugars and organic acids in wine by HPLC analysis, applied for the characterization of northeast Brazil wines. The proposed method was applied over seven red wines and one white wine sample. In red wines tartaric acid was obtained in the range 630-4390 mg/L, malic acid in the range 7-1590 mg/L and lactic acid in the range 1240-3400 mg/L. For the white wine sample of Chenin Blanc results were slightly different being their concentrations 770, 3180 and 130 mg/L, respectively. The results of the analysis of DOCG wine samples were found almost completely in accordance with these ranges of concentrations. The major differences were in the results of white wines since higher concentrations (except for malic acid) were obtained in the DOCG wine samples under investigation, being the mean values 2487.4±329.9 mg/L for tartaric acid, 229.7±148.9 mg/L for malic acid and 805±267.8 mg/L for lactic acid.

Ivanova-Petropulos *et al.* (2020) performed the study of organic acids in six red Merlot wines and six Chardonnay white wines from Macedonia, by HPLC-DAD analysis. The results obtained by the authors are quite in accordance with the results obtained in the present study. Indeed, tartaric acid was obtained in the range 2450-3240 mg/L in white wines and 1850-339 mg/L in red wines and, in the DOCG wine samples, it was obtained in the ranges 1810-2980 mg/L and 1290-2740 mg/L, respectively. Shikimic acid was obtained in the range 1119-3280 mg/L for Macedonian wines, while in DOCG wine samples it was obtained in lower concentrations, being in the ranges 15.3-40.9 mg/L in white wines and in the range 37.1-62.3 mg/L in DOCG red wine samples. This can be attributed to several reasons, including the different grape varieties, different winemaking processes and

different geographical areas involved in the two studies. Concerning malic acid, it was obtained with an opposite trend. Indeed, Ivanova-Petropulos *et al.* obtained this acid in the range 1750-2280 mg/L in white wines and 20-920 mg/L in red wines. In the DOCG wine samples, instead, it was obtained in lower concentrations in white wines (the range 94.79-474.60 mg/L) when compared to red wines (363.28-1440.88 mg/L). As expected, lactic acid was obtained by Ivanova-Petropulos *et al.* in lower concentrations in white wines (240-990 mg/L) when compared to red wines (640-3140 mg/L). Finally, succinic acid was obtained in higher concentrations in the DOCG wine samples here investigated, when compared to the mentioned study, where the organic acid was obtained in the ranges 190-1000 mg/L.

Concerning wines produced in Marche region, Boselli *et al.* (2004) reported the chemical and sensorial characterization of five DOC red wines of different vintages, including different samples ”Cònero” and sweet “Vernaccia di Serrapetrona”. The wines were analysed by taking into consideration different chemical parameters, including organic acids. The results were reported in terms of chromatographic peak areas obtained by the HPLC-MS analysis and the two wines typologies showed a high variability in their organic acid content. In particular, malic and lactic acids were more abundant in “Vernaccia di Serrapetrona” wines, when compared to “Cònero”. In the DOCG wine samples under investigation a different trend was observed. Succinic acid was, indeed, more homogeneously distributed among the samples without pointing out a clear difference between the two wine typologies. Furthermore, malic acid was obtained with slightly higher concentrations in “Cònero” wine samples (518.6-1210.2 mg/L) when compared to “Vernaccia di Serrapetrona” wines (312.9-676.9 mg/L).

5.3.2 Statistical analysis

5.3.2.1 Principal component analysis

The PCA was performed considering the concentrations of the seven organic acids as variables and the 18 DOCG wine samples under investigation, in order identify homogenous groups of data. The first two components (PC1 and PC2) were able to explain a high percentage of the total variance (84.4%) and a clear grouping of samples was obtained, as reported in the biplot plot in **Figure 4**.

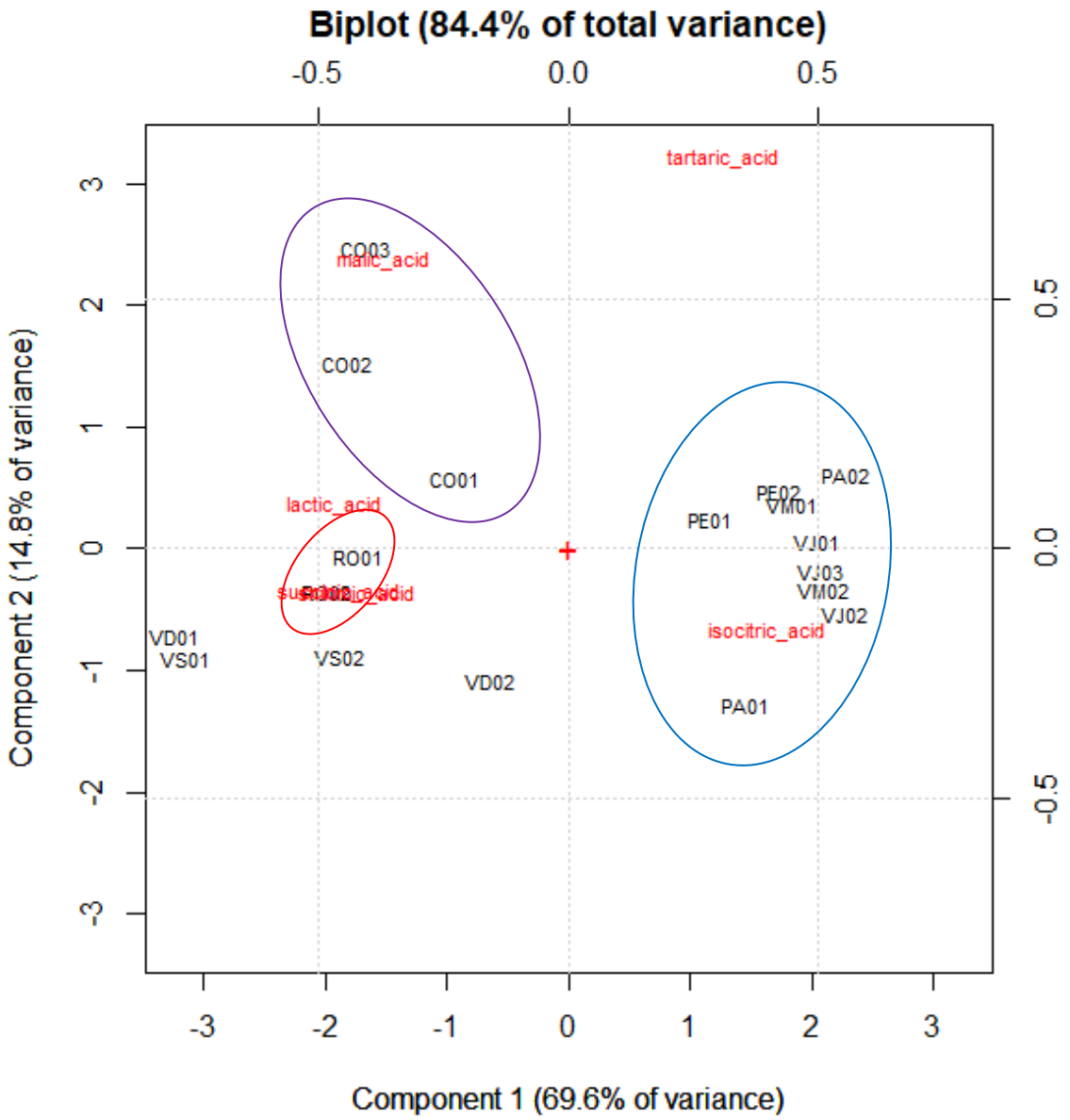

Figure 4. Principal component analysis and related biplot plot, obtained using the concentrations of six organic acids as variables (in red), and the DOCG wine samples investigated (in black), as scores. VM: "Verdicchio di Matelica Riserva"; VJ "Castelli di Jesi Verdicchio Riserva"; PE: "Offida Pecorino"; PA: "Offida Passerina"; RO: "Offida Rosso"; CO: "Cònero"; VD: "Vernaccia di Serrapetrona" sweet; VS: "Vernaccia di Serrapetrona" dry. Violet, blue and red circles indicate the different grouping of wine samples. Overlapped words in red are "succinic_acid" and "shikimic_acid".

The PC1 was able to discriminate between red and white wine samples, meaning that this was the most important parameter able distinguish the samples in terms of organic acids abundances. Indeed, while all red wine samples are located in the left part of the biplot, the white wines are in the right part. Among the red wine samples, in the axe of PC2 a clustering was obtained between the "Cònero" (violet circle) and "Offida Rosso" (red circle) were obtained.

The biplot confirmed that the white wine samples were more enriched in isocitric acid, while red wines in malic, shikimic, succinic and lactic acids. In particular "Cònero" wines seemed to be

characterized by a higher content of malic acid, while “Offida Rosso” and “Vernaccia di Serrapetrona” in shikimic and succinic acids. Differently, tartaric did not play an important role in the discrimination between samples being positioned distant, in the upper part of the biplot.

5.3.2.2 One-way ANOVA

The results obtained by the PCA suggested that the five DOCGs were characterized by a peculiar and distinct organic acid profile. In order to better investigate this aspect, the analysis of variance (ANOVA) coupled to Tukey’s test for pairwise comparison was performed on the results by dividing the samples in eight different groups, since the five DOCGs were in turn divided in subgroups. “Vernaccia di Serrapetrona” DOCG samples were distinguished in sweet and dry and, in the same way, Offida DOCG samples were divided in three different groups: “Offida Pecorino”, “Offida Passerina” and “Offida Rosso”. The ANOVA was then performed to assess significant differences (P <0.05) in the organic acids composition among the eight DOCG wines groups and the results are reported in **Table 4**.

Table 4. Average concentration (mg/L) of the organic acids in the different DOCG samples, divided into eight groups (distinguishing Vernaccia DOCG wines into two different groups: dry and sweet; and Offida DOCG into three groups: Pecorino, Passerina and Offida Rosso).

	Verdicchio di Matelica Riserva	**RSD %**	**Caselli di Jesi Verdicchio Riserva**	**RSD %**	**Offida Pecorino**	**RSD %**	**Offida Passerina**	**RSD %**	**Offida Rosso**	**RSD %**	**Cònero**	**RSD %**	**Vernaccia di Serrapetrona sweet**	**RSD %**	**Vernaccia di Serrapetrona Dry**	**RSD %**
tartaric acid	2591.4^{a}	6.4	2435.4^{a}	7.4	2552.2^{a}	6.2	$2396.9^{a,b}$	34.9	1781.2^{b}	7.5	2632.1^{a}	4.1	1571.8^{b}	7.8	1545.8^{b}	23.2
shikimic acid	20.3^{a}	24.1	17.6^{a}	12.0	36.0^{a}	19.4	32.8^{a}	14.1	41.9^{b}	11.3	48.3^{b}	21.4	53.5^{b}	23.0	54.4^{b}	3.0
isocitric acid	$1884.6^{a,b}$	92.5	2031.6^{a}	8.2	3050.7^{a}	21.4	3229.0^{a}	25.3	244.7^{b}	20.0	413.5^{b}	56.4	618.3^{b}	70.4	269.6^{b}	28.1
lactic acid	869.6^{a}	40.0	683.7^{a}	59.4	904.1^{a}	19.8	823.2^{a}	16.2	4024.5^{b}	4.5	3473.5^{b}	33.5	3805.6^{b}	40.9	4259.9^{b}	47.7
succinic acid	1031.8^{a}	13.3	954.3^{a}	7.5	1025.1^{a}	12.2	986.2^{a}	12.1	1666.8^{b}	9.1	1541.9^{b}	11.8	1625.0^{b}	28.2	1862.4^{b}	8.6
malic acid	131.6^{a}	26.9	132.2^{a}	40.7	472.4^{b}	0.7	231.1^{a}	30.0	625.6^{b}	1.3	877.1^{b}	39.5	494.9^{b}	52.0	515.7^{b}	41.8

RSD% is the percentage relative standard deviation obtained among samples belonging to the same DOCG class. Letters indicate significant differences between the concentrations (mg/L) in the 8 different DOCG wines groups (One-way ANOVA, $P < 0.05$, Tukey's test for pairwise comparison).

The heatmap was also designed on the same dataset to obtain a graphical representation of the organic acid profile in the eight DOCG groups (**Figure 5**).

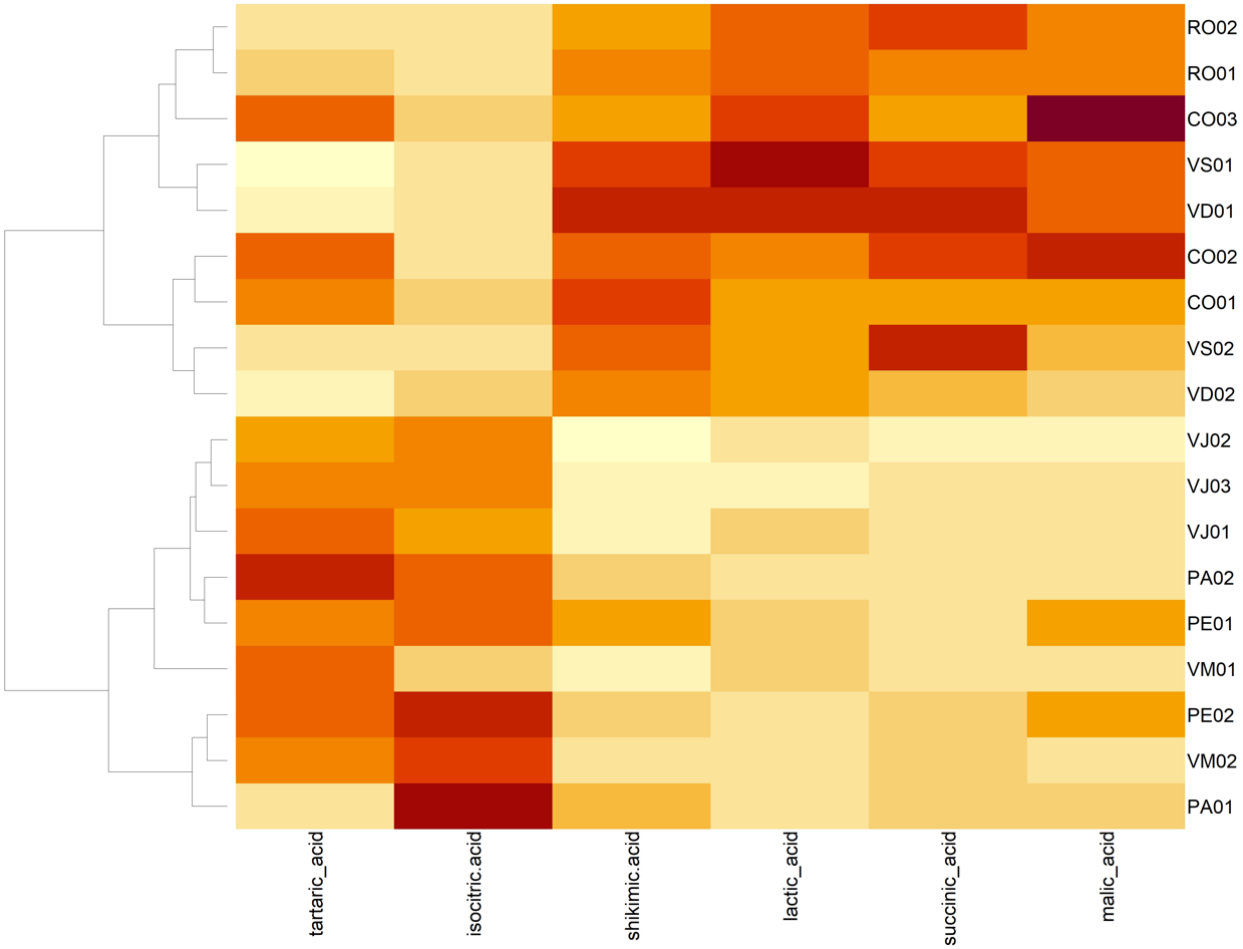

Figure 5. Heatmap of the eight organic acids concentrations in DOCG wine samples investigated (variables clustered on the vertical axes). VM: "Verdicchio di Matelica Riserva"; VJ: samples of "Castelli di Jesi Verdicchio Riserva"; PE: "Offida Pecorino"; PA: "Offida Passerina"; RO: "Offida Rosso"; CO: "Cònero"; VD: "Vernaccia di Serrapetrona" sweet; VS: "Vernaccia di Serrapetrona" dry.

The ANOVA confirmed the presence of significant differences (P <0.05) mainly dividing white and red DOCGs. Tartaric acid was indeed more abundant in white wine samples, even if "Cònero" DOCG showed a content of the acid comparable to those of white DOCGs (**Table 4**) showing a more peculiar behaviour. Concerning the other organic acids, isocitric acid was also generally more abundant in white DOCGs, while the other acids (shikimic, succinic, malic and lactic acids) were more abundant in red DOCGs.

The ANOVA considering only white or red DOCGs did not point out significant differences among the DOCGs but, at the same time, some organic acids showed peculiarities which can derive from the grape variety or to the geographical production area. Isocitric acid, for example, was obtained in both Verdicchio DOCGs with very similar concentrations, slightly lower compared to

those of white Offida DOCGs, which showed in turn comparable amounts of the compound. The latter are produced with the homonymous and thus different grape varieties, but in the same geographical area, which may be the reason why their abundances are quite similar.

Also shikimic acid showed a behaviour which seemed to recognize DOCGs according to grape variety or production area. In fact, the two Verdicchio DOCGs were characterized by a very similar quantity of the acid (20.3±5.0 and 17.6±2.1 mg/L). The same can be underlined for the "Offida" white DOCGs (36±7.0 mg/L in "Offida Pecorino" and 32.8±4.6 mg/L in "Offida Passerina") but also for "Offida Rosso" and "Cònero" DOCGs (41.9±4.7 and 48.3±10.4 mg/L, respectively) which are produced with the same minimal amount of Montepulciano grape variety and for "Vernaccia di Serrapetrona" sweet and dry (53.5±12.3 and 54.4±1.6 mg/L, respectively) which are produced in the same circumscribed area and with the same grape variety (Vernaccia Nera) Statistically significant differences (P <0.05) only raised in the content of the acid only between white and red DOCGs but due to the characteristic concentrations obtained for every DOCG, this acid should be better investigated. Shikimic acid has been used by some authors to discriminate wines according to grape variety. As an example, Nyitrainé Sárdy *et al.* (2022) performed the H^1-NMR spectroscopic analysis on Hungarian wine samples in order to distinguish four varieties (Cabernet Sauvignon, Blaufränkisch, Merlot, and Pinot Noir) and their geographical origin. They observed that shikimic acid, together with other target compounds, was able to discriminate Cabernet Sauvignon wines from the others, showing in this wine typology the hhighest concentrations of the acid.

Finally, "Offida Pecorino" DOCG showed concentrations comparable to those obtained in red DOCGs for malic and citric acids.

5.4 Conclusions

The HPLC-DAD analysis to determine seven organic acids (tartaric, malic, lactic, citric, isocitric, shikimic and succinic) in the 18 DOCG wine samples under investigation was performed. Tartaric and isocitric acids were the most abundant organic acids in white wine samples, while malic, lactic, succinic and shikimic acids were more abundant in red DOCG wines. Citric acid was found in concentrations much higher that the limit dictated by legislation (of 1 g/L) probably due to coelution with interfering compounds.

Different statistical analyses were then performed to interpret the results and the ANOVA carried out on the samples divided according to the belonging DOCG group did not point out significant differences in the organic acids content. Anyway, when comparing the overall organic acids profile, every DOCG seemed to be characterized by a peculiar outline.

Isocitric acid was the compound showing the most distinguishing behaviour. Indeed, it was obtained with very similar values when considering the two DOCGs produced with Verdicchio grape variety ("Verdicchio di Matelica Riserva" and "Castelli di Jesi Verdicchio Riserva") or DOCG wines produced in the same geographical area, such as in "Offida Pecorino" and "Offida Passerina" DOCGs. The same can be highlighted for shikimic acid which showed a certain degree of discrimination when considering grape variety or geographical origin.

Hence, beyond the fact that non-statistically significant differences raised among the organic acids composition of the DOCG wines investigated, every DOCG showed a peculiar overall organic acids profile. This should be further investigated by performing the analysis on more samples (produced by diverse wineries) and considering also different vintages, to confirm the fingerprint characterizing these wines protected by the most restrictive designation of origin and that could be possibly used to assess their authenticity.

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6: Sensory analysis and correlation with the chemical fingerprint

The sensorial analysis of the 18 DOCG wine samples was performed by a panel of 6 people, experts in the field of winemaking and/or oenologists. They evaluated the samples describing them with specific features for the appearance, olfactory attributes and gustative perception. These attributes were then linked to the chemical fingerprint obtained by the analysis previously reported.

It is important to underline that wine aroma and flavour does not come from the mathematical sum of individual compounds. Indeed, volatile odorant compounds can interact and show either additive or competitive effects, which may turn into synergic or antagonistic results. The relationships between the volatile composition and the sensory descriptors of wines have been reported by several authors and, among the hundreds of different volatiles identified, only a limited subset was likely to be actively involved in the flavour (Lund *et al.*, 2009, Barbe *et al.*, 2008; Vilanova *et al.*, 2010).

For example, it has been observed as furaneol and homofuraneol, strongly characterized by caramel aromas, have an important role in the enhancement of red-berry fruit aroma. Also, for yellow fruity aroma, correlated compounds such as isoamyl acetate (banana) or ethyl butyrate are masked by isoamyl alcohol. This is able to explain why red wines are not usually described by this kind of fruity aromas. Indeed, in wine samples under investigation, isoamyl alcohol was one of the most abundant alcohols, together with phenyl ethyl alcohol in red wine samples. Hence, this can partially explain the red fruit aromas associated to red wines. The complex interactions between aromatic compounds have been widely studied and investigated by authors (Ferreira *et al.*, 2016). Furthermore, the overall aroma can also derive from the interaction of volatile and non-volatile compounds present in the matrix.

Concerning the colour, while for white wines it has been attributed to the presence of flavan-3-ols, such as (+)-catechin, which are able to form yellow pigments, for red wines among flavonoids the most abundant pigment in grape skin are anthocyanins, such as malvidin-3-glicoside, which is responsible for red ruby (Yuan, *et al.*, 2022). Peonidin is also considered an important pigment in wine, whose presence makes wine colour more garnet. Depending on the relative concentrations of these two major compounds and to co-pigmentation processes that take place, wine can take different red colour tones and shadows, and this has been widely investigated by different authors (Gomez-Miguez *et al.*, 2006; Boulton, 2001). As expected, in the DOCG white wine samples, malvidin-3-glicoside was always below the limit of detections by contrary, for red samples it was the most abundant anthocyanin. A significant difference was also obtained between the red samples “Cònero” and “Offida Rosso” (CO01, CO02, CO03, RO01 and RO02) and the samples “Vernaccia di

Serrapetrona" (VS01, VS02, VD01 and VD02) and this gap is the main responsible for the different colour that distinguishes the DOCGs. The formers are produced with Montepulciano vine (minimal amount of 85%) and were found more enriched in maldivin, which explains the more intense ruby red colour. Instead, "Vernaccia di Serrapetrona" DOCG, is produced with Vernaccia Nera grape variety (the samples investigated are monovarietal, made with 100% of the vine) and it is characterized by lower concentrations of this compound, which results in a more garnet red. Moreover "Vernaccia di Serrapetrona" DOCG is a sparkling wine, and it is produced with a very characteristic and unique winemaking process, which involves three different fermentation steps. This can explain the difference in the phenolic compound composition when compared to other DOCG red wines made with "more classic" vinification techniques.

The descriptions of aspect, scents and taste are reported for samples divided into seven DOCG groups. "Offida" DOCG, indeed, has been divided into three subgroups ("Offida Pecorino","Offida Passerina" and "Offida Rosso"). A brief comparison between the description reported in wine consortium sites (available online for the wine belonging to IMT consortium at: https://imtdoc.it/vini-delle-marche/) or production disciplinary and the attributes described by the panel is performed.

When a plausible correlation, linking sensorial attributes to specific chemical compounds is identified, a brief explanation and hypothesis are reported.

"Verdicchio di Matelica Riserva"

Appearance: The aspect of "Verdicchio di Matelica Riserva" was generally described as golden yellow, which is the colour that characterizes the DOCG according to the production disciplinary and the sensorial analysis reported by the IMT.

Olfactory attributes: The aroma perceived in the samples under investigation were in accordance with the scents characterizing the DOCG, which were mainly described as dried green, apricot, anise, laurel and ripe cedar.

Verdicchio grape variety belongs to the non-aromatic vine, hence methoxypyrazines, which are usually associated to green and herbaceous aroma, were not detected by the SPME-GC-MS analysis. Anyway, other compounds are considered to impart green aroma, such as hexanol and they are associated to vegetable and grass notes. Anise notes have been already attributed to the presence of methyl salicylate, a compound which characterized Verdicchio wines as previously reported by Carlin *et al.* (2019). Some authors also found a correlation between anise scent and ethyl hexanoate (Gómez-Míguez *et al.*, 2007). Laurel has been found by authors to be positively associated to the

presence of decanoic acid, which is a compound detected in the samples. Fruity aroma, such as apricot can be attributed to the presence of ethyl esters. A study performed by Nandorfy *et al.* (2021) evidenced the positive correlation between apricot aroma and terpenes, so the synergic effect of the identified linalool and (*E*)-geraniol, together with other terpenes, can be supposed. Finally, the perception of cider scents can be attributed to ethyl 2-methylbutyrate, which, in the past, has been correlated to fruity and lemon scents (Gómez-Míguez *et al*., 2007). Also, ethyl butyrate has been associated to citrus scents.

Taste: The taste of Verdicchio di Matelica was described as savoury, acidic and complex with one sample being characterized by a light vanilla aftertaste (VM01), while the other by a more bitter finish (VM02). The acidity can be explained by tartaric acid, which is the most abundant organic acid in white wines.

"Castelli di Jesi Verdicchio Riserva"

Appearance: The aspect of "Castelli di Jesi Verdicchio Riserva" is characterized by a straw yellow, intense, golden colour.

Olfactory attributes: From the olfactory perception point of view this wine has been described with different and variable scents. Indeed, its aroma is generally characterized by honey, vanilla, citrus and tropical cedar scents together with more green aromas associated to balsamic such as menthol or dried aromatic herbs, sage, save and thyme. A sample (VJ02) was characterized by a more tertiary coffee and roasting scents.

Honey aroma has been associated to the synergic effect of phenyl ethyl alcohol and ethyl 9-decenoate by authors (Tang *et al.,* 2019; Slaghenaufi *et al.*, 2021). Cedar was previously correlated to the presence of ethyl 2-methyl butyrate together with ethyl butyrate. Anyway, the compounds were not detected in all the samples. Methyl salicylate, previously mentioned to be correlated to anise notes, has also been correlated to more balsamic notes by some authors (Slaghenaufi *et al.*, 2021). Aromatic herbs, save and thyme scents can be attributed to the presence of sesquiterpenes that could not be detected with the used method. Citrus and tropical fruits notes are associated to varietal aromas, such as terpenes and thiols (Cannon & Ho, 2018), even if for the DOCG, (*E*)-geraniol was the only terpene detected. Coffee, roasting and menthol nuances are tertiary aromas which originate during wine aging. Coffee scents, for example, has been correlated to the varietal thiol furfuryl thiol, even if the compound was not detected by the SPME-GC-MS analysis.

Taste: The taste of this DOCG was described as savoury, salty**,** with, in some cases, a light vanilla and toasted or vanilla and almond aftertastes.

"Offida Pecorino"

Appearance: The production disciplinary describes this DOCG with attributes which were found in the samples under investigation, being characterized by a straw yellow with golden shadows colour.

Olfactory attributes: Regarding olfactory attributes, even in this case the aroma of the samples was generally in accordance with the scents of yellow pulp fruit (ananas), floral notes and aromatic herbs associated to the DOCG. Indeed anise, cedar, citrus, medlar and yellow peach flavour have been perceived.

As mentioned, anise note is associated to methyl salicylate, even if in this sample this compound was not detected. Anyway, some authors found a correlation between this attribute and ethyl hexanoate (Gómez-Míguez *et al*., 2007). Peach and fruity scents are usually mainly attributed to ethyl esters of organic acids, abundantly present in the samples. Indeed, a study performed by Slaghenaufi *et al.* (2021) correlated the peach aroma to ethyl octanoate and ethyl hexanoate, the first being the most abundant ethyl esters in the "Offida Pecorino" samples.

Taste: Taste of "Offida Pecorino" is described as fresh, mineral and savoury, with a lasting aftertaste as encountered by the panel in the samples investigated. One sample showed a ginger hint aftertaste (PE02).

"Offida Passerina"

Appearance: The aspect of "Offida Pecorino" was described as bright straw yellow, which is in accordance with the description reported on the production disciplinary of this DOCG wine.

Olfactory attributes: Concerning the olfactory attributes usually described as citrus or yellow pulp fruits, the samples "Offida Passerina" under investigation were described as more floral, camomile dried flower, citrus, dried hay and mandarin peel (PA02).

Floral and camomile scents have been associated with the presence of 2-phenylethyl acetate and ethyl benzoate, respectively (Yang *et al.*, 2022), with the first one being detected in the samples.

Furthermore, floral aroma can be also attributed to compounds such as phenyl ethyl alcohol and linalool or geraniol, with phenyl ethyl alcohol being one of the most abundant alcohols in the samples investigated. Citrus notes have been associated to ethyl 2-methylbutyrate or ethyl butyrate but also to α-terpineol and linalool (Gómez-Míguez *et al.*, 2007).
Mandarin peel, being a fruity aroma can be explained by the abundant presence of ethyl esters. In particular, orange has been linked to ethyl octanoate (Yang *et al.*, 2019). Nerolidol and citronellol can also play a role in this olfactory attribute.

Taste: This DOCG is generally described with fresh and mineral taste and indeed, the samples were described as acid given that acidity plays an important role in the freshness taste of white wines. The samples were also characterized by more peculiar citrus (PA01) or bitter (PA02) aftertastes.

"Offida Rosso"

Appearance: "Offida rosso" DOCG was described with the typical ruby colour with garnet shadows which characterized it. The sample (RO02) "Offida Rosso" was characterized by the highest amount of malvinidin-3-glucoside, and the same sample was indeed described as the samples with the most intense red colour. This is in accordance with the phenolic compounds analysis, since the concentration of malvidin-3-glucoside in the sample was the highest (54 mg/L) when compared to all the other samples.

Olfactory attributes: "Offida Rosso" aroma is characterized by chocolate, liquorice and red berries scents as reported by its production disciplinary. The samples under investigation were described with more red fruits, jam, toasting, in spirit cherries, red fruits, plums and cooking jam scents.

Red fruits aromas are associated to different compounds, including mainly ethyl esters together with compounds such as isoamyl alcohol or isoamyl acetate as reported by some authors (Cabaroglu *et al.* 2004). Hence the fruity and jam aromas in the sample can be a sum of the compounds able to impart this kind of aroma.
Taste: The taste of this DOCG is described as smooth, complex and with a lasting aftertaste. One of the two samples investigated was found more astringent and tannic (RO01), while the other was characterized by wood, liquorice and vanilla tastes (RO02).

The sample characterized by the vanilla aftertaste was also the one that showed a very high concentration of vanillic acid, which derives from vanillin oxidation (Mourtizinos *et al.*, 2009). Hence

the presence of vanillic acid may be attributed to a high presence of vanillin which would explain the characteristic vanillin aftertaste encountered in the sample.

"Cònero"

Appearance: "Cònero" wines were described with the characteristic red ruby colour.

Olfactory attributes: The production disciplinary describes this DOCG with characteristic clove, plum, vanillin and berries scents. Indeed, the samples investigated were generally described with berries, blue and black berries and cherries in spirit attributes. Single samples were more characterized by clove, nutmeg, undergrowth and chocolate scents.

The more specific olfactory attributes, such as clove or nutmeg, are normally linked to characteristic molecules, but in this case these molecules were not detected by the SPME-GC-MS analysis. Chocolate in fact, can arise from vanillin (Selli *et al.*, 2004), while clove in wine has been associated to 4-ethylguaiacol and 4-vinylguaiacol (Mo *et al.*, 2010). Also, black fruit aroma was found to be a result of norisoprenoids presence.

Taste: Concerning taste, the characteristics of this DOCG are in accordance with those encountered by the panel performing the sensorial analysis. In fact, the samples were tannic, astringent and savoury, with jam and sour cherries notes. The investigated samples were also characterized by undergrowth, balsamic, fresh, menthol and balsamic aftertastes.

The balsamic and fresh taste can be also attributed to the high abundance of tartaric and malic acid. The former was indeed obtained with concentrations comparable to those of white wines, while the latter with the highest concentrations when compared to all the other DOCGs.

"Vernaccia di Serrapetrona" sweet and dry

Appearance: These DOCG samples were described according to the characteristic garnet red colour.

Olfactory attributes: These wines are characterized by red fruit and jam attributes, together with cinnamon, cherry and floral scents. The description provided by the production disciplinary is indeed in accordance with the attributes perceived by the panel.

Sweet: ripe fruit, sour cherries, red berries, plums, jam geranium.

Dry: cinnamon, chocolate, withered flowers, high volatile acidity, cherries in spirit.

Ethyl esters are the main responsible of fruity aroma in red wines, as previously discussed. Geranium is usually associated to (*E*)-geraniol but a positive correlation with hexanoic acid has been also found as reported by Vilanova (2010). Both compounds were detected by the GC-MS analysis in "Vernaccia di Serrapetrona" sweet sample characterized by this scent. The floral aroma of "Vernaccia di Serrapetrona" wines has been associated with phenyl ethyl alcohol (Fiorini *et al.*, 2014). Cherries in spirit an liquors attributes can be linked to compounds such as isoamyl alcohol. Cinnamon and chocolate are tertiary aromas which evolve during wine ageing.

Taste: This DOCG is characterized by a smooth, astringent with a delicate tannic taste. Floral and cinnamon tastes are typical of the wine.
Sweet: red berries or metallic, bitter, astringent.
Dry: incense, harmonic or more pungent.

6.1 Conclusions

The search for new reliable and feasible technologies aimed to assess wine authenticity has challenged researchers over the years, being an aspect of huge importance for both consumers and winemakers. The quality is known to be influenced by many factors, comprising grapevine varieties, origins, growing condition and vinification techniques and the designation of origin entails that the characteristics of the products are almost exclusively due to the production geographical location.

The organoleptic characteristics are strictly related to their chemical fingerprint and, indeed, the different chemical analysis performed underlined trends and profiles which may be used as authenticity markers for the DOCG wines of Marche region.

In this section some of the organoleptic characteristics, which have been evidenced by a panel of experts, have been associated to determined molecules (or sum of molecules), by hypothesizing the correlation that may exist between the chemical fingerprint and the sensorial analysis of the samples investigated. This was performed to characterize the DOCG wines produced in Marche region which, indeed, showed peculiar profiles.

Starting with "Verdicchio di Matelica Riserva" and "Castelli di Jesi Vericchio Riserva", these DOCGs were described by fruity aromas and, indeed, they were generally enriched in ethyl esters, a class of aroma compounds which have been attributed to this kind of scents by several authors. Also, the presence of methyl salicylate able to impart the characteristic anise note was identified in the DOCGs.

The "Offida" white wines, even if produced in the same geographical area, are produced with different vines and were described by diverse olfactory and taste attributes. "Offida Pecorino" for example, was described by peach and fruity scents which are usually attributed to ethyl esters, abundantly present in the samples. By contrary, "Offida Passerina" samples were described by more floral and camomile scents, that have been associated to the presence of 2-phenylethyl acetate. The aroma of "Offida Rosso" DOCG was generally described by fruit and jam scents, that has been attributed to the synergic behaviour of compounds such as isoamyl acetate and isoamyl alcohol. The sample "Offida rosso" RO02 showed the highest colour intensity and, indeed, it was also characterised by the highest concentration of malvidin-3-glicoside, an anthocyanin known to be an important pigment in red wines.

"Cònero" was more described by aromas, such as clove and vanillin. The balsamic and fresh tastes of the DOCG compared to the other red wines, was linked to the high concentrations of both tartaric and malic acids.

Finally, “Vernaccia di Serrapetrona” sweet and dry wines were generally described by scents such as floral, geranium and cherry in spirit, which can be linked to compounds such as phenyl ethyl alcohol and isoamyl alcohol. This DOCG was also described by tertiary aromas such as cinnamon and chocolate.

The sensorial analysis performed on the DOCG wine samples produced in Marche region, pointed out characteristic features, which were able to discriminate one DOCG over the other, highlighting the unicity of these wines protected by the most restrictive designation of origin. Interestingly, some wine samples showed peculiarities that were not associable to the DOCG, but that may arise from the different oenological practises of wineries, able to produce wines with distinct and unique characteristics.

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Chapter II: Ultra-selective solid phase isolation of mercaptans using Cu(I) salts. Application to the selective isolation of five polyfunctional mercaptans from wine - *Project carried out during 6 months staying in Spain as visiting PhD student*

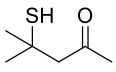
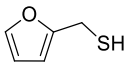
2.1 Introduction

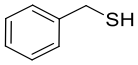
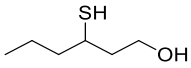
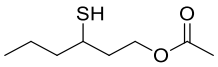
2.1.1 The role of mercaptans in wine aroma

Mercaptans are sulphur containing molecules that play important roles in both fresh (vegetable, fruit, plants, etc.) and processed foods (coffee, wine, roasted, etc.), due to the characteristic aromatic impact they can give (Harsch & Garden, 2013; Tominaga *et al.*, 1998). However, they are considered as a double-edged sword. In fact, while certain sulphur volatile compounds, such as hydrogen sulphide, are able to impart very negative rotten egg-like aromas, others can contribute with pleasant flavours playing essential roles in food products such as grapefruit, passion fruit, onions or wine (Buettner & Schieberle, 1999; Werkhoff, 1998; Granyogl *et al.*, 2004; Swiegers & Prietorious, 2007). Moreover, most of them have very low threshold levels, in the magnitude of ng/L, meaning that they can be smelled at tiny concentrations and their aromatic contribution can also become more or less attractive or repulsive depending on their absolute and relative concentrations.

In wine, mercaptans are characterized by powerful and penetrating odours and, while some of them are known for their putrefaction-related unpleasant odours, others are responsible for positive flavour nuances (Mateo-Vivaracio *et al.*, 2010; Chen *et al.*, 2019). Some of them have been found amongst the most relevant odorants in wine, like 4-mercapto-4-methyl-2-pentanone (MP), furfurylthiol (FFT), benzyl mercaptan (BM), 3-mercaptohexanol (MOH) and 3-mercapto hexyl acetate (MHA). The chemical structure together with the main sensorial characteristics are listed in **Table 1**.

Table 1. Chemical structures, olfactory descriptors, ranges of concentrations and odor thresholds of the main mercapatans in wine (Capone *et al.*, 2015).

Compound	Structure	Olfactory descriptor	Range of concentrations in wine (ng/L)	Odor threshold (ng/L)[e]
MP		box tree, passion fruit, broom, black currant[a]	<0.6-87.9	0.8
FFT		roast coffee[b]	<0.5-225	0.4

BM		smoked	<0.5-131	0.3
MOH		passion fruit, grapefruit, gooseberry, guava[c]	10-7256	60
MHA		passion fruit, grapefruit, box tree, gooseberry, guava[d]	< 2-591	4

[a]Darriet *et al.*, 1995.[b]Tominaga *et al.*, 2000. [c]Tominaga *et al.*, 1998; Dubourdieu *et al.*, 2006. [d]Tominaga *et al.*, 1996; Dubourdieu *et al.*, 2006.[e]Capone *et al.*, 2015.

The first studies concerning mercaptans, such as MP, were published by Darriet *et al.* (1995) and Bouchilloux *et al* (1996). In particular, this thiol was identified as an important characteristic varietal aroma in Sauvignon wines, where it could reach relatively high concentrations, up to 15 ng/L.

Subsequently, Guth (1997) reported the essential role of MP on a Scheurebe wine overall flavour when compared to other wines. Indeed, while in Scheurebe wine it was present in quiet high concentration (400 ng/L), it was much lower in the Gewürztraminer wine (< 10 ng/L), and this was one of the main reasons for the flavour differences between the two wines.

MH and MHA were also the subject of studies of different authors, due to their importance in the sensorial quality of certain wines. Lund *et al.* (2009) found out that the two compounds were characteristics flavours in Sauvignon blanc wines, even if produced in different parts of the world.

Concerning MH, it was found to play an essential role in the aroma of rosé wines, and it was also identified by more than one author as the key aromatic compounds of botrytized Sauternes wine, being present even at concentrations up to 5000 ng/L (Campo *et al.*, 2008a, 2008b; Thibon *et al.*, 2009; Sarrazin *et al.*, 2007; Ferreira *et al.*, 2002; Masson & Schneider, 2009). Concerning instead MHA, Campo *et al.* (2008a, 2008b) demonstrated that the tropical fruit nuance of Verdejo wines was a distinct characteristic imparted by the presence of this sulphur compound.

FFT is another important aromatic mercaptan and its presence in wine has been attributed to oak wood (Blanchard *el al.*, 2001). This volatile compound, together with BM, were firstly identified and quantified by Tominaga *et al.* (2003a, 2003b) in old Champagnes and in some white wines, such as Chardonnay, Sauvignon blanc and Sémillon where the highest concentrations (reaching 400 ng/L) were encountered in 15 years aged Champagnes samples, but also in red wines such as Merlot and Cabernet Sauvignon.

Thiols are released during alcoholic fermentation from their non-odorant precursors and the concentrations in which they can be found is strictly related to the grape variety and to the yeast assimilable nitrogen (YAN) (Coetzee & du Toit, 2012; Chone *et al.*, 2006).

For MP, MOH and MHA three biogenesis pathways are commonly used to explain their release, namely the cysteinylated precursors, the glutathionylated precursors or the (*E*)-2-hexenal pathways, which are summed up in **Figure 1** and explained in detail in the review published by Roland *et al.* (2011).

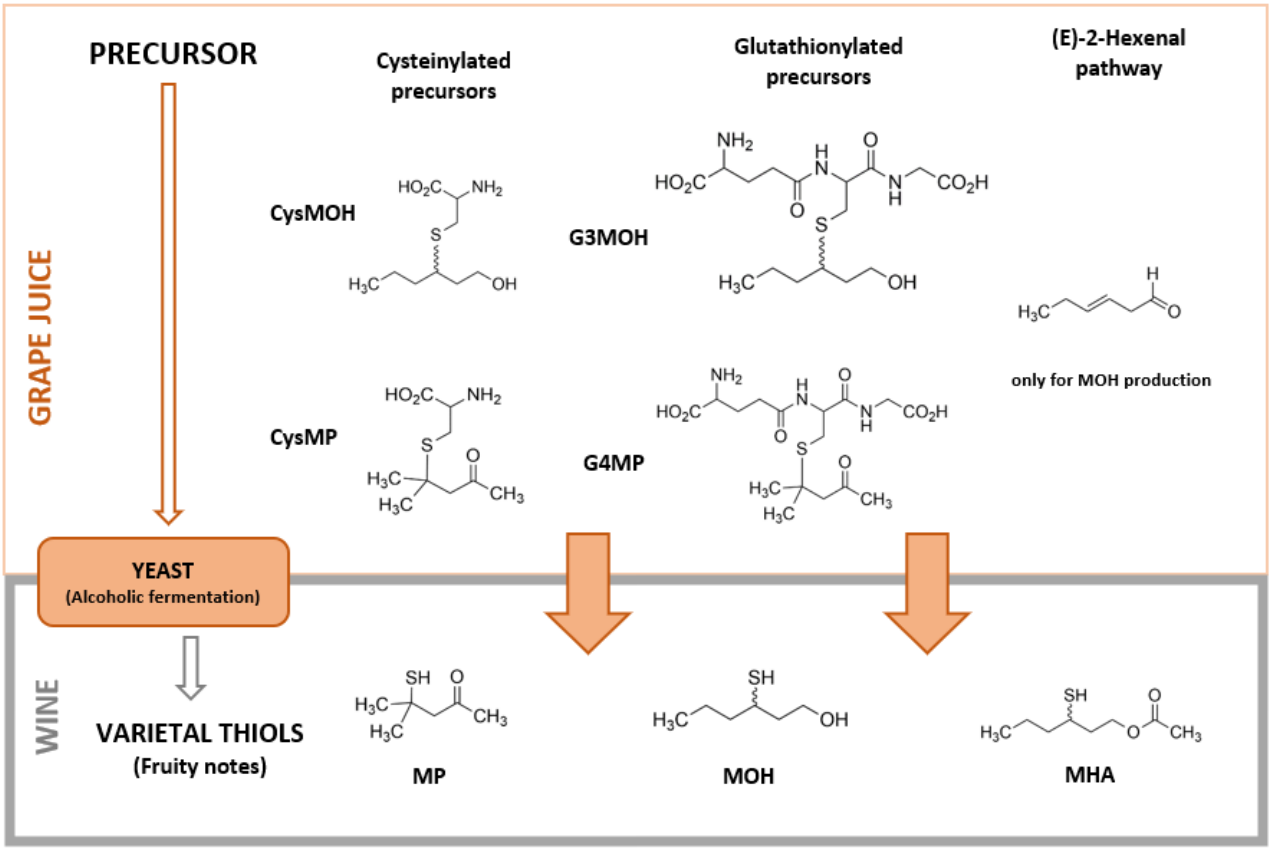


Figure 1. Release pathways for MP, MOH and MHA (Roland *et al.*, 2011).

The formation pathways of FFT is different. This compound is usually present only in barrel-fermented white wines and it derives from the furfural released by toasted staves (Blanchard *et al.*, 2001). Indeed, it was reported by some authors that the FFT content in wine was considerably affected by the container used. Wines fermented in new barrels had, in fact, higher FFT content than those fermented in new barrels, while no FFT was detected in wines fermented in stainless steel vats and this suggested the presence of its precursor in toasted wood of new barrels. This precursor was found to be furfural since a positive correlation raised between the concentration of this compound in must and the concentration of FFT formed. The presence of this thiol in white wines is thus closely related to the yeast's metabolism.

Finally, the presence of BM in wines has been associated to wine aging in bottle and whose final concentration was found to be influenced by the degree of oxygen in the bottle (Ugliano, 2013).

This compound has been found to significantly contribute to the aroma of certain wines, such as Sauvignon Blanc, Semillon or Chardonnay where its concentrations was found to be 30-100 times higher than its perception threshold (Tominaga *et al*., 2003).

2.1.2 Methods for the analysis of PFMs in wine

The analysis of polyfunctional mercaptans is very challenging. In fact, they are known to be poorly detectable and the relative mass spectra lack of characteristic ions with high m/z. Moreover, the chromatographic properties are also poor due to the adsorptive characteristics of the thiol function. In fact, this causes tailing peaks in some stationary phases or in the chromatographic systems with residual active sites. Mercaptans are also quite instable and are easily oxidized by oxygen or other oxidants and can also form insoluble complexes with many metal ions, which cause their precipitation (Hofmann *et al.,* 1996). For these reasons, most of the current methods for their analysis make use of their derivatization to form more highly detectable derivatives, exploiting the complexing properties of the thiol group. Thiol-specific extraction methods based on the strong affinity of thiols for metal ions (such as, Hg^{+} or Ag^{+}) has been exploited for their determination through different extraction techniques, such as liquid-liquid extraction (LLE) (Darriet *et al.*, 1995). Also, methods involving solid-phase micro extraction (SPME) with derivatisation steps have been reported by some authors (Mateo-Vivaracho *et al*., 2006, 2008).

2.1.3 The use of SPE for the extraction of PFMs from wine

Solid-phase extraction (SPE) is an extraction technique that was invented in the 70s and that spread very fast due to its exploitation in different domains of research (Bielicka-Daszkiewicz & Voelkel, 2009). It has numerous advantages since it is fast and easy, involves the use of small quantities of solvents, is selective and relatively inexpensive. Its use comprises different steps as reported in **Figure 2**.

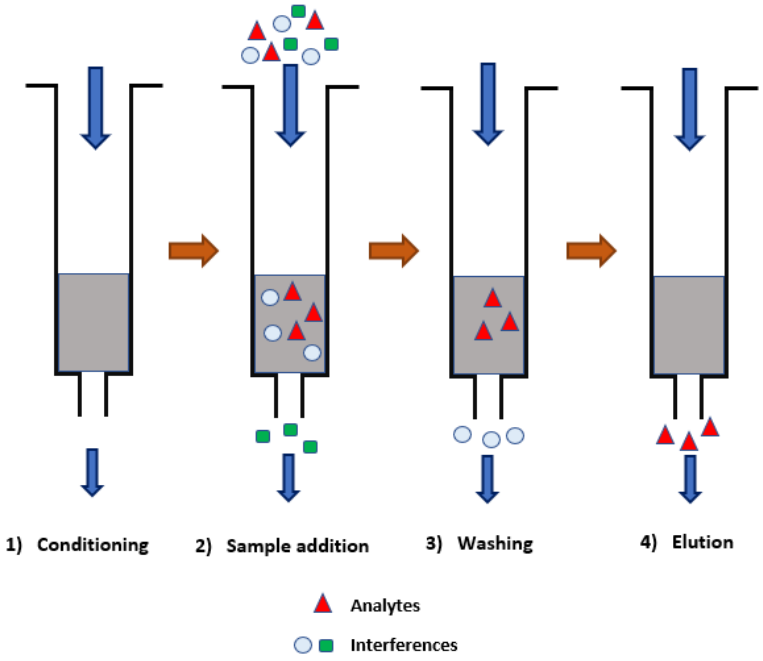


Figure 2. Steps of solid-phase extraction.

A first conditioning of the SPE cartridge is performed to reduce interferences and to increase the effective surface area of the cartridge. This is followed by loading of the sample, where the analytes, but also some interferences, are adsorbed and retained in the adsorbent bed. Then, the use of SPE finds the advantage of an additional cleaning step which enables to further remove interferences, obtaining more clean extracts. Finally, the analytes of interest are eluted from the cartridge using the proper strong solvent, such as dichloromethane, hexane, etc.

The SPE technique permits the isolation of different compounds from very complex matrices, thus comprising grapes and wine, where it has found different applications, mainly focused on the isolation of volatile substances (Sánchez-Palomo *et al.*, 2009). This extraction technique has been also exploited for the study of other compounds in wine. Mercolini *et al.* (2008), for example, used the SPE in order to quantify melatonin and resveratrol isomers from both white and red wines, followed by HPLC analysis coupled with fluorimetric detection (HPLC-F). Pérez-Magariño *et al.* (2008), instead, optimized an SPE method in order to isolate phenolic compounds in red wines, subsequently analyzed by HPLC – diode array detection (HPLC-DAD).

Concerning its use in the analysis of thiols in wine, some authors reported their determination, by exploiting derivatization (Herbst-Johnstone *et al.*, 2013). Matepo-Vivaracho *et al.* (2008), for example, reported the improvement of a previously developed method which used 2,3,4,5,6-pentafluorobenzyl bromide (PFBBr) as deriving agent. The method proposed consisted in the

derivatization reaction performed directly in the solid-phase extraction sorbent by adding 50 µL of a PFBBr solution after sample loading in the cartridge, followed by gas-chromatography-negative chemical ionization-mass spectrometry (GC-NCI-MS) analysis.

Capone *et al.* (2015) optimized a method for the determination of MOH, MHA, FFT, BM and MP in the form of 4,4-dithiodipyridine (DTDP) derivatives, by directly spiking the wine samples with the derivatizing agent, followed by their determination trough HPLC-MS/MS. DTDP was also used later by Mafata *et al.* (2018) for the determination of thiols in single cultivar south African red wines.

Previous works have made use of mercury salts, as reported by Schneider *et al.* (2003). Subsequently, Tominaga & Dubourdieu (2006) reported a novel method for the isolation and quantification of 2-methyl-3-furanthiol and 2-furanemethanthiol from wine by their release using a cysteamine solution applied in reverse flow to the sample percolation on the basis of *p*-hydroxymercuribenzoate-volatile thiol conjugate, which is formed by directly spiking the wine sample (Tominaga & Dubourdieu, 2006). More recently Chen & Darriet (2022) proposed a method for the quantitative screening of thiols by silver ion (Ag^+)-based sorbent SPE with heart-cutting multidimensional GC-MS-olfactometry (GC-MS-O).

However, these methodologies are usually lengthy and involve the use of toxic reagents. Derivatives are also odorless, which rends these methods useless for the qualitative identification of unknown mercaptans, and the yields of the derivatizations are extremely matrix-dependent, so that the use of isotopomers as internal standards becomes crucial. From both reasons, having at hand selective isolation procedures becomes essential.

2.1.4 Aim of the work

All the studies present in literature suggest that the five polyfunctional mercaptans surely play outstanding roles in the aroma of wines.

In the present study, we explore the known ability of Cu(I) salts to form strong complexes with mercaptans and take advantage of the possibilities of solid phase extraction to remove all other unretained compounds. Therefore, the main aims of this work were to undergo a thorough study of all the different aspects and variables affecting the selective SPE isolation of mercaptans and specifically develop an optimal procedure for the selective isolation and subsequent SBSE extraction TD-GC-GC-MS determination of the five main PFMs of wine. Initial experiments investigated the best way of forming and extracting the Cu(I)-SR complexes. Once determined that they will be formed on the mercaptans extracted in the SPE bed, the breakthrough volume for the five PMFs in

wine was assessed to evaluate the amounts of sample that can be safely loaded in the cartridge (15 mL). Then the concentration, composition and volume of the CuCl aqueous solution used to complex the mercaptans in the cartridge was investigated. Subsequently, cleaning, washing and elution steps using different solvents and solutions were studied and optimized. The washing step was found to be the most critical step and hence many different combinations of solvents and antioxidants using different conditions were tested. Finally, for the quantitative determination of the five underivatized main wine polyfunctional mercaptans, a procedure using methanol as elution solvent, further diluted with water and extracted with SPME or twister extraction was further developed. The most sensitive conditions were achieved by twister extraction (SBSE, PDMS 1 cm) and further TD-GC-GC-MS. The SBSE step and the TD-GC-MS-MS chromatographic methods are still under optimization in order to lower the sensitivity of the method (in terms of limits of detection (LOD) and quantification (LOQ)) with the aim to reach a suitable sensitivity to apply the procedure for the quantification of important odorant thiols in wine.

2.2 Materials and method

2.2.1 Reagents and standards

Ethanol, methanol (Suprasolv) and dichloromethane (DCM) (Suprasolv) were purchased from Merck (Darmstadt, Germany). Hydrochloric acid (37%) and (+)-tartaric acid (99%) were from PanReact (Barcelona, Spain). Copper(I) chloride (99.9%), L-cysteine hydrochloride anhydrous (99%) and 1,4-dithiothreitol (DTT, 97%) was purchased from Aldrich (Steinheim, Germany).

4-Mercapto-4-methyl-2-pentanone (MP) and 3-mercaptohexyl acetate (MHA) were from Oxford Chemical (Hartlepool, UK). Furfurylthiol (FFT), benzyl mercaptan (BM), 3-mercaptohexanol (MOH) were from Lancaster (Strasbourg, France). Reservoir SPE test tubes (1 mL) and FTPE frits were purchased from Merck (Darmstadt, Germany). ISOLUTE ENV+ absorbent phase was obtained from Biotage and the semi-automated SPE VAC ELUT 20 station was purchased from Varian (Walnut Creek, CA, USA). Ultrapure water was obtained from Milli-Q purification system (Millipore, Billerca, MA, USA). The pHmeter was a Crison micropH 2002 (Crison Instruments, Barcelona, Spain).

2.2.2 Preparation of standards and CuCl solution

To preserve the integrity of sulphur stock solutions, all of them were prepared by weighting, in sealed vials protected from light, with solvents and vials purged with nitrogen and always kept at low temperatures (-20°C solutions). The standard solution containing the five PFMs was prepared by dissolving the appropriate amount of every stock solution (which ranged from 3500 to 9000 mg/L) in ethanol, reaching a final volume of 8 mL in a volumetric flask, to obtain a solution containing each analyte at a concentration of 100 mg/L.

Pure standard and CuCl 500mg/L solutions were always handled in the anoxic chamber to avoid oxidation. The CuCl solution was daily prepared by dissolving the proper quantity in ultrapure water buffered at pH 2 with HCl. The solution was taken out from the anoxic chamber few minutes before its use.

For the preliminary essays, 2-octanol was used as internal standard (IS) at a concentration of 250 µL/L, which was prepared from a concentrated stock solution (1000 µL/L in ethanol) by dilution with ethanol in a volumetric flask.

2.3.3 Synthetic wine and wine samples

A hydroalcoholic wine model solution was prepared to perform the procedure optimization with 12% ethanol (v/v) and by dissolving cysteine hydrochloride (5 mg/L), (+)-tartaric acid (5 g/L) in ultrapure water and then buffered at pH 3.5 with NaOH 4N. The wine use used for the work was a young white wine of Verdejo (2021).

2.3.4 Cu(I)-complexes formation in the cartridge after sample loading

After preparing the SPE cartridge with 65 mg of ISOLUVE ENV+ absorbent phase, it was conditioned and then the sample (10 mL spiked with 200 ppb of PFMs) was loaded. Subsequently, the solution of CuCl was percolated through the cartridge in order to complex PFMs under the form of Cu(I)-S-R complexes. Two fractions (1 mL) of methanol where collected, diluted to 5 mL with water and then extracted with 1 mL of dichloromethane after adding 1 g NaCl and SI (2-octanol 250 ppm, 20 µL), for 1h.

2.3.5 CuCl solution condition evaluation

In order to assess the optimal quantity of CuCl in terms of concentration and volumes, the assays were performed by following the procedure previously reported and varying the CuCl solution concentrations (100 and 500 mg/L) and volume (2 or 4 mL).

2.3.6 Cartridge preparation

ISOLUTE ENV (65 mg) + SPE cartridges were prepared in 1 mL standard polypropylene SPE reservoirs. The absorbent phase was conditioned with 2 mL of DCM and 2 mL of methanol. Then it was rinsed with 2 mL of 12 % ethanol water solution helped by vacuum pump.

2.3.7 Proposed SPE procedure

An aliquot of wine sample (5 mL) is loaded through a previously conditioned SPE cartridge, followed by 4 mL of a CuCl (500 mg/L) aqueous solution (buffered at pH 2 with HCl). Once the mercaptans are fixed in the cartridge, the impurities are removed by loading 2 mL of methanol. The cartridge is then rinsed with 4 mL of an aqueous cysteine solution (50 g/L) containing DTT (5 g/L) and a further wash is performed with 10 mL of a DTT solution (3 g/L). The cartridge is then dried under nitrogen flux and, finally, the extracted mercaptans are eluted with 1.3 mL of a methanol solution containing DTT (2 g/L) (or 0.6 mL DCM).

2.3.8 GC-MS analysis for the optimization of SPE procedure

Preliminary assays were performed to optimize the SPE procedure and analysed by directly injecting the DCM extract in a GC-2010 Shimadzu coupled with a single quadrupole mass spectrometer QP2010 from Shimadzu (Kyoto, Japan). The injection (1 µL) was performed in splitless mode, and the injector temperature was maintained at 250 °C. The carrier gas was He at an initial flow rate of 1.26 mL/min The column was a polyethylene glycol (DB-WAXetr length 30m, i.d. 0.250mm, film thickness 0.50µm; Agilent Technologies, Santa Clara, USA). The oven temperature was maintained at 45 °C for 2 min, then raised at 30 °C/min until reaching 120 °C, then raised again at 10 °C/min until 240 °C, temperature which was keep for 5 min, for a total run time of 23.77 min. The detector temperature was set at 240 °C. The mass analysis was performed in SIM mode (8.50-8.85 min for MP, 8.90- 9.75 min for FFT, 12.10-13.05 min for BM, 13.10-14.55 min for AMH and 14.60-16.10 min for MOH). The identification of the compounds was performed by comparing the

retention times and their mass spectra to those of authentic standards. The m/z used for the identification of analytes were: 132 for MP, 81 for FFT, 91 for BM, 116 for AMH and 100 for MOH.

2.3 Results and discussion

2.3.1 CuCl immobilization capacity in SPE cartridge

At first, the retention capacity of CuCl in the SPE cartridge was assessed in order to determine where the formation of Cu(I)-S complexes was more efficient for the extraction of PFMs. To this purpose, the breakthrough volume (BV) of the CuCl solution was assessed by percolating a 100 mg/L solution through the SPE cartridge previously conditioned and collecting 0.5 mL fractions. The fractions where then diluted with a bathocuproine desulphonated (BCDA) aqueous solution (85 mg/L) of and the presence of Cu(I) was determined using a UV/vis- spectrophotometer at 484 nm wavelength (**Figure 3**).

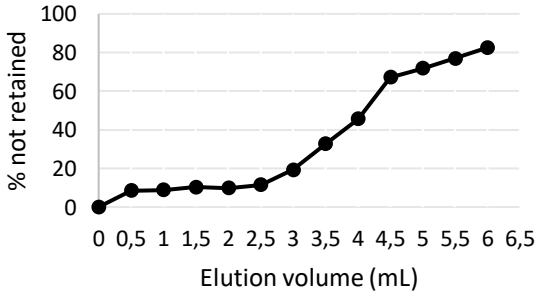


Figure 3. BV curve of Cu(I) to assess its retention capacity in the SPE cartridge.

Bathocuproine desulphonated is able to form 2:1 characteristic colored complex with Cu(I) and this absorbance characteristic was indeed used in the past for the quantification Cu(I) in different matrixes, such as serum or proteins, since the formed complexes showed a good stable, sensitivity and reproducibility (Zak, 1958; Rapisarda *et al.*, 2002).

This initial experiment showed that CuCl can be weakly retained in the cartridge, with breakthrough volume as low as 7 mL, which precludes the use of CuCl-loaded SPE cartridges for the preconcentration of mercaptans contained in hydroalcoholic solutions. For this reason, subsequently, experiments were performed to investigate if the formation of Cu(I)-S complexes should be performed in the wine sample before percolating it in the SPE cartridge or directly in the cartridge after loading the sample.

2.3.2 Breakthrough volumes to assess the best way of forming Cu(I)-complexes

At first, the retention properties for the five PFMs in the 65 mg ISOLUTE ENV + bed was assessed by defining their breakthrough volumes (BV), which refers to the maximal volume of sample which can be loaded in the cartridge, without any loss of the analytes of interest. This was performed by adding real wine, spiked with 200 ppb of PFMs, with a sufficient amount of CuCl (20 mg/L). The sample (100 mL) was then passed through the previously conditioned cartridge containing 65 mg of ISOLUTE ENV+ absorbent phase. Five mL fractions were collected and extracted in 1 mL DCM after adding SI (25 µL, 2-octanol 250ppm), 0.5 mL cysteine 50 g/L aqueous solution and 1 g NaCl, for 1 h.

It was observed that Cu-mercaptans already formed in wine were just poorly retained in the cartridge, with non-ideal retention kinetics suggesting that the complexes could not have access to the sorbent microporous particles (**Figure 4**).

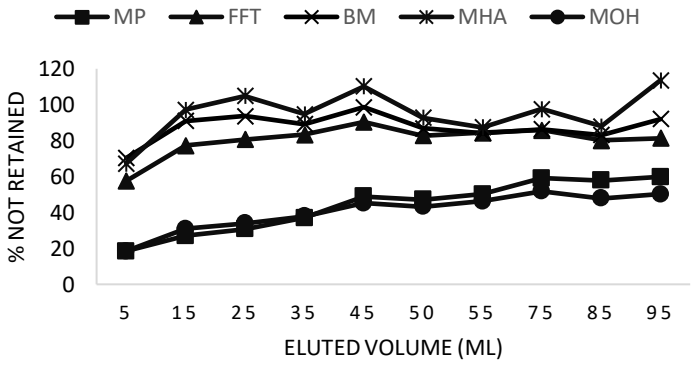


Figure 4. BV curves for the analytes by direct spiking of wine with CuCl.

Because of that, it was hypnotized that the maximum advantage of the selectivity introduced by Cu(I) could be achieved if the complexes were formed once the mercaptans were extracted in the SPE cartridge.

For this reason, the BVs of the five polyfunctional mercaptans were determined on real wine spiked with 200 ppb of PFMs, after loading the SPE cartridge with 4 mL of a CuCl 500 mg/L aqueous solution and the results obtained are shown in **Figure 5**.

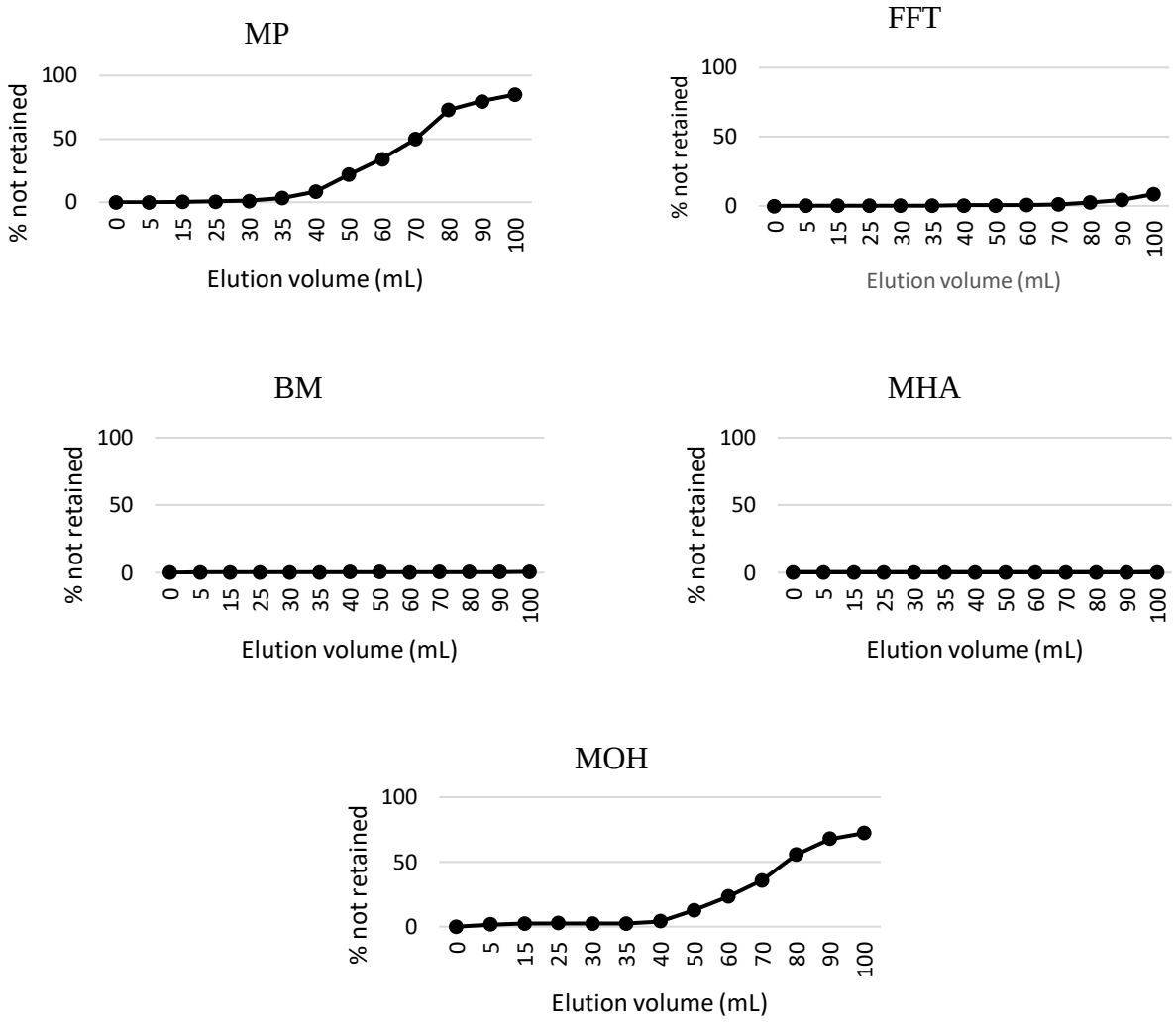


Figure 5. BV curves obtained by formind Cu(I)-S complexes in the cartridge.

As it can be observed, for FFT, BM and MHA no losses were observed even after 100 mL of sample loading, confirming that these analytes are able to be strongly retained in the cartridge under the form of Cu(I)-S complexes. Concerning MP and MOH, the BV was observed to be above 30 mL. Some hypotheses can be done on the different behaviour of the two mercaptans. The poor ability of MP to form derivative was previously observed by Mateo-Vivaracho *et al*. (2008) during the improvement of a SPE procedure for the isolation of polyfunctional mercaptans, comprising MP, under the form of PFBBr derivatives. This compound was the one showing the lowest reaction rate and the oddest behaviour when compared to the other mercaptans in the mentioned study. This was explained since this is a tertiary mercaptan and thus some side reactions related to the possible formation of tertiary carbocation would be promoted. Furthermore, the proximity of the oxygen of the carbonyl group and the hydrogen of the thiol function may result in the formation of an intra-

molecular hydrogen bond. The formation of the cyclic structure was considered to be the most relevant cause able to explain the poor yields.

Considering the overall results, a final volume of 15 mL of sample was kept as the optimal to be loaded in the cartridge and to continue with the further procedure optimization steps.

2.3.3 Removal of interfering compounds

Different cleaning solvents were further studied. To this purpose, 10 mL of synthetic wine spiked with 200 ppb of PFMs were used. The rinsing step for removing the major wine volatiles in SPE cartridge, after sample loading, has been previously performed by using 2 mL of an aqueous solution containing 30% methanol and 1% $NaHCO_3$ (Mateo-Vivaracho *et al*., 2009). In this study 100% hexane, 100% methanol and a 1:1 H_2O:methanol solution were evaluated. Results revealed that Cu-mercaptan complexes are so strongly retained in the cartridge, that more than 6 mL of MeOH can be used for the washing step without eluting detectable amounts of mercaptans. However, 2 mL of MeOH were selected as optimal, since this volume can elute nearly 100% of the volatile material retained in the sorbent.

2.3.4 CuCl solution optimization

The optimal CuCl solution conditions were assessed by evaluating different concentrations (100 mg/L and 500 mg/L) and volumes (2 mL and 4 mL) of the solution. This was performed in order to study the best manner for complexing the PFMs already present in the cartridge, to avoid significant PFMs losses during the impurities cleaning step and to assess preliminary recovery performances of the eluted obtained by the isolation procedure. To see if relevant losses of PFMs were due to the rinsing step, also a few fractions eluted during this step were investigated.

Hence to control possible losses in the rinsing step 2 subsequent 1 mL methanolic fractions were then diluted 1:5 with water and extracted in 1 mL DCM, followed by GC-MS analysis.

The percentages of mercaptans lost during the rinsing step and recovered in the final DCM eluted, performed by percolating 0.6 mL of the solvent through the cartridge, were calculated and the results are reported in **Figure 6**.

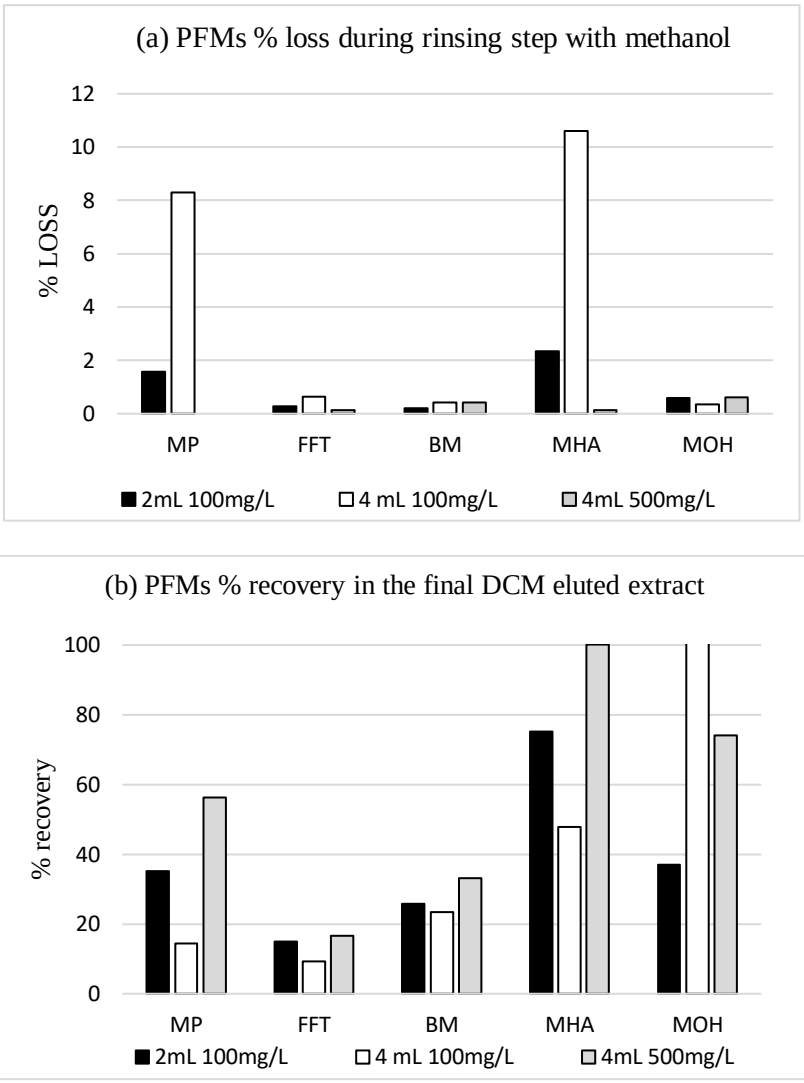


Figure 6. (a) PFMs percentage loss in 2 mL of cleaning methanol; (b) % PFMs recovery in DCM. The percentage of the two 1 mL MeOH fractions are shown summed, since the second fraction contained negligible amounts of mercaptans compared to the first.

As shown in **Figure 6**, the use of 2 mL of CuCl 100 mg/L gave little losses of MP and MHA (1.6 and 2.3 %, respectively) and negligible for the other three PFMs. At the same time, very poor recoveries of PFMs in the final elution step were obtained (in the range 15-75.1%). When using 4 mL of the same concentrated solution, the losses were higher showing a weak possibility to efficiently control the formation of Cu(I)-S complexes in the cartridge at these conditions. In particular, MP and MHA again gave the worst results in terms of losses (8.3 and 10.6%, respectively). Using a higher amount, it was expected to obtain better results, but the final recovery of PFMs the results were not satisfactory, given that they were in the range 9.4-100%, but 100% observed only for MOH.

For this reason, stronger conditions, i.e., using 4 mL with a higher concentration of CuCl (500 mg/L), were evaluated which gave satisfactory results in terms of losses of PFMs, being always below

0.6 %. Wine naturally contains cysteine, hence, Cu(I) may interact not only with PFMs but also with other thiols, for this reason the use of a higher amount of CuCl was found to be advisable. Hence, to proceed and to optimize the final recovery of the PFMs, which was still not satisfactory (ranging from 16.6 to 74.0% with only MHA showing 100%), these final conditions were kept.

2.3.5 Cu(I)-S complexes cleavage and quantitative extraction of analytes

Then, the cleavage of the Cu(I)-S complexes and further elution of the mercaptans were finally investigated.

First assays performed by using 4 mL of a cysteine aqueous solution (50 g/L) followed by elution with DCM offered consistently poor recoveries and acceptable results were obtained only for MP (73%) and AMH (89%) as shown in **Figure7**.

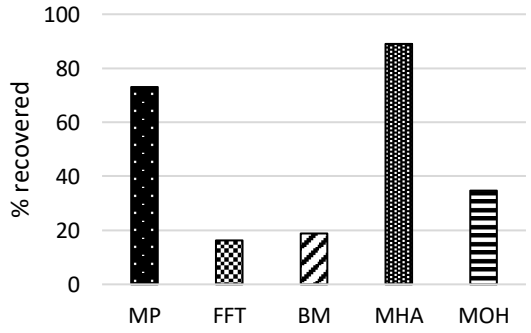


Figure 7. Final % recovery using cysteine 50g/L for cleavage and DCM as elution solvent.

A thorough research of the factors affecting this, revealed that the problem was not related to a lack of complex cleavage, of solvent strength or of elution volume, but to oxidation. For FFT, which was the most challenging to recover, it was supposed that it may form bis(2-furfuryl)disulphide by chemical oxidative dimerization, which may occur under oxidative conditions (Huynh-Ba, *et al.*, 2003). For this reason, it was decided to investigate the use of antioxidant agents. Results much improved when antioxidants such as TCEP and, in particular, 1,4-dithiothreitol (DTT) were incorporated in the procedure (as reported in **Table 2**), confirming that the main problems were probably due to oxidation happening in the SPE bed.

Initially the use of TCEP was investigated. It was used as a further cleavage reagent (10 mL 3 g/L) after 4 mL of cysteine (50 g/L) (**Figure 8**, A). A great improvement was observed for all the analytes, except for MHA which was already obtained with a good recovery with the previous conditions. MP improved from 73.1% to 100%, FFT from 16.3% to 54.7%, BM from18.8 to 86.9%

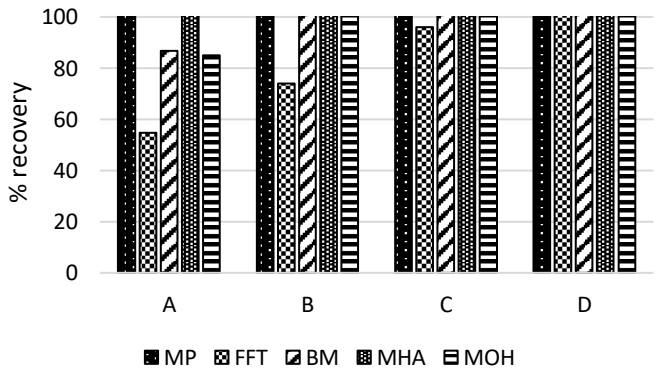
and MOH from 34.7 to 84.9%. Hence, recovery was still below 100% for all the analytes, with FFT still being the most challenging compound.

Then, it was decided to optimize the amount of TCEP to be used by adding this reducing agent also in the final elution step. **Figure 8** (B) shows the results obtained when the elution of PFMs from the cartridge was performed by spiking DCM with 1.5 g/L of TCEP. A further improvement was achieved since a total recovery was obtained for almost all compounds except of FFT, that increased to 73.9 %.

Then the use of DTT was investigated and it enabled to greatly improve the recovery of FFT, from 73.9 to 96.1% (**Figure 8,** C). Better results were obtained by using the reducing agent also in the cysteine solution with a concentration of 2.5g/L, followed by a 10 mL containing DTT at a concentration of 1.5g/L and in the final elution solvent (at a concentration of 1 g/L).

Table 2. Conditions used with antioxidant agents to improve PFMs recoveries.

	Cu(I)-S complexes cleavage	additional cleavage	elution
A	4 mL cysteine (50 g/L)	10 mL TCEP (3 g/L)	0.6 mL DCM (5% MeOH)
B	4 mL cysteine (50 g/L)	10 mL TCEP (3 g/L)	0.6 mL DCM (5% MeOH) + TCEP (1,5 g/L)
C	4 mL cysteine (50 g/L) + DTT (2.5 g/L)	10 mL DTT (1,5 g/L)	0.6 mL DCM (5% MeOH) + DTT (1 g/L)
D	4 mL cysteine (50 g/L) + DTT (5 g/L)	10 mL DTT (3 g/L)	0.6 mL DCM (5% MeOH) + DTT (2 g/L)



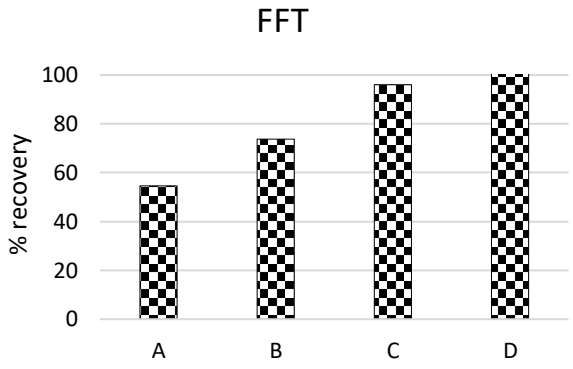


Figure 8. (a) Recoveries for analytes with use of TCEP and DTT. (b) Recovery trend for FFT.

At the end, by doubling the DTT quantities in all three subsequent steps, outstanding results were obtained and a 100% recovery for the five PFMs was observed. Hence, it was obtained by using 4 mL cysteine 50 g/L spiked with DTT 5 g/L, followed by a further 10 mL percolation of a DTT aqueous solution (3 g/L) (**Figure 8**, D). Mercaptans could be further quantitatively eluted out of the cartridge by using 0.6 mL of DCM containing also DTT (2 g/L).

2.3.6 Gas chromatography-mass spectrometry analysis of the eluted substances to check SPE procedure optimization

The results of the optimization of the SPE procedure were initially monitored by analyzing DCM extract by direct injection in GC-MS. The isolation procedure finally developed was highly selective and very clean extract were obtained. The chromatographic peaks, referred to the specific m/z ratio for each compound obtained in the DCM eluted are reported in **Figure 9**.

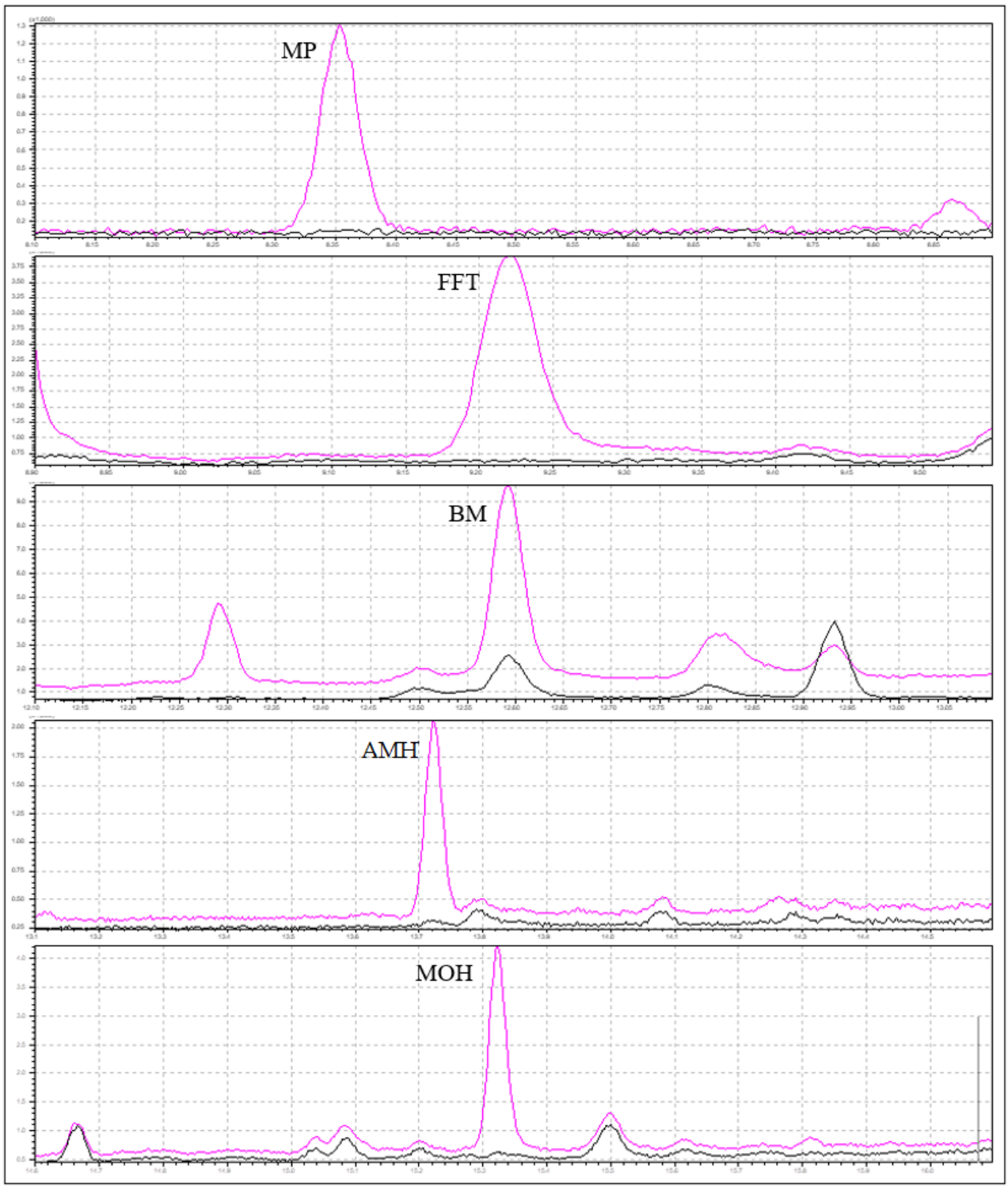


Figure 9. Chromatograms obtained in the GC-MS analysis of a real wine sample containing 200 ppb PFMs, analysed by direct injection of DCM extract in GC-MS in SIM mode. MP: 4-mercapto-4-methly-2-pentanone, FFT: furfurylthiol, BM: benzyl mercaptan, AMH: mercapto hexyl acetate, MOH: 3-mercaptohexanol.

2.3.7 Enhancement of method sensitivity and further developments

Since PFMs are usually present in wine at very tiny concentrations, the sensitivity of the method was still not the ideal for a further quantification of the mercaptans in wine. Indeed, LOD and LOQ values, were initially calculated by analyzing a standard mixture of mercaptans at 10 ppb to assess that the sensitivity of the method was suitable to proceed with the isolation procedure optimization. These limits were estimated by considering the peak areas corresponding to 10 and 3 times the signal to noise ratio and the results are reported in **Table 3**.

Table 3. LOD and LOQ initially calculated by analyzing a 10 ppb mixture standard solution of mercaptans by GC-MS.

Compound	LOD (µg/L)	LOQ (µg/L)
MP	834.5	2778.8
FFT	219.1	729.7
BM	154.2	513.5
AMH	694.1	2311.5
MOH	615.3	2049.1

Obtained values were low enough when the optimized procedure was used and a real wine or wine model were spiked with 200 ppb of PFMs.

Very low LOD values have been obtained in the past by some authors. Mateo-Vivaracho *et al.* (2009), for example, were able to obtain LOD values in the range 1.5-13.0 ng/L.

To obtain a sufficient sensitivity in the analytical determination of underivatized PFMs, the elution with MeOH was selected followed by dilution with water to evaluate a further extraction with solid phases, by initially investigating the use of SPME. Previous studies which involved the use of SPME for the extraction of thiols from wine (but comprising previous derivatization steps), permitted to obtain very good sensitivity. Rodríguez-Bencomo *et al*. (2009), for example, obtained LOD values in the range 0.03-1.29 ng/L.

The replacement of DCM with methanol (diluted with water) was performed due the hydrophobicity of mercaptans, with the aim to enhance their affinity and, thus, extraction in solid phases. Furthermore, the volume used to elute PFMs from the SPE system (0.6 mL) was not enough to perform SPME in immersion mode, which was the extraction mode initially investigated. Anyway, after the first experiments, the extraction method was found to be unable to provide enough concentration factors and reproducibility.

Finally, SBSE was considered, coupled with a thermal desorption system (TD) and followed by GC-GC-MS analysis. This extraction made allowed to reach detection limits in the ppt range. Indeed, preliminary assays suggested that LOD could be obtained the concentrations shown in **Table 4**.

Table 4. Limits of detection (LOD) expected by performing the SBSE-GC-GC-MS condition optimization.

Compound	**Expected LOD (ng/L)**
MP	< 0.3
FFT	< 0.2
BM	< 0.8
MHA	< 15
MOH	< 1.5

By reaching these LOD values, a suitable sensitivity would be achieved which permit the further determination of PFMs in wine samples.

2.4 Conclusions

Cu(I) can be satisfactorily used to complex and stabilize mercaptans previously retained in a polymeric SPE cartridge. The fixation is strong enough to achieve a complete separation of fixed mercaptans from any other volatile retained in the SPE bed. By using strong reducing agents during complex cleavage and elution, quantitative recoveries of completely isolated mercaptans were achieved. For the automated analysis of underivatized mercaptans, best sensitivities were obtained by using MeOH elution, and further SBSE extraction followed by TD-GC-GC-MS. The optimization of SBSE and TD-GC-GC-MS conditions are still under investigation with the aim to lower the sensitivity of the method to the expected values and thus to validate the method to finally apply it for the determination of the five polyfunctional mercaptans in wine samples.

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