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New Analytical Methods for teh Study of Wine Aroma. Applications to the Characterizaton of the Headspace Evoluction an Ultra-trace Analysis

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NEW ANALYTICAL METHODS FOR TEH STUDY OF WINE AROMA. APPLICATIONS TO THE CHARACTERIZATON OF THE HEADSPACE EVOLUCTION AN ULTRA-TRACE ANALYSIS

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New Analytical Methods for the Study of Wine Aroma

Applications to the Characterization of the Headspace Evolution and Ultra-trace Analysis

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Wen Yan

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CERTIFICAN:

Que la presente memoria, titulada "New analytical methods for the study of wine aroma. Applications to the characterization of the headspace evolution and ultra-trace analysis" correspondiente al plan de investigación aprobado por la Comisión de Doctorado del Departamento de Química Analítica y presentada para optar al grado de doctora en Ciencia Analítica en Química, ha sido realizada bajo nuestra dirección por Dª. Wen Yan, autorizando su presentación para proseguir los trámites oportunos y proceder a su calificación por el tribunal correspondiente.

Zaragoza, 8 de octubre de 2018

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Also, wines analyzed in Chapter 7 were provided by the project “Valorización aromático de los vinos del piedemonte pirenaico (VALOVITIS). VALOVITIS es un proyecto de colaboración transfronteriza entre España y Francia cofinanciado por el Fondo Europeo de Desarrollo Regional (FEDER) a través del Programa Interreg V-A España-Francia-Andorra (POCTEFA 2014-2020), destinado a reforzar la integración económica y social de la zona fronteriza España-Francia-Andorra.

This doctoral work has published two scientific articles:

- Wen, Y., Lopez, R., & Ferreira, V. (2018). An automated gas chromatographic-mass spectrometric method for the quantitative analysis of the odor-active molecules present in the vapors emanated from wine. *Journal of Chromatography A, 1534*, 130-138.
- Wen, Y., Ontañon, I., Ferreira, V., & Lopez, R. (2018). Determination of ppq-levels of alkylmethoxypyrazines in wine by stirbar sorptive extraction combined with multidimensional gas chromatography-mass spectrometry. *Food Chem, 255*, 235-241.

The following works are under preparation:

- The release of volatile compounds from wine: different effects of physical conditions, wines and time.
- Volatile and sensory evolution of wine headspace: a study of their correlations.

The work included in Chapter 7 awarded the Poster Third Prize at *In Vino Analytica Scientia 2017*.

- Determination of ultra-trace levels of alkylmethoxypyrazines in wine by stir bar sorptive extraction combined with multidimensional gas chromatography-mass spectrometry.

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To Mama

“People like us, who believe in physics, know that the distinction between past, present, and future is only a stubbornly persistent illusion.”

—Albert Einstein

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PRESENTATION

PRESENTATION

The present doctoral thesis contains a series of studies focusing on the development of new analytical methodologies to study wine aroma, including (1) a fast dynamic headspace sampling method for studying volatile compounds and their temporal changes in the headspace above wine over time, and (2) an innovative method to determine the concentration of impact odor-active compound at sub-ng/L level in wine.

The thesis is basically divided into two parts. The first one, which is the most extensive, is focused on the volatile compounds and their time-dependent evolution in the headspace under the conditions close to real wine tasting. The purpose is to expand the knowledge of the sensory changes perceived during wine tasting and their relationship with the chemical composition. The second part is aimed at the development of a sensitive method for analyzing alkylmethoxypyrazines in different wines of numerous grape varieties.

The thesis is presented in the following parts:

- Summary: in English and Spanish.
- Introduction: a brief description of the applications of analytical techniques for wine aroma analysis, including headspace sampling techniques and innovative techniques for separation and quantitation.
- Objectives: the proposed objectives for the whole thesis.
- Chapters: the thesis contains 7 chapters.

Chapter 1 proposed and validated an automated dynamic headspace sampling method (DHS), which was able to take a snapshot of the headspace composition above wine during wine tasting. From Chapter 2 to 5, the developed strategy in Chapter 1 was applied to study the release and temporal evolution of volatile compounds presented in the headspace of wine over time, under different conditions and matrices. Chapter 6 discussed the relationship between sensory changes and the headspace evolution during real wine tasting period. Chapter 7 developed a method to quantify alkylmethoxypyrazines in wine and applied it to a large number of wines.

- Conclusions: the section summarizes the most important results and conclusions of the thesis.

SUMMARY

SUMMARY

One of the main challenges in wine chemistry is to answer the question that whether flavor perception can be explained or even predicted by the determination of the chemical composition or not. Although knowing the general volatile composition in the liquid phase of wine is essential, it is not enough for explaining the aroma nuances that are more related to the headspace above the wine. It is clear that interpreting the role played by aromatic stimuli in the overall perception needs to involve other aspects, such as the integration of the perceptual processes, the changes associated to the release of volatiles and their temporal modifications. Another important aspect is that some impact odorants can dominate the aroma profile of a particular variety of wine even at ultra-trace levels, requiring more sensitive quantitative strategies. The present thesis has investigated the characterization of the release of volatile compounds and their evolution in the headspace above wines and also has researched the quantitative analysis of ultra-trace alkylmethoxypyrazines in wines by developing new analytical methodologies.

With the consideration to study the headspace composition of wine under non-equilibrium conditions that are more closely to the headspace of real wine tasting, an automated dynamic headspace (DHS) method combined with thermal desorption (TD) and gas chromatography-mass spectrometry (GC-MS) has been developed in **Chapter 1**. Thanks to this fast dynamic sampling strategy, the method could provide a snapshot of the contents in the wine vapors

of up to 40 aroma compounds, including methanethiol, sulfur dioxide, aldehydes, fusel alcohols or volatile phenols.

In **Chapter 2**, the validated DHS method has been applied to assess the changes in the wine headspaces with time, monitoring the levels of 34 odorants emitted to the headspace by 4 different wines during five consecutive time points. Three patterns of behavior within the evolution of the aromas were found. These patterns corresponded to the physicochemical characteristics of volatile compounds and the potential interactions with wine matrix components, which suggests that prediction of the aroma impact in these cases should include an estimation of the odorant-matrix interactions in wine.

Chapter 3 presents the application of the validated DHS method to study the potential influences of physical parameters on the release of volatile compounds from wine, which occur during tasting period, including agitation, evaporation, oxidation and degasification that could occur during wine drinking. Furthermore, the trends of the headspace evolution of a red and a white wine were studied during a 30 min period. The physicochemical characteristics of the volatile compounds were crucial to explain their release behavior.

Chapter 4 and **Chapter 5** mainly studied the impact of the potential volatile-matrix interactions on the release behavior of volatile compounds, using the DHS method to detect the contents of given volatile compounds in the headspace within different evaporation times. **Chapter 4** reconstituted 7 model wines by adding a standard aroma solution to deodorized wines with distinct matrices.

Chapter 5 applied the same strategy but to the study of the headspace changes relating to macromolecules that were oenological additives, such as polysaccharides and polyphenols.

Chapter 6 was devoted to the comprehensive study of two premium category wines. In this case, both the analytical data of the volatile components in the liquid phase and those of the headspace at different time points were combined with a complete simultaneous sensory study carried out by a panel of tasters. The sensory data at different moments of wine tasting were correlated with the corresponding concentration data in the headspace of volatile compounds that were relevant to each aroma descriptor.

Alkylmethoxypyrazines are potent odorants in many food products, as well as in wines. Usually, they are presented at extremely low concentration levels. In **Chapter 7**, a new method for the identification and quantitation of 3-isobutyl-2-methoxypyrazine, 3-isopropyl-2-methoxypyrazine and 3-sec-butyl-2-methoxypyrazine has been developed and applied to wine. According to the validated method, the analytes were extracted from 5 mL of wine using stirbar sorptive extraction followed by thermal desorption and multidimensional gas chromatography-mass spectrometry analysis in a single oven. The method is not only automatable and environmentally friendly but also provides the best limits of detection for these compounds published to date. The method has been applied to the analysis of 111 Spanish and French wine samples produced with minor and rare grape varieties.

RESUMEN

RESUMEN

Uno de los principales desafíos en la Química del vino es contestar a la pregunta de si la percepción sensorial del mismo puede se explicada o incluso predicha mediante la determinación de su composición química. Aunque conocer la composición en volátiles de la fase líquida del vino es imprescindible, no es suficiente para explicar las notas aromáticas más relacionadas con el espacio de cabeza que se encuentra sobre el vino. Está claro que interpretar el papel jugado por los estímulos aromáticos en la percepción global debe implicar otros aspectos, tales como la integración de los procesos perceptuales, los cambios asociados a la liberación de los volátiles y sus modificaciones temporales. Otro aspecto importante es que algunos odorantes impacto pueden dominar el perfil aromático de una variedad de vino particular, incluso encontrándose en niveles de ultratraza requiriendo, por tanto, estrategias de análisis cuantitativo más sensibles. La presente tesis ha caracterizado la liberación de compuestos volátiles y su evolución en el espacio de cabeza de diferentes vinos y también ha investigado el análisis de alquilmetoxipirazinas en vino a niveles ultratraza mediante la aplicación de nuevas metodologías analíticas.

Con la consideración de estudiar la composición del espacio de cabeza del vino en condiciones de no-equilibrio, más cercanas a una cata real del vino, en el **Capítulo 1** se ha desarrollado un método de análisis basado en la automatización del espacio de cabeza dinámico (DHS) combinado con desorción térmica (TD) y cromatografía gas con detección espectrométrica (GC-MS). Gracias a esta estrategia

rápida y dinámica de muestreo, el método proporciona una "instantánea" de la composición de los vapores emanados del vino, ya que permite conocer la concentración de hasta 40 compuestos aromáticos, incluyendo el metanotiol, el dióxido de azufre, los alcoholes de fusel o los fenoles volátiles.

En el **Capítulo 2**, el método DHS validado anteriormente se ha aplicado para evaluar los cambios en los espacios de cabeza del vino y sus cambios temporales, monitorizando los niveles de 34 odorantes emitidos al espacio de cabeza desde 4 vinos diferentes durante 5 puntos en el tiempo. Se han encontrado tres tipos de patrones en cuanto al comportamiento de la evolución de dichos aromas. Estos patrones corresponden a las características físico-químicas de los compuestos volátiles y a las interacciones con los componentes matriciales, lo que sugiere que la predicción del impacto aromático de estos componentes debería incluir una estimación de sus interacciones odorante-matriz en ese vino particular.

El **Capítulo 3** recoge la aplicación del método DHS, validado previamente, al estudio de las potenciales influencias de los parámetros físicos en la liberación de los compuestos volátiles desde el vino. Estos fenómenos que ocurren durante el periodo de cata incluyen la agitación, evaporación, oxidación y desgasificación. Además, las tendencias de evolución del espacio de cabeza en un vino tinto y otro blanco fueron estudiadas durante 30 min. Durante los experimentos, las características físico-químicas de los compuestos estudiados fueron la clave para explicar su comportamiento en la liberación al espacio de cabeza.

Los **Capítulos 4 y 5** se dedican fundamentalmente al estudio del impacto de las potenciales interacciones compuesto volátil-matriz en el comportamiento de liberación de dichos compuestos, usando el método DHS para detectar los contenidos en un volátil determinado a diferentes tiempos de evaporación. El **Capítulo 4** presenta los resultados de la reconstitución de 7 vinos modelo mediante la adición de una mezcla estándar de aromas a vinos desaromatizados con matrices muy diferenciadas. En el **Capítulo 5** se aplica la misma estrategia pero al estudio de los cambios en el espacio de cabeza relacionados con la presencia de macromoléculas usadas frecuentemente como aditivos enológicos (diversos polisacáridos y polifenoles).

El **Capítulo 6** está dedicado al estudio exhaustivo de dos vinos de categoría Premium. En este caso, se combinaron tanto los datos analíticos de los componentes volátiles en fase líquida y fase gas, como los datos obtenidos de un estudio sensorial llevado a cabo por un panel de catadores, en ambos casos en diferentes puntos del proceso de cata. Los datos sensoriales en los diferentes momentos del consumo del vino se correlacionan con la correspondiente concentración en el espacio de cabeza de los compuestos volátiles que son relevantes para cada descriptor de aroma.

Las alquilmetoxipirazinas son odorantes muy potentes de muchos alimentos y también en algunos vinos. Normalmente se encuentran presentes en dichos vinos en concentraciones extremadamente bajas. En el **Capítulo 7**, se desarrolla y valida un nuevo método para la identificación y cuantificación de 3-isopropil-2-metoxipirazina, 3-*sec*-butil-2-metoxipirazina y 3-isobutil-2-

metoxipirazina. De acuerdo con el método validad, los analitos se extraen de 5 mL de vino usando una barra de extracción SBSE seguido por una desorción térmica y una cromatografía gas multidimensional. El método es automatizable, respetuoso con el medioambiente y proporciona los mejores límites de detección para estos compuestos publicados hasta la fecha (en el rango de las ppq). El método se ha aplicado a la determinación de estos analitos en 111 vinos españoles y franceses elaborados con variedades de uva minoritarias.

INTRODUCTION

INTRODUCTION

1. Introduction

The aroma profile of wine is one of the most important quality parameters for consumers' acceptance. As a very complex mixture, thousands of wine components have been identified and quantified following remarkable developments in analytical techniques, especially volatile compounds that could directly contribute to the aroma characteristics (Ferreira & Cacho, 2009; Robinson, Boss, Solomon, Trengove, Heymann, & Ebeler, 2013). The past decades witnessed the most significant expansion in knowledge of wine aroma chemistry, such as the explanation of the formation of most aroma compounds or the existence of aroma compounds at concentration levels from ng/L to mg/L (Ebeler & Thorngate, 2009; Polaskova, Herszage, & Ebeler, 2008; Waterhouse, Sacks, & Jeffery, 2016). Among them, some key odorants or impact compounds could powerfully determine the aroma profile of special varietal wines at ultra-trace concentration levels but with very low odor thresholds, i.e. rotundone, thiols and alkylmethoxypyrazines (Darriet, Tominaga, Lavigne, Boidron, & Dubourdieu, 1995; Ferreira & Cacho, 2009; Guth, 1997; Kotseridis, Anocibar Beloqui, Bertrand, & Doazan, 1998; Tracey, Siebert, Wood, Elsey, & Pollnitz, 2008). Despite these particular cases, most wines have a very similar constitution in volatile compounds, what usually differentiate one exceptional wine to another are not only variations in the concentrations of aroma compounds, but also (perhaps more importantly) their efficient delivery from the liquid phase to the headspace.

In common with many researches on sensation, perceptual interactions between volatile compounds exist and could cause masking or synergistic effects on the expression of wine aroma characteristics in simple solutions or in the more complex reconstituted wines (de-la-Fuente-Blanco, Saenz-Navajas, & Ferreira, 2016; Saenz-Navajas, Campo, Cullere, Fernandez-Zurbano, Valentin, & Ferreira, 2010; Sébastien, Gérard, & Thierry, 2018). With the compositional complexity of real wines, various types of interactions may occur that can impact the volatility and further generate the variation of perception of aroma compounds. For instance, ethanol and macromolecules have been proved to interact with volatile compounds via various chemical bindings (Jung, de Ropp, & Ebeler, 2000; Pozo-Bayón & Reineccius, 2009; Anthony L. Robinson, Ebeler, Heymann, Boss, Solomon, & Trengove, 2009; Rodríguez-Bencomo, Muñoz-González, Andújar-Ortiz, Martín-Álvarez, Moreno-Arribas, & Pozo-Bayón, 2011; Taylor, Tsachaki, Lopez, Morris, Ferreira, & Wolf, 2010). The nature of all the interactions mentioned above highly depends on the physicochemical characteristics of both aroma compounds and matrix compounds (Taylor, 1998; Villamor & Ross, 2013; Voilley & Lubbers, 1998). Otherwise, wine tasting is a time-consuming and dynamic process that makes the mass transfer of volatile compounds challenging to predict. Furthermore, the available knowledge is limited by the practical difficulty of mimicking a wide variety of wine components. Considering all these parameters, wine flavor chemists have started to turn their focus more on the sensory relevance of volatile compounds regarding the nature of their mass transfer to the headspace, rather than devote more to determine every

compound in the liquid phase which is a task almost finished. Progress in the field of wine headspace has been driven by the developments in sampling techniques, gas chromatography (GC) and mass spectrometry techniques (MS).

2. Headspace sampling techniques

As a preconcentration step, headspace sampling techniques will extract volatile compounds from the vapor phase of a liquid or solid matrix, and then sample them to gas chromatography (GC) for separation. Generally, a liquid or solid sample is placed in a well-sealed container under specific conditions depending on the used technique and analytical purpose, then the gaseous mixtures will be transferred to a GC-MS for identification or quantification analysis. Expectedly, the extracted mixtures contain few matrix components, thereby more efficiently reflect the sensory contribution of aroma compounds than using the total concentration in a liquid or solid. Also, these HS based techniques are environmental-friendly and now are practically automated by using modern autosamplers (Snow & Slack, 2002; Soria, García-Sarrió, & Sanz, 2015). Therefore, headspace sampling techniques are now routinely used by analytical chemists to enhance the knowledge of volatile components in different fields.

To a better understanding of the sensory relevance and real aroma contributions of many volatile compounds present in wine, headspace sampling techniques are widely applied by wine chemists to identify the vapor composition and to measure the effective concentrations of volatile compounds in the headspace, as well as

their dynamic changes (Hirson, Heymann, & Ebeler, 2012). Besides the more traditional static and dynamic sampling techniques, other modern sampling techniques are available, such as solid-phase microextraction (SPME) and stir-bar sorptive extraction (SBSE) with greater concentration capacity, dynamic analysis by using mass-spectrometric techniques like atmospheric pressure chemical ionization mass spectrometry (APCI-MS) and proton transfer reaction mass spectrometry (PTR-MS) are also employed (Muñoz-González, Rodríguez-Bencomo, Moreno-Arribas, & Pozo-Bayón, 2011).

2.1. Static headspace sampling techniques (SHS)

As the most traditional technique of headspace sampling, static headspace sampling technique is usually carried out to analyze volatile compounds at the relative high-ppb concentration level due to its limited sensitivity. The vapor phase of the sample above the liquid phase will be usually sampled by a gas-tight syringe or heatable transfer line when the thermodynamic equilibrium between the vapor phase and the liquid phase is reached under a given condition. A typical SHS process is shown in Fig. 1. Basically, experimental parameters like incubation temperature, equilibrium time, sampling volume, extracting speed and injection temperature should be pre-optimised to reach a satisfactory sensitivity. Although SHS also could be operated under nonequilibrium condition, experimental parameters should be more carefully controlled for the sake of reproducibility (Kolb & Ettre, 2006; Sithersingh & Snow, 2012; Snow & Slack, 2002).

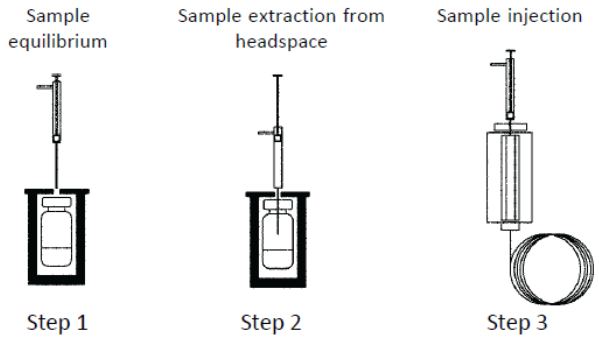

Fig. 1. The SHS process of a typical gas-tight syringe system. Figure reproduced from the technical material of Restek (United States).

Fundamentally, the distribution of an aroma compound between the vapor phase and matrix phase is determined by its partition coefficient (k) that represents the ratio of its equilibrium concentrations in the two phases. SHS sampling has been routinely applied to quantify concentration in the vapor phase in order to further study the k value of aroma compounds (Kolb & Ettre, 2006). Specifically, the vapor phase calibration (VPC) and liquid calibration static headspace (LC-SH) with external calibration and the phase ratio variation (PRV) without external calibration are the most commonly applied ones (Athès, Peña y Lillo, Bernard, Pérez-Correa, & Souchon, 2004; Ayed, Lubbers, Andriot, Merabtine, Guichard, & Tromelin, 2014; Kolb & Ettre, 2006; Taylor, 1998). These mentioned approaches are very practical to study the volatile-macromolecule interactions in food chemistry (Jouquand, Ducruet, & Giampaoli, 2004; Merabtine, Lubbers, Andriot, Tromelin, & Guichard, 2010; Terta, Blekas, & Paraskevopoulou, 2006; Xu, He,

Zeng, Li, Qin, Wang, et al., 2017). Initially, the SHS method coupled with GC was used by Voilley et al. (1991) to access the volatile-macromolecule interaction in a model wine system. Chalier and coworkers (2007) applied the SHS sampling method to study the interaction between selected volatile compounds and mannoproteins from yeasts and reported a global retention effect for volatile compounds. More recently, Lorrain et al. (2013) have studied the variable influences of polyphenols on the volatility and perception of red wine esters by SHS technique. In addition, another group of researchers has proposed a method to study the volatile-volatile interaction of wine esters and high alcohols in the headspace by determining partition coefficient using static headspace sampling technique (Cameleyre, Lytra, & Barbe, 2018).

Due to its long-period equilibrium, poor sensitivity and selectivity, SHS sampling is currently applied to quantify the most volatile compounds in sub-ppb concentration range with some particular concerns. For instance, a quantitative method based on SHS sampling has been validated to evaluate the free-format volatile sulfur compounds under a minimum equilibrium condition in our laboratory to reflect the real potential of volatile sulfur compounds and avoid the reversibility of their combined-format in wine (Franco-Luesma & Ferreira, 2014). Moreover, Martí and coworkers (2003) applied SHS aiming for fast screening of 2,4,6-trichloroanisole in wines. Furthermore, new SHS techniques have been improved in instrumentation to enhance the versatility, such as the application of programmed temperature vaporization inlet, which has been well reviewed recently (Snow & Bullock, 2010).

2.2. Dynamic headspace sampling techniques (DHS)

In dynamic headspace sampling techniques, a flow of inert gas drags out volatile compounds from the product and will be subsequently directed to a sorbent or cryogenic trap, in which volatiles are retained. The vapors produced with these techniques are more similar to those observed in real olfaction than those obtained by using equilibrium methods such as static headspace (Escudero, San-Juan, Franco-Luesma, Cacho, & Ferreira, 2014). Also, the detection limit of DHS techniques is 10 to 100 times smaller than that of SHS, which makes it a better option for quantifying very volatile compounds in wines (Aznar & Arroyo, 2007; Garciajares, Garciamartin, & Celatorrijos, 1995; Marquez, Serratosa, Merida, Zea, & Moyano, 2014). However, experimental parameters should be well optimized, such as the type of sorbent, gas purging volume, trapping time and temperature for trapping conditions, and desorption temperature and time if the thermal desorption instrumentation is applied (Soria, García-Sarrió, & Sanz, 2015). The application of this technique has been well reviewed by Soria and others (2015). It is worth mentioning that DHS has a more complicated instrumental setup than that of SHS or SPME (Fig. 2). Compared with those techniques the total volume of the sample that should be introduced in the column is far more substantial. Peak broadening in DHS is avoided with the use of an additional trap inside the injector, with a cryo-focalization system, or usually with both at the same time.

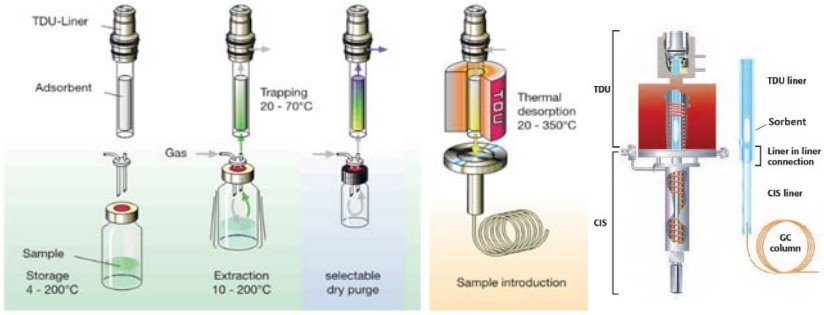

Fig. 2. The DHS process from extraction to sample introduction in Gerstel TDU-CIS system (similar to the one used on this work). Figure reproduced from the technical material of Gerstel GmbH (Germany).

Real wine tasting is always a dynamic process under nonequilibrium conditions. Also, the volatility changes of odorants due to volatile-macromolecule interactions and the temporal dimension of aroma release during tasting should be taken into consideration. Therefore, dynamic headspace sampling is more promising than SHS to study the vapor composition above the wine glass.

Dynamic headspace can be used not only for strictly quantitative purposes, but also for sensory applications. By combining with gas chromatography-olfactometry (GC-O), purge and trap strategy could provide a more representative headspace composition than that obtained with liquid or SPE extractions. DHS can be tuned to obtain a vapor profile most similar to that of wine, including even the high-polarity compounds that are often underestimated (Ferreira & Cacho, 2009; Ferreira, San-Juan, Escudero, Culleré, Fernández-Zurbano, Saenz-Navajas, et al., 2009). According to a well-validated

methodology in our laboratory, aroma compounds above the liquid phase are continuously purged by a gentle stream of nitrogen and further trapped by LiChrolut EN resins, which could more efficiently retain the less volatile compounds. It is worth noticing that gas purging in this strategy is as gentle as not to disturb the liquid surface. Although others reported that gas bubbling could rapidly achieve the equilibrium between vapor and liquid phases (Taylor, 2002), it is far more concentrated than the real headspace in the wine glass, which may cause the over-estimation of the less polar compounds (Escudero, San-Juan, Franco-Luesma, Cacho, & Ferreira, 2014; San-Juan, Pet'ka, Cacho, Ferreira, & Escudero, 2010). Instead of using thermal desorption process, solvent elution is applied to provide a single extract that is sufficient for a sensory panel and allows multiple injections (Escudero, San-Juan, Franco-Luesma, Cacho, & Ferreira, 2014; Ferreira & Cacho, 2009). The drawback of this strategy is the co-elution of some of the most volatile components with the solvent. For that type of compounds, a solvent-less strategy should be used. Some modern desorption systems provide an alternative to deal with the problem of reinjection by capturing part of the desorbed compounds in an additional trap located inside the system. In this way, one extraction process could allow multiple olfactometries, which is useful for scarce or limited samples.

According to the fundamentals of dynamic sampling (Kolb & Ettre, 1991; Soria, García-Sarrió, & Sanz, 2015), the amount of volatiles that DHS sampling method extracts will follow an exponential decay trend with time (stepwise sampling process) after the continuous sweeping process. This strategy, similar to multiple

headspace extractions, will be applied in Chapter 2 to study the release behavior changes of volatiles from different wines over time. Besides, DHS sampling strategy has been widely applied by wine chemists to study the interaction between volatile compounds and macromolecules in wines, which has been reviewed by several research groups (Muñoz-González, Rodríguez-Bencomo, Moreno-Arribas, & Pozo-Bayón, 2011; Pozo-Bayón & Reineccius, 2009; Villamor & Ross, 2013; Voilley & Lubbers, 1998). Although the knowledge is still limited, all these researches devoting into wine analysis provide valuable information to explain why wines with similar composition may have different aroma characteristics.

2.3. Other headspace sampling techniques

Solid-phase microextraction (SPME) was developed in the 1990s and is widely used now in wine analysis (Arthur & Pawliszyn, 1990; Pawliszyn, 1997). Essentially, SPME uses a modified syringe device to protect a retractable fiber coated with polymeric materials (Fig. 3). The coated fiber could extract analytes by directly immersing into the sample matrix or extract the volatile compounds in the headspace above the matrix (Kataoka, Lord, & Pawliszyn, 2000; Pillonel, Bosset, & Tabacchi, 2002). However, the latter one (HS-SPME) has been more widely performed in wine analysis in order to avoid the interference causing by the numerous matrix components (Muñoz-González, Rodríguez-Bencomo, Moreno-Arribas, & Pozo-Bayón, 2011; Robinson, Boss, Solomon, Trengove, Heymann, & Ebeler, 2014). After extraction, the volatiles on the fiber will be transferred from the extraction unit (usually an autosampler) to a GC or another separation instrument for further

analysis. Compared with the headspace sampling techniques mentioned above, SPME has a relatively higher extraction capacity and could be applied to analyze a wide range of volatile compounds due to its large number of commercial fibers. Due to the low amount of extracting material in the fiber, the desorption takes place in a very short time, which makes for the absence of a cryo-focusing system. The same low amount of sorbent causes, however, a limited dynamic range and competitive adsorption phenomena on the SPME fiber. Experimental conditions need to be optimized to achieve the analytical aims and avoid matrix effects (Kataoka, Lord, & Pawliszyn, 2000; Pawliszyn, 2000; Wardencki, Michulec, & Curyło, 2004).

Due to its satisfactory sensitivity and easy to use characteristics, HS-SPME has been widely used for quantifying almost any volatile wine aroma compound, whether trace or major. Examples are numerous: volatile sulfur compounds, alkylmethoxypyrazines or aldehydes among many others (Bueno, Zapata, & Ferreira, 2014; Ferreira, Herrero, Zapata, & Escudero, 2015; Franco-Luesma & Ferreira, 2014; Godelmann, Limmert, & Kuballa, 2008; Vinholes, Coimbra, & Rocha, 2009). To overcome the drawbacks mentioned above, complicated calibration strategies are commonly applied to minimize the matrix effect. On the one hand, surrogates or multiple internal standards corresponding to the target analytes are helpful as reported by Bueno et al. (2014) for quantifying aldehydes and Ferreira et al. (2015) for analyzing a wide range of aroma compounds. On the other hand, the stable isotope dilution assay (SIDA) is also frequently applied for the quantification of aroma compounds by using isotopically labeled internal standards with

similar chemical structure as the compounds of interest (Petrozziello, Borsa, Guaita, Gerbi, & Bosso, 2012; T. E. Siebert, Smyth, Capone, Neuwöhner, Pardon, Skouroumounis, et al., 2005; Swiegers, Capone, Pardon, Elsey, Sefton, Francis, et al., 2007). Other applications of HS-SPME in quantification have recently been reviewed by Azzi-Achkouty and coworkers (2017).

Also, researchers have applied HS-SPME aiming to study the interactions between volatile compounds and macromolecules, which has been reviewed by Muñoz-González and others (2011). Among these studies, Ebeler's group used very short extraction time (only 1 min) to screen the composition in the vapor phase to represent the "true" headspace above wine. Although the number of volatile compounds was limited due to the short sampling time, the volatile-macromolecule interactions were proved in their experimental system (Jung & Ebeler, 2003). Recently, a new technology SPME-arrow is commercially available (Kremser, Jochmann, & Schmidt, 2016a, 2016b). It has similar instrumentation as SPME, but instead is an arrow-shaped device, that magnifies the sorption phase surfaces and volumes by widening the outer diameter (shown in Fig. 3). With the enhancement of sensitivity and the advantage of full automatization, it could be promising to be applied to analyze trace compounds of wine, although as far as we know, there are no scientific publications.

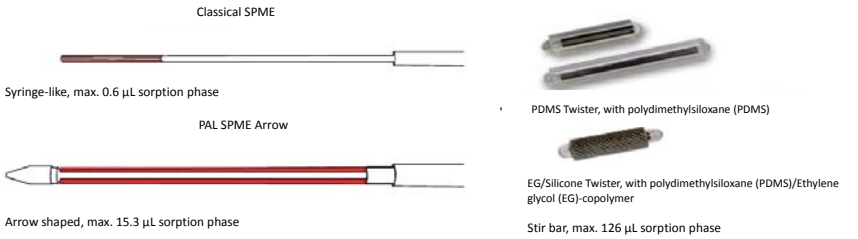

Fig. 3. Comparison of classical SPME fiber, SPME-arrow, and Twister (stir bar). Figure reproduced from the technical material of PAL system (Switzerland) and Gerstel GmbH (Germany).

Stir-bar sorptive extraction (SBSE) was developed with the same fundamentals of SPME (Baltussen, Sandra, David, & Cramers, 1999). SBSE enhances analytical sensitivity by increasing the volume of the coated fiber using a stir bar instead, although only two stir bar types are now commercially available (Fig. 3). Briefly, the extraction could be performed by both immersion and headspace sampling, and the extracts are further desorbed by solvent or thermal desorption (Callejon, Clavijo, Ortigueira, Troncoso, Paneque, & Morales, 2010; Perestrelo, Nogueira, & Câmara, 2009; Prieto, Basauri, Rodil, Usobiaga, Fernández, Etxebarria, et al., 2010). However, the whole procedure of SBSE is not fully automated, bringing more inconveniences of applying HS-SBSE than HS-SPME. Consequently, SBSE has been more frequently used to quantify trace/ultra-trace aroma compounds that are odor-active in wines by stirring in the liquid phase (David & Sandra, 2007; Fang & Qian, 2006), such as alkylmethoxypyrazines (Franc, David, & de Revel, 2009).

Over the past decades, needle-based microextraction techniques have been developed on the base of SPME, like in-tube extraction (ITEX) and solid-phase dynamic extraction (SPDE), and are now applied to wine analysis (Laaks, Jochmann, Schilling, Molt, & Schmidt, 2014; Malherbe, Watts, Nieuwoudt, Bauer, & Du Toit, 2009; Zapata, Lopez, Herrero, & Ferreira, 2012). Such techniques improve the sorption capacity and overcome the fiber fragility of SPME by replacing the material of needle, plunger or fiber core (Kędziora-Koch & Wasiak). Take ITEX as an example, a typical ITEX extraction procedure is presented in Fig. 4. Notably, the extraction could be multiplied by moving the plunger of the gas-tight syringe up and down to absorb the sample headspace dynamically (Zapata, Lopez, Herrero, & Ferreira, 2012; Zapata, Mateo-Vivaracho, Lopez, & Ferreira, 2012). Therefore, ITEX could reach similar sensitivity to purge and trap system while needs less instrumental effort due to complete automation.

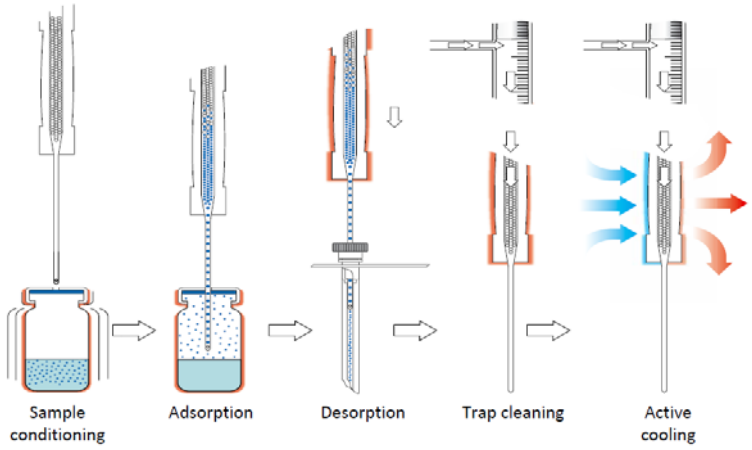

Fig. 4. A typical ITEX dynamic headspace extraction procedure. Figure reproduced from the technical material of PAL system (Switzerland).

Another approaches such as direct atmospheric pressure chemical ionization mass spectrometry (APCI-MS) (Jublot, Linforth, & Taylor, 2005; Taylor, Linforth, Harvey, & Blake, 2000) or proton transfer reaction mass spectrometry (PTR-MS) (Frank, Appelqvist, Piyasiri, Wooster, & Delahunty, 2011; Lindinger, Hansel, & Jordan, 1998) have been proposed for the determination of the aroma compounds present in the headspaces emanated from a given product. These strategies are able to create reactive ions H_3O^+ softly and consistently in an ionization source by an electric discharge between water and nitrogen. Meanwhile, the generated ions will react with aroma molecules allowing the quantitative analysis of real-time aroma release. Instead of combing with GC for separation, the sampling air is directly introduced into a mass spectrometer for identification. The analysis time is thus dramatically shortened and make it an adequate method to analyze aroma release in vivo or in vitro from nose and mouth (Cayot, Dury-Brun, Karbowiak, Savary, & Voilley, 2008; Harvey & Barra, 2003; Ingham, Linforth, & Taylor, 1995). Also, researchers have applied the strategy to study the effect of ethanol and macromolecules on the behavior of aroma release (Aznar, Tsachaki, Linforth, Ferreira, & Taylor, 2004; Linforth, Baek, & Taylor, 1999; Muñoz-González, Rodríguez-Bencomo, Moreno-Arribas, & Pozo-Bayón, 2011; Spitaler, Araghipour, Mikoviny, Wisthaler, Via, & Märk, 2007; Taylor, Tsachaki, Lopez, Morris, Ferreira, & Wolf, 2010). According to the principle of these techniques, the detection of aroma compounds (R) is based on the identification of the protonated molecular ion, which mass corresponds to the addition of one proton (RH^+) (Taylor, Linforth, Harvey, & Blake, 2000). Therefore, APCI-MS and PTR-MS require

the analyzed aroma compounds to be different in molecular mass to avoid ambiguity. Otherwise, they are not sensitive enough for the direct monitoring of aroma compounds present at low levels. These drawbacks limit their applicability to the study of products containing relatively large amounts of volatile compounds such as wine, since wine headspaces are much enriched in ethanol, fusel alcohols and other major wine volatiles. New developments in the field had led to the use of high-resolution mass spectrometry PTR-MS to improve selectivity. However, the cost and complexity of such systems prevents is general usage in flavor laboratories.

3. Separation and detection techniques for analyzing volatile compounds in wine

So far, more than 1000 volatile compounds have been identified and quantified, and their concentrations range from mg/L (e.g., ethyl acetate) to ng/L (e.g., alkylmethoxypyrazines and trichloroanisole) (Polaskova, Herszage, & Ebeler, 2008). The significant variations in concentration results in analytical challenges in determining and quantifying different volatile compounds by a single methodology. Therefore, it is necessary to have an array of analytical methods differentiated in techniques of pre-concentration, separation or detection if one study concerns a comprehensive aroma analysis (Escudero, Campo, Fariña, Cacho, & Ferreira, 2007; Ferreira & Cacho, 2009). A system of gas chromatography-flame ionization detector (FID) would be sensitive enough for quantifying the solvent extracts of major volatile compounds at high concentration level (Ortega, López, Cacho, & Ferreira, 2001). However, the quantitation

of minor or trace compounds need more powerful pre-concentration techniques, such as solid phase extraction, headspace sampling techniques, SPME and SBSE, as well as sensitive detectors, therefore mass spectrometer is commonly applied in this case (Franc, David, & de Revel, 2009; Lopez, Aznar, Cacho, & Ferreira, 2002; Petrozziello, Borsa, Guaita, Gerbi, & Bosso, 2012). Furthermore, the quantification of some particular classes of volatile compounds, like volatile sulfur compounds, requires specialized detectors due to their high volatility, reactivity and relatively low abundance. Therefore, specific sulfur detectors such as pulsed flame photometric detector (pFPD) and sulfur chemiluminescence detector (SCD) are thus applied (Franco-Luesma & Ferreira, 2014; Siebert, Solomon, Pollnitz, & Jeffery, 2010).

Apart from the advances in the techniques mentioned above, the latest innovations in gas chromatography (GC) and mass spectrometry (MS) make remarkable progress in analyzing volatile compounds at sub-ng/L concentrations in wines with a complex matrix. In a traditional one dimensional GC-MS system (1D-GC-MS), volatile compounds are separated in the gas phase with a temperature gradient due to differences in boiling point and polarity of analytes, then the MSD detects the ionized molecules (by electron ionization mode or chemical ionization mode) based on the corresponding mass-to-charge ratio. Multidimensional gas chromatography (MDGC) employs two or more gas chromatographic separations in sequence, thereby it has much greater resolving power than the 1D-GC (Prebihalo, Berrier, Freye, Bahaghighat, Moore, Pinkerton, et al., 2018; Seeley & Seeley, 2013). In addition, the developments of the mass spectrometers, such as

time-of-flight MS (TOF-MS) and tandem mass spectrometry (MS/MS), enhance the selectivity, sensitivity and the identification capacity (Mayr, Capone, Pardon, Black, Pomeroy, & Francis, 2015). All these state-of-art approaches make the GC-MS system more robust for both qualitative and quantitative wine analysis.

The majority of applications are 2D-GC with two columns, which are categorized into heart-cutting 2-D GC and comprehensive 2D-GC (GC×GC). In the heart-cutting 2D-GC system, only the target analytes will be transferred to the second column, which could efficiently improve the peak capacity only for the volatile compounds of interest. Thus it could advance the GC-O strategy in screening new odorants in wine, which are usually co-eluted with other compounds or at low concentration level (Campo, Ferreira, López, Escudero, & Cacho, 2006; Ochiai & Sasamoto, 2011). A comprehensive 2D-GC system usually uses a short second column, then the whole effluent of the first column will be rapidly transferred to the second one in a continuous and sequential mode. Therefore, it usually combines with a detector that could operate at high speed, such as TOF-MS (Muñoz-González, Rodríguez-Bencomo, Moreno-Arribas, & Pozo-Bayón, 2011; Nicolli, Biasoto, Souza-Silva, Guerra, dos Santos, Welke, et al., 2018).

Taking the recent analysis of alkylmethoxypyrazines (MP) for example, the advanced GC techniques or MS techniques have been applied to determine this ultra-trace odor-active volatile compounds in special wines or grapes. In 2005, Ryan et al. (2005) described the application of GC×GC coupled with TOF-MS or NPD for the determination of IBMP. Similarly, GC×GC coupled with TOF-MS

was used for the determination of IPMP and IBMP in wine grapes (Ryona, Pan, & Sacks, 2009). Also in wine grapes, Legrum et al. (2015) recently applied Enantio-GC×GC–MS for the enantiodifferentiation of SBMP in different species. The same research group previously applied GC×GC–MS to the analysis of MP in wine (Schmarr, Ganß, Koschinski, Fischer, Riehle, Kinnart, et al., 2010). Despite its very high separation efficiency, GC×GC is probably not the most straightforward approach for a limited number of target analytes as is the case with MP. Due to its simpler experimental setup and easiness for data processing, heart-cutting 2D-GC has also been applied to the analysis of MP in wine. Culleré et al. (2009) used MDGC combined with SPE to achieve very low detection limits for MP in wine. While MP extraction with HS-SPME- MDGC was faster, it provided slightly higher detection limits (Botezatu, Pickering, & Kotseridis, 2014; Kögel, Botezatu, Hoffmann, & Pickering, 2014). Even more selectivity could be obtained combining on-line liquid chromatography with the MDGC–MS technique (Schmarr, et al., 2010), or through tandem mass spectrometry MDGC–MS/MS (Godelmann, Limmert, & Kuballa, 2008; Legrum, Gracia-Moreno, Lopez, Potouridis, Langen, Slabizki, et al., 2014). Ochiai et al. (2011) proposed a MDGC–MS combined with olfactometry and with preparative fraction collection for the determination of IBMP among other off-flavors. In Chapter 7, a method for the determination of this compounds, based on a good pre-concentration technique (SBSE) combined with heart-cutting 2D-MS (as shown in Fig. 5), will be developed and validated.

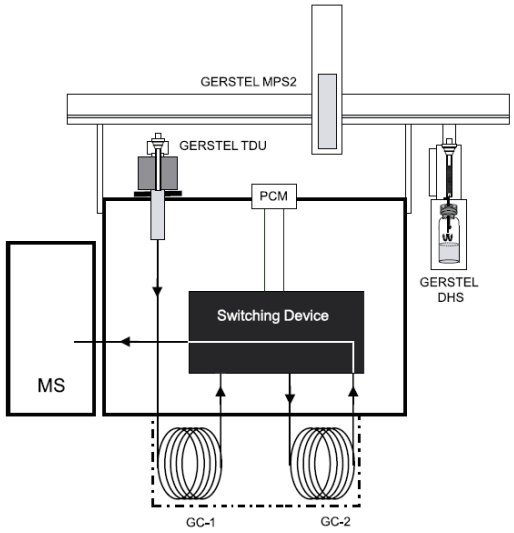

Fig. 5. An example of the GC×GC system combined with a thermal desorption unit, which is similar to the one we used in Chapter 7 for analyzing alkylmethoxypyrazines. Figure reproduced from the technical material of Gerstel GmbH (Germany).

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OBJECTIVES

OBJECTIVES

One of the main concerns of wine chemists is to answer the question of whether flavor perception can be explained by the determination of the chemical composition or not. In this regard, the analytical resources expended during the last decades have been considerable. Nowadays, there is a common thought among researchers that considers that, although knowing the contents of aroma compounds in wine is essential, it is not enough for answering such question. It is clear that interpreting the role played by aroma chemicals in the overall perception needs to involve other aspects, such as the integration of the perceptual processes, the changes associated to the release of volatiles and their changes with time.

Considering all the mentioned above, the primary objective of this thesis is to develop novel strategies to analyze the wine headspace composition, in particular to the applications of the Dynamic Headspace Sampling (DHS) method for further understanding the evolution of volatile compounds in the headspace, in an attempt to explain the sensory changes during wine tasting. Additionally, the development of new ultra-trace analytical methods is also included in this work. The specific objectives are presented as follows:

1) To develop a fast DHS method for analyzing volatile compounds in the headspace under the non-equilibrium conditions closed to wine tasting.

2) To study the consecutive release of volatile compounds over time from wine by using the DHS method.

3) To study the effect of physical conditions on the release of volatile compounds from different wines.

4) To study the effect of different wine matrices on the release behavior of volatile compounds over different evaporation times.

5) To study the effect of selected commercial wine macromolecules on the release behavior of volatile compounds over different evaporation times.

6) To study the characterization of sensory and headspace evolutions, and the possible relationship between them.

7) To propose and validate a selective, sensitive and fast method to determine alkylmethoxypyrazines in a large number of different types of wines.

CHAPTER 1

An Automated Gas Chromatographic-Mass Spectrometric Method for the Quantitative Analysis of Volatile Compounds Present in the Vapor Emanated from Wine

CHAPTER 1

An automated gas chromatographic-mass spectrometric method for the quantitative analysis of volatile compounds present in the vapor emanated from wine

1. Introduction

The characteristic odors and flavors elicited by a product are related to the aroma composition of the headspaces that reach the olfactory receptors during the action of smelling or eating the product. In the case of wine, there is strong evidence that some aroma compounds can bind to different compounds or structures forming the non- volatile matrix of wine (Bueno, Zapata, & Ferreira, 2014; Franco-Luesma & Ferreira, 2014; Robinson, Ebeler, Heymann, Boss, Solomon, & Trengove, 2009). The existence of these interactions suggests that the odor activity of those odorants in a given wine will be related not only to the concentrations of the odorants, but to the amount and type of "aroma-binders" present in that wine. This means that two wines with the same aroma composition could produce headspace vapors differing in composition, depending on the level and type of "aroma-binders" specifically present in each wine (Zapata, Lopez, Herrero, & Ferreira, 2012). This could explain why the same aroma extract reconstituted in different wine non-volatile matrixes can produce markedly different aroma perceptions (Saenz-Navajas, Campo, Cullere, Fernandez-Zurbano, Valentin, & Ferreira, 2010).

The existence of odorant with matrix interactions potentially responsible for aroma changes has been previously addressed in

wine (Aznar, Tsachaki, Linforth, Ferreira, & Taylor, 2004) and other products, notably solid or semisolid food products (Baek, Linforth, Blake, & Taylor, 1999; Delahunty & Piggott, 1995; Ingham, Linforth, & Taylor, 1995). In these last cases, it is evident that the levels of aroma chemicals released from the product are strongly dependent on the specific composition of the solid or semisolid matrix.

Several approaches have been proposed for the determination of the aroma compounds present in the headspaces emanated from a given product. The most direct strategy is the continuous monitoring of the composition of the headspace with methods such as direct atmospheric pressure chemical ionization mass spectrometry (APCI-MS) (Jublot, Linforth, & Taylor, 2005; Taylor, Linforth, Harvey, & Blake, 2000) or proton transfer reaction mass spectrometry (PTR-MS) (Frank, Appelqvist, Piyasiri, Wooster, & Delahunty, 2011; Lindinger, Hansel, & Jordan, 1998). These strategies are, however, not sensitive enough for the direct monitoring of aroma compounds present at low levels, which limits their applicability to the study of products containing relatively large amounts of volatile compounds. By contrast, in many natural food products, including wine, aroma properties can be strongly influenced by powerful aroma compounds present at very low concentrations. In the particular case of wine and other alcoholic beverages, selectivity also becomes a problem, since wine headspaces are much enriched in ethanol, fusel alcohols and other major wine volatiles.

A second possibility is trapping the aroma compounds present in the headspace in a sorbent or cold trap in order to gain sensitivity, and to analyze the concentrated odorants by GC-MS, to gain

selectivity. The obvious drawback of these strategies is that monitoring will become discontinuous. It should be noted, however, that most reports using these strategies do not intend to analyze the headspace, but the volatiles present in the product. In this context and because of its simplicity, solid phase micro-extraction (SPME) is frequently used (Jelen, Majcher, & Dziadas, 2012), although other headspace sampling techniques have also been widely applied (Soria, Garcia-Sarrio, & Sanz, 2015). In dynamic Headspace (DHS) techniques, a flow of inert gas drags out volatile compounds from the product and is subsequently directed to a sorbent or cryogenic trap, in which volatiles are retained. The vapors produced with these techniques are more similar to those observed in real olfaction than those obtained by using equilibrium methods such as static headspace or headspace SPME sampling (Escudero, San-Juan, Franco-Luesma, Cacho, & Ferreira, 2014).

There are several reports proposed DHS techniques for wine aroma analysis. In most of them, volatiles are dragged out by an inert gas bubbled through the wine (Aznar & Arroyo, 2007; Garciajares, Garciamartin, & Celatorrijos, 1995; Ortega-Heras, Gonzalez-SanJose, & Beltran, 2002; Salinas, Alonso, & Estebaninfantes, 1994) or streamed on the wine headspace (Marquez, Serratosa, Merida, Zea, & Moyano, 2014), but as was aforementioned, these methods were designed for the quantitative analysis of the aroma compounds present in the liquid phase of the wine rather than to monitor the changes in concentrations in wine headspaces. In the present work, our main aim is to develop a fast and simple DHS method able to provide a "snapshot" of the headspaces emanated from wine in conditions close to those found during wine tasting. For that, the

headspace of unstirred wine will be dragged by a gentle stream of nitrogen during a relatively short time.

2. Materials and methods

2.1. Reagents and chemicals

Ethanol was supplied by Merck (Darmstadt, Germany) and tartaric acid 99% was obtained from Panreac (Barcelona, Spain). The internal standards (methyl 2-methylbutyrate, 2,6-dichloronisole) and standards of the aroma compounds were obtained from Aldrich, Fluka (Madrid, Spain).

2.2. Wine samples

Four white wines, two red wines and a rosé wine with diverse characteristics (regarding grape variety, alcoholic content and aging) from Spain were used to validate and develop the method. The synthetic wine contained 5 g/L of tartaric acid, adjusted to pH 3.4 with 1 M NaOH, and ethanol content of 12% vol.

2.3. Proposed method

Five mL of sample was pipetted into a 20 mL standard headspace vial, then 20 μL of the internal standard solution was added to reach a concentration level of 200 μg/L. The vial was then closed and placed in the Gerstel MPS2 auto-sampler (Mülheiman der Ruhr, Denmark) where the DHS sampling was automatically carried out under the conditions detailed in Table 1. Thermal desorption and cryo-focusing were carried out using a Thermo Desorption Unit (TDU) and Cooling Injection System (CIS4) also supplied by Gerstel. Solvent venting mode was used to perform the desorption.

Detailed experimental conditions are shown in Table 1.

Gas chromatography-mass spectrometry analysis was performed with a 7890 Agilent GC system coupled with a 5975C Agilent quadrupole mass spectrometer (Santa Clara, CA, USA). A J&W DB-Wax column was used (60 m × 0.25 mm i.d. × 0.25 μm film thickness, Agilent). The temperature program was: initial oven temperature 35 °C held for 3 min, then raised to 220 °C at 10 °C /min, and 7 min of final hold time. The carrier gas was helium at a constant flow of 1 mL/min. The chromatograms were collected in both full scan and SIM mode. Ionization was carried out in electron impact mode at 70 eV. The ion source temperature was 230 °C. Spectra were recorded both in scan mode from 33 to 250 m/z and in selected ion monitoring. Selected ions for particular compounds are shown in Table 2.

Table 1. Experimental parameters of the DHS system.

Parameters			
Incubation time	5 min	Initial TDU temperature	20 °C
Incubation temperature	30 °C	End TDU temperature	300 °C
Purge volume	100 mL	Rate TDU	200 °C/min
Purge flow	25 mL/min	Initial CIS temperature	-100 °C
Purge temperature	40 °C	End CIS temperature	250 °C
Dry volume	50 mL	Rate CIS 1	16 °C/s
Dry flow	10 mL/min	Rate CIS 2	12 °C/s
Dry temperature	40°C	Sample volume	5 mL
Sorbent material	Tenax TA	No stirring	

2.4. Method validation

2.4.1. Internal standards

Two compounds which potentially should provide a headspace concentration independent of the wine specific composition were tested (methyl 2-methylbutyrate and 2,6-dichloroanisole). For that, a synthetic wine, 4 whites, 2 reds and 1 rosé, all made from different grape varieties were spiked with 200 μg/L of both components and were analyzed in duplicate and on 3 different days.

2.4.2. Precision

Method precision was studied over a four-month period. Four bottles (from the same batch) of a Spanish red Crianza wine from La

Rioja were kept refrigerated at 10 ◦C. Each month, one bottle was opened in a glove box from Jacomex (Dagneux, France) with oxygen levels under 0.002%. Immediately after opening, each bottle was aliquoted in 4 20 mL SPME vials to be analyzed on 4 different days within the same week. The vials were kept in the glove box until the analysis.

2.4.3. Linearity and limits of detection

Method linearity was evaluated using a set of 9 different volatile compounds found in wine representing different chemical families, as detailed in Table 4. These compounds were dissolved in ethanol and were further spiked at five different levels to a Spanish red wine from La Rioja. The ethanol content was adjusted to maintain the same level in all calibration samples. All samples were prepared in duplicate and were analyzed following the procedure described in Section 2.3. The areas of each compound in Table 4 were normalized by those of the IS (MBM), corrected by subtracting the relative area obtained for that compound in the unspiked wine and fitted to an unweighted least-squares regression model.

Table 2. Acquisition mode and selected ions for the determination of target compounds in the study.

Compound	Retention time (min)	Scanning mode[a]	Ions (m/z)
Methanethiol	2.983	SIM	47, 48
Dimethyl sulfide	3.418	SIM	62, 47, 61
Sulfur dioxide	7.151	SIM	64, 48
Ethyl acetate	5.191	full scan	74
Ethyl propanoate	6.632	full scan	102
Ethyl butyrate	8.321	full scan	88
Ethyl hexanoate	12.257	full scan	99
Ethyl octanoate	15.730	full scan	88
Ethyl decanoate	18.799	full scan	101
Ethyl isobutyrate	7.209	full scan	116
Ethyl 2-methylbutyrate	9.029	full scan	115

Compound	Retention time (min)	Scanning mode[a]	Ions (m/z)
Ethyl 3-methylbutyrate	9.218	full scan	115
Ethyl lactate	12.332	full scan	45
Diethyl succinate	17.582	full scan	129
Isobutyl acetate	8.057	full scan	73
Isoamyl acetate	9.986	full scan	70
Phenethyl acetate	19.497	full scan	104
Acetaldehyde	2.880	SIM	42, 43, 44
Diacetyl	6.386	full scan	86
Isobutanal	4.123	SIM	72, 41
Methylbutanal	5.822	SIM	58, 57, 71
2,3-Pentanedione	8.617	full scan	100
Acetoin	11.466	full scan	88
Furfural	14.216	full scan	96
Benzaldehyde	15.459	SIM	105, 106
β-Damascenone	20.246	SIM	121, 190
Isobutanol	8.095	full scan	74
1-Butanol	9.190	full scan	56
Isoamyl alcohol	10.153	full scan	70
cis-3-Hexenol	13.091	full scan	82
2-Phenylethanol	20.473	SIM	122, 91, 92
Linalool	15.827	SIM	121, 93
Acetic acid	13.900	full scan	60
Butyric acid	16.344	SIM	60, 88
Hexanoic acid	19.105	SIM	60, 87
γ-Butyrylactone	16.742	full scan	86
trans-Whiskeylactone	20.281	SIM	99, 71
cis-Whiskeylactone	21.267	SIM	99, 69
4-Ethylphenol	21.570	SIM	107, 122
4-Ethylguaiacol	22.931	SIM	137, 152

[a] SIM: selected ion monitoring

Method sensitivity was assessed by estimation of the limits of detection. These were defined as the amount of analyte in the liquid phase of wine that produces with the proposed method a peak with a height equivalent to three times the average standard deviation of the baseline in the surrounding area to the ion peak. The concentration of the different compounds in the liquid phase of the wine was estimated by using previously validated methods as is described in Section 2.5.

2.5. Quantitative analysis of compounds in the liquid phase

The quantitative analysis of major volatile compounds contained in wine was carried out using the method proposed and validated in our laboratory (Ortega, Lopez, Cacho, & Ferreira, 2001). By this method, 3 mL of wine containing the internal standards (2-butanol, 4-methyl-2-pentanol, 4-hydroxy-4-methyl-2-pentanone, and 2-octanol) and 7 mL of water were salted with 4.5 g of ammonium sulfate and extracted with 0.2 mL of dichloromethane. The extract was then analyzed by GC with FID detection. The area of each analyte was normalized by that of its corresponding internal standard and was then interpolated in the corresponding calibration plot built by applying the same analytical method as that applied to synthetic wines containing known amounts of the analytes covering the natural range of occurrence of these compounds.

The quantitative analysis of minor and trace compounds in the liquid phase of wine was carried out using the method proposed and validated in our laboratory (Lopez, Aznar, Cacho, & Ferreira, 2002) with the following changes in the procedure: standard solid phase extraction (SPE) cartridges (1 mL, total volume) filled with 200 mg of LiChrolut EN resins were placed in the vacuum manifold extraction system (Varian Sample Preparation Products), and the sorbent was conditioned by rinsing the cartridges with 4 mL of dichloromethane, 4 mL of methanol, and, finally, with 4 mL of a water-ethanol mixture (12%, v/v). The cartridges were then loaded with 50 mL of wine sample and 26 μL of a surrogate standards solution (recovery standard) containing 3-octanone, β-damascone, and heptanoic acid (all at 200 μg/g of ethanol). This mixture was

passed through the SPE cartridges (2 mL/min), followed by a washing step using 5 mL of 30% methanol in water and 1% NaHCO3 solution. The resins were then dried by letting air pass through them (negative pressure of 0.6 bar, 10 min). Analytes were recovered in a 2 mL vial by elution with 1.6 mL of dichloromethane. Thirty-four µL of an internal standard solution (300 mg/L of 4-hydroxy-4-methyl-2-pentanone and 2-octanol) was added to the eluted sample. The extract was analyzed by GC with ion trap mass spectrometry (MS) detection (GC-450 gas chromatograph fitted to a Varian Saturn 2200 ion trap-MS).

3. Results and discussion

3.1. DHS method

The present method seeks to provide a reliable snapshot of the composition of the vapors emanating from wine when it is smelled or consumed at a given time. For this reason, it is important to fulfill two conditions:

1st The purging time has to be the smallest possible ensuring acceptable sensitivity.

2nd The purging process has to produce headspaces with compositions equivalent to those produced during real olfaction or consumption.

Regarding the first condition, the total volume of gas used to drag the wine headspace was limited to 100 mL in 4 min with the sample thermostat at 30 °C and without stirring. This relatively short sampling time and gas sampling volume also ensure that ethanol

does not saturate the Tenax trap, which would reduce breakthrough volumes, and that even the most volatile compounds are retained.

Regarding this second condition, it should be noted that bubbling through the liquid facilitates mixing and the transport to the headspace of all compounds present in the liquid phase. In those conditions, the stream of vapors produced would have a composition close to those observed in the headspace in equilibrium with the liquid phase (San-Juan, Pet'ka, Cacho, Ferreira, & Escudero, 2010). However, such conditions are far from those observed during real tasting and consumption, where the vapor composition is determined by the kinetics of mass transfer from the liquid to the gas (Tsachaki, Gady, Kalopesas, Linforth, Athes, Marin, et al., 2008; Tsachaki, Linforth, & Taylor, 2009). If instead, the purging gas is used only to drag the headspace of the unstirred liquid, the headspace is quickly diluted and impoverished in the most volatile compounds which cannot be satisfactorily transferred from the bulk of the unstirred liquid to the headspace.

The optimized experimental parameters of the DHS system are listed in Table 1. A typical GC-MS chromatogram can be seen in Fig.1. The method allows to study 40 wine aroma compounds in a wide range of volatilities (from methanethiol to 4-ethylphenol), concentrations (from μg/L to >200 mg/L) and polarities (from acetic acid or sulfur dioxide to ethyl decanoate). The other operative conditions, such as the drying volume or solvent split at the TDU were chosen in order to minimize problems with water and column overloading. Once these optimal conditions were found, the method was evaluated for different quality parameters.

3.2. Internal standards

Finding an internal standard whose instrumental response can correct for changes in the instrument sensitivity is of paramount importance for the method. Only with such an internal standard could a comparison between different wines can be achieved. The ideal internal standard for the present method is a compound whose concentration in the headspace is always constant and independent from the wine matrix, implying that it should exert a minimum interaction with the matrix components. According to previous work carried out in our laboratory (Bueno, Zapata, & Ferreira, 2014), methyl 2-methylbutyrate (MBM) and 2,6-dichloroanisole (DCA) were suitable candidates. Their potential usefulness was experimentally checked by repeatedly analyzing batches of different commercial wines (n = 7) and synthetic wine models containing this compounds at fixed concentrations. The results revealed that both compounds could be used as internal standards. MBM performed better with a global relative standard deviation (RSD) for the absolute ion peak areas of 10%. Additionally, the difference between the average area measured in real wines coincided closely with the average area in synthetic wine (-3.5%), confirming that the volatility of this compound was almost independent of the matrix composition. Therefore, this compound was used to normalize the areas of the analytes and to correct potential variations in the trapping system or the instrumental response. The DCA performance was slightly worse with a 16% global RSD, but it was retained in the internal standard solution for additional quality controls.

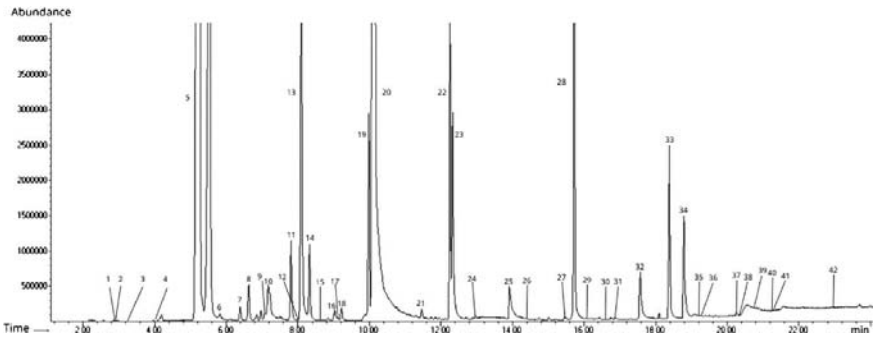

Fig.1. GC-MS chromatogram (SCAN mode) of a wine sample: (1) acetaldehyde; (2) methanethiol; (3) dimethyl sulfide; (4) isobutanal; (5) ethyl acetate; (6) 2- & 3-methylbutanal; (7) diacetyl; (8) ethyl propanoate; (9) sulfur dioxide; (10) ethyl isobutyrate; (11) methyl 2-methylbutyrate (IS); (12) isobutyl acetate; (13) isobutyl alcohol; (14) ethyl butyrate; (15) 2,3-pentanedione; (16) ethyl 2-methylbutyrate; (17) 1-butanol; (18) ethyl 3-methylbutyrate; (19) isoamyl acetate; (20) isoamyl alcohol; (21) acetoin; (22) ethyl hexanoate; (23) ethyl lactate; (24) cis-3-hexen-1-ol; (25) acetic acid; (26) furfural; (27) benzaldehyde; (28) ethyl octanoate; (29) linalool; (30) butyric acid; (31) γ-butyrolactone; (32) diethyl succinate; (33) 2,6-dichloroanisole (IS); (34) ethyl decanoate; (35) hexanoic acid; (36) phenethyl acetate; (37) β-damascenone; (38) trans-whiskeylactone trans-whiskeylactone; (39) β-phenylethanol; (40) cis-whiskeylactone; (41) 4-ethylguaiacol; (42) 4-ethylphenol.

3.3. Precision, linearity and detection limits

Precision was measured regarding method reproducibility and method repeatability. The repeatability was estimated as the within-batch variability (same sample, different days within the same week), while reproducibility added the inter-month and sample bottle variability and hence is not an appropriate measurement of the method performance. As can be seen in Table 3, repeatability was in general satisfactory, particularly considering that the measurements took place during one week. Even if wines were kept as stable as possible within an anoxic glove chamber, some inevitable changes would occur during a week, affecting mainly to highly volatile or reactive compounds. This suggests that the values obtained for

repeatability in Table 3 represent a worst-case scenario. As can be seen, the worst results were obtained for methanethiol. This poor result can be partly attributed to the low levels at which it was present in the wine used in the study (3.5 μg/L), but also to the fact that the concentration of this elusive molecule can change substantially during the experiments because of its high volatility, lability to oxygen and because of the existence of different non-volatile species in equilibrium with the volatile form (Bueno, Zapata, & Ferreira, 2014; Franco-Luesma & Ferreira, 2014). Relatively poor repeatabilities obtained for acetaldehyde and methyl butanal could also be related to the ability of these compounds to form stable complexes with SO_2. In the cases of acetaldehyde and DMS, their high volatility and poor retention in the Tenax trap can also explain the outcome. Acetic acid seems to be particularly poorly retained in Tenax. Leaving aside these cases, most compounds can be quantified with a worst-case reproducibility better than 10%, which can be considered acceptable taking into account the conditions of the experiment.

The detection limits were estimated considering the concentrations of the compounds in the wine used for validation. These concentrations were determined by different headspace, liquid-liquid or solid phase extraction strategies (see methods). The results are given in Table 3. As expected, the detection limits are strongly related to the volatility of compounds in the wine matrix. Accordingly, the lowest detection limits (0.1–0.3 μg/L) were found for various non-polar ethyl esters, such as ethyl 3-methylbutyrate, while the highest was found for the most soluble compounds such as sulfur dioxide, acetaldehyde, acetoin or acetic acid. Fortunately, the

method makes it possible to determine many relevant wine aroma compounds at the concentrations at which they are present in normal wines.

Another key validation parameter was linearity. In order to have a realistic estimation of this quality parameter, a red wine was spiked with known amounts of a small group of selected analytes representative of the different chemical families of volatile compounds found in wine. This approach guarantees that the intrinsic volatilities of the compounds do not change as a consequence of changes in the matrix polarity caused by increases in the levels of non-polar compounds. As can be seen in Table 4, in all cases linear dynamic ranges spanned at least 2 or 3 orders of magnitude with determination coefficients better than 0.99 in all cases. The study of the residuals did not show the existence of any particular trend. These data prove that in the proposed DHS method, any change in the composition of the headspace causes a proportional change in the signal.

Table 3. Precision and detection limits of the DHS method.

	RSD (%)		Concentration	Detection
	Repeatability	Reproducibility	in wine (μg/L) [a]	limit (μg/L)
Methanethiol	28	30	3.46 ± 0.08	0.34
Dimethyl sulfide	14	48	20.0 ± 0.7	0.19
Sulfur dioxide	10	12	13900 ± 800	1.83
Ethyl acetate	8	15	87300 ± 3500	15.3
Ethyl propanoate	1	8	220 ± 10	0.74
Ethyl butyrate	5	7	120 ± 6	0.16
Ethyl hexanoate	8	13	357 ± 14	0.11
Ethyl octanoate	6	15	233 ± 9	0.05
Ethyl decanoate	10	17	79.2 ± 1.8	0.07
Ethyl isobutyrate	9	9	141 ± 12	0.35
Ethyl 2-methylbutyrate	6	8	27.4 ± 2.8	0.21
Ethyl 3-methylbutyrate	4	5	51.3 ± 0.9	0.35
Ethyl lactate	9	11	17500 ± 3000	41.3
Diethyl succinate	7	7	19400 ± 600	5.50
Isobutyl acetate	11	14	8.10 ± 0.14	0.33
Isoamyl acetate	7	14	333 ± 12	0.06
Phenethyl acetate	6	11	27.3 ± 1.1	0.23
Acetaldehyde	13	29	6491 ± 410	356

	RSD (%)		Concentration	Detection
	Repeatability	Reproducibility	in wine (µg/L) [a]	limit (µg/L)
Diacetyl	11	16	990 ± 46	1.97
Isobutanal	8	13	45.5 ± 2.4	0.79
Methylbutanal	13	20	21.0 ± 1.1	0.38
2,3-pentanedione	11	12	300 ± 17	0.87
Acetoin	10	11	22300 ± 600	4.52
Furfural	11	18	343 ± 79	6.43
Benzaldehyde	10	12	11.1 ± 0.2	0.02
β-damascenone	8	8	1.86 ± 0.04	0.04
Isobutanol	5	12	35400 ± 400	35.9
1-Butanol	7	7	718 ± 17	5.35
Isoamyl alcohol	5	11	245000± 4000	17.9
cis-3-Hexenol	7	9	180 ± 4	2.51
2-Phenylethanol	10	10	40900 ± 2400	2.82
Linalool	1	2	6.74 ± 0.35	0.04
Acetic acid	12	17	451000± 26000	354
Butyric acid	8	13	968 ± 22	22.9
Hexanoic acid	3	7	2290 ± 130	16.5
γ-Butyrolactone	7	13	17000 ± 400	241
trans-whiskeylactone	4	5	25.2 ± 0.6	0.13
cis-whiskeylactone	10	9	171 ± 3	22.8
4-Ethylphenol	7	9	340 ± 8	0.88
4-Ethylguaiacol	5	6	14.4 ± 0.2	0.75

[a] Uncertainty expressed as the standard error of the mean (n=3).

Table 4. The linearity of the proposed DHS method.

Compound	Concentration range (µg/L)	Slope	R^2
Dimethyl sulfide	20 - 566	5.00×10^{-5}	0.9998
Acetaldehyde	1550 - 16400	5.35×10^{-4}	0.9983
Ethyl acetate	2100-41000	1.29×10^{0}	0.9998
Ethyl butyrate	120 - 2450	2.08×10^{0}	0.9945
Ethyl decanoate	80 - 1460	1.51×10^{0}	0.9999
1-Butanol	720 - 14900	6.22×10^{-1}	0.9986
2-phenylethanol	18000-112000	1.10×10^{-2}	0.9971
Butyric acid	970 - 17800	1.05×10^{-2}	0.9952
4-Ethylphenol	340 - 6270	6.85×10^{-2}	0.9995

4. Conclusions

The proposed DHS-TD-GCMS method provides quantitative data of up to 40 different relevant aroma compounds in the vapors emanating from wine. In summary, the method showed satisfactory validation parameters and can be used to assess the content of up to

these relevant aroma compounds in the headspaces emanating from wine and hence to study how these headspaces change in response to the different matrix and environmental parameters.

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CHAPTER 2

Study of the Changes in Wine Headspace with Time by Multiple Dynamic Headspace Sampling Strategy

CHAPTER 2

Study of the changes in wine headspace with time by multiple dynamic headspace sampling strategy

1. Introduction

Wine aroma is one of the essential characteristics of wine quality. As a sequential process, the olfactory perception firstly relies on the released contents of odorants in the vapor phase rather than their initial concentrations in the liquid phase (Ferreira, 2012), then this emanated headspace is perceived by olfactory receptors and finally generate the overall figure of wine aroma (Muñoz-González, Rodríguez-Bencomo, Moreno-Arribas, & Pozo-Bayón, 2011; Taylor, 2002). To study the first step, large numbers of researches have devoted to the complexity of wine matrix components and the variations in physicochemical properties of volatile compounds (Ayed, Lubbers, Andriot, Merabtine, Guichard, & Tromelin, 2014; Bueno, Zapata, & Ferreira, 2014; Robinson, Ebeler, Heymann, Boss, Solomon, & Trengove, 2009). This uncertain type of odorant with matrix combinations powerfully affect the aroma release, producing evolutionary headspace composition above wine, and also enhancing the difficulty to predict the final aroma profile of a particular wine even with well-known chemical data of the liquid phase (Rodríguez-Bencomo, Muñoz-González, Andújar-Ortiz, Martín-Álvarez, Moreno-Arribas, & Pozo-Bayón, 2011; Saenz-Navajas, Campo, Cullere, Fernandez-Zurbano, Valentin, & Ferreira, 2010; Villamor & Ross, 2013). Other concerns of the study are the non-equilibrium condition in a real wine glass and the difficulties to

reproduce real tasting factors in the laboratory (Escudero, San-Juan, Franco-Luesma, Cacho, & Ferreira, 2014; Hirson, Heymann, & Ebeler, 2012). Owing to all the factors, it stresses the importance of studying effective concentration at which the different aroma compounds release from the matrix to the headspace to achieve a better understanding of the analytically relevant information between aroma perception and chemical data, rather than the focusing on the total aroma composition.

Various headspace sampling techniques have been applied to study the aroma release from wine matrix as mentioned in the previous chapter (Escudero, San-Juan, Franco-Luesma, Cacho, & Ferreira, 2014; Jelen, Majcher, & Dziadas, 2012; Jublot, Linforth, & Taylor, 2005; Soria, Garcia-Sarrio, & Sanz, 2015; Taylor, Linforth, Harvey, & Blake, 2000). Besides, our colleagues have combined multiple headspace analysis with in-tube extraction to study aroma release behavior from different wines (Zapata, Lopez, Herrero, & Ferreira, 2012). Their findings have pointed out the relative volatility of odorants due to complicated interactions among wine components. However, in that case, the time-dependent change of headspace during real wine tasting was not taken into consideration. Therefore, in this chapter, we attempt to achieve a preliminary understanding of the volatile release from red wines at different vintages by using the validated DHS-TD-GC-MS method in Chapter 1. A modified strategy of multiple headspace extractions will be applied. Instead of obtaining quantitative data of volatile compounds, our purpose is to monitor the compositional changes in the wine headspace potentially experimented during the time that the wine is kept in the glass during

consumption.

2. Materials and methods

2.1. Regents and chemicals

The internal standards with purity above 99% in all cases were obtained from Aldrich, Fluka (Madrid, Spain). Dichloromethane, methanol and ethanol of HPLC quality were purchased from Merck (Darmstadt, Germany). Standard solid phase extraction cartridges and Lichrolut EN resins were supplied by Merck (Darmstadt, Germany).

2.2. Wine samples

Four Spanish red wines with different aging times were selected: a 1 year old young red wine without barrel ageing (coded as "YOUN1"), two different 4 year old red wines (coded as "AGED1" and "AGED2") and a 7 year old red wine (coded as "AGED3"). Detailed information about the wines is included in Table 1 and 2. The wines were prepared at room temperature and adjusted to 14.5% ethanol content.

Table 1. Detailed information about the studied red wines.

Wine code	Commercial name	Grape varieties [a]	Vintage	Age in barrel	Alcohol (% v/v)	pH	TPI [b]	Free SO_2 (mg/L)	Total SO_2 (mg/L)
YOUN1	Borsao selección	Gre., Sy, Tem.	2015	0	14.5	3.45	56.2	19.2	32.0
AGED1	Hacienda Lopez de Haro	Tem., Gre., Gra.	2011	18	13.5	3.67	47.7	17.6	44.8
AGED2	Viña pomal	Tem.	2011	12	13.5	3.64	51.2	33.6	51.2
AGED3	Coto de Imaz	Tem.	2008	18	13.5	3.47	57.3	4.8	33.6

[a] Gre.: Grenache, Tem.: Tempranillo, Gra.: Graciano, Sy: Syrah. [b] TPI: total polyphenol index.

Table 2. Concentrations (µg/L) of volatile compounds in the four studied wines.

Compound	YOUNG 1	AGED 1	AGED 2	AGED 3
LINEAL ETHYL ESTERS (fruity)				
Ethyl propanoate	<L.D.	197.46	<L.D.	241.79
Ethyl butyrate	92.56	107.75	73.28	105.44
Ethyl hexanoate	332.66	352.86	296.49	371.61
Ethyl octanoate	141.97	204.19	271.19	227.86
Ethyl decanoate	29.21	59.50	60.79	84.39
BRANCHED ETHYL ESTERS (fruity)				
Ethyl isobutyrate	98.24	85.59	183.73	262.01
Ethyl 2-methylbutyrate	11.82	8.76	19.96	43.09
Ethyl isovalerate	23.68	17.64	34.91	79.95
ACETATES (fruity and flowery)				
Ethyl acetate	64459.31	72537.90	71195.11	108432.02
Isoamyl acetate	301.21	187.02	185.74	115.40
Hexyl acetate	<L.D.	<L.D.	<L.D.	<L.D.
Isobutyl acetate	7.30	7.77	10.11	7.57
Butyl acetate	2.60	1.00	7.86	1.88
Phenylethyl acetate	17.24	9.31	12.05	6.95
MISCELLANEOUS ESTERS (no relevant from an aromatic point of view)				
Ethyl lactate	80911.18	170058.61	153368.73	179752.63
Diethyl succinate	17156.03	24455.79	22180.37	35060.00
ALCOHOLS (fusel and green)				
Isobutanol	32629.12	35055.18	60226.70	34813.19
1-Butanol	1456.74	992.68	963.53	1160.14
Isoamyl alcohol	219160.31	235298.97	316939.03	246378.89
1-Hexanol	2121.40	2051.32	2101.18	1972.24
c-3-Hexenol	75.42	238.12	288.62	170.17
Metionol	1680.03	2047.01	2964.24	2496.88
Benzylic alcohol	<L.D.	<L.D.	<L.D.	<L.D.
β-Phenylethanol	41927.28	45711.93	68273.04	44491.42
CARBONYL COMPOUNDS (oxidative, lactic)				
Acetaldehyde	156.85	228.19	170.09	460.29
Diacetyl	614.79	450.35	569.66	196.41
Acetoine	21873.72	17676.21	17945.49	32695.91
Benzaldehyde	6.99	19.57	7.43	1.79
ACIDS (cheese, except the vinegar aroma for the acetic acid)				
Acetic acid	509437.22	502438.89	565882.71	566712.45
Butyric acid	1013.07	1084.06	885.89	1219.57
Isobutyric acid	2019.32	2756.32	3971.08	2857.78
Isovalerianic acid	1280.26	1120.85	1950.29	1240.02
Hexanoic acid	2923.60	3164.33	2607.98	2427.39

Compound	YOUNG 1	AGED 1	AGED 2	AGED 3
Octanoic acid	3449.25	3533.30	2496.66	2332.66
Decanoic acid	344.06	300.01	212.99	<L.D.
MONOTERPENOLS (green, citric)				
Linalool	11.27	6.66	5.63	0.42
Linalool acetate	<L.D.	<L.D.	<L.D.	<L.D.
α-Terpineol	7.99	4.05	4.33	2.41
β-Citronelol	6.95	<L.D.	<L.D.	<L.D.
Geraniol	<L.D.	<L.D.	<L.D.	<L.D.
NORISOPRENOIDS (flowery, sweety)				
β-Damascenone	1.14	1.19	0.79	0.53
α-Ionone	0.75	1.09	0.43	1.02
β-Ionone	<L.D.	<L.D.	<L.D.	<L.D.
PHENOLS (animal, leather)				
Guaiacol	6.33	8.20	8.16	12.75
o-Cresol	1.45	1.29	1.28	0.74
4-Ethylguaiacol	0.36	12.14	22.57	20.74
m-Cresol	0.25	0.60	0.53	0.88
4-Propylguaiacol	<L.D.	0.48	1.52	1.67
Eugenol	2.85	14.51	18.94	34.49
4-Ethylphenol	0.83	168.15	209.05	176.22
4-Vinylguaiacol	51.17	27.28	17.13	17.96
E-Isoeugenol	7.79	12.32	10.73	7.95
2,6-Dimethoxyphenol	19.69	27.72	31.60	56.24
4-Vinylphenol	6.08	10.18	9.47	12.16
4-Alyl-2,6-dimethoxyphenol	6.13	17.82	16.34	23.23
CINNAMATES (flowery)				
Ethyl dihidrocinnamate	<L.D.	<L.D.	<L.D.	<L.D.
Ethyl cinnamate	0.37	0.47	0.36	0.43
LACTONES (coconut, peach)				
γ-Butyrolactone	15285.85	17786.00	21668.93	13103.81
t-Whiskylactone	<L.D.	30.14	17.79	59.53
c-Whiskylactone	<L.D.	128.40	184.38	77.28
γ-Nonalactone	18.75	12.06	10.72	7.96
γ-Decalactone	38.91	18.14	28.45	58.58
VANILLIN DERIVATIVES (sweety)				
Vanillin	4.20	33.85	71.80	26.02
Methyl vanillinate	24.18	6.09	5.65	3.86
Ethyl vanillate	197.84	157.88	163.81	125.88
Acetovanillone	104.15	74.83	80.36	70.84
Siringaldehyde	0.26	0.12	0.03	0.04

2.3. Dynamic headspace detection of wine volatile compounds

The validated DHS-TD-GC-MS method comprehensively described in Chapter 1 was applied to analyze the composition of headspace. Briefly, 5mL of the sample was transferred into a 20 mL standard headspace vial and then 20 µL of internal standards solution was also added. The thermal desorption was performed by TDU-CIS system (Gerstel, Denmark) and was programmed as described before. Gas chromatography-mass spectrometry analysis was performed with a 7890 Agilent GC system coupled with a 5975C Agilent quadrupole mass spectrometer (Santa Clara, CA, USA). The temperature program was: initial oven temperature 35 °C held for 3 min, then raised to 220 °C at 10 °C/min, and 7 min of final hold time. Ionization was carried out in electron impact mode at 70 eV. Spectra were recorded both in scan mode from 33 to 250 m/z and in selected ion monitoring.

2.4. Quantitative analysis of compounds in the liquid phase

Total volatile sulfur compounds were quantified by using the method proposed and validated in our laboratory (Franco-Luesma & Ferreira, 2014). First, 10 mL of brine was added to a 20 mL standard headspace vial. The vial was then capped and Argon bubbled through the septum for 2 min to eliminate oxygen. Next, 200 µL of wine sample and 20 µL of internal standards were added to the vial, and the prepared sample was analyzed immediately by SPME-GC-pFPD. The quantitative analysis of major and trace volatile compounds contained in wine was carried out using the method proposed and validated in our laboratory (Lopez, Aznar, Cacho, &

Ferreira, 2002; Ortega, Lopez, Cacho, & Ferreira, 2001). Total and free sulfur dioxide was determined by the aspiration/titration method (Rankine method recommended by the OIV, International Organization of Vine and Wine) (OIV-MA-AS323, 2009). All analyses were performed in triplicate as supplementary chemical information (shown in Table 1 and Table 2).

2.5. Changes in wine headspace with time

To assess the changes in the headspace concentrations of the different analytes with time, the headspaces of each wine sample were analyzed with the proposed DHS method five consecutive times. For that, the wines were prepared in the vials as described in the method, analyzed, and after 70 min the same vial was re-analyzed following the procedure. The vials were kept closed in the vials at 25 °C between extractions. The analysis was duplicated for each wine.

2.6. Statistical analysis

Data from each wine were normalized to the level of compounds in the first sampling point, then 2-way ANOVA was applied to assess the significance of the factors wine, time (injection number) and their interaction by SPSS 19.0 (SPSS Inc., USA). F-test was applied to analyze significant differences in Lnβ by Excel 2013 (Microsoft, USA).

3. Results and discussion

3.1. Changes in wine headspace with time

The validated DHS method has been applied to study how the

headspaces emanated from four different wines change with time as consequence of evaporation, shifts in chemical equilibria or other phenomena that can take place during the time in which the wine is served in a glass. However, in this experiment, the wines were kept in a closed vial during the experiment (see methods). As will be shown, the levels of nearly a half of the studied aroma compounds decayed with time, and the rates of decay were directly related to the fraction of compound emitted to the headspace, suggesting that evaporation is the primary cause of the observed changes. It should also be noted that in the present study decay curves are not used to obtain unbiased estimators of the concentration of compound in the original matrix, as done in previous works (Ezquerro, Pons, & Tena, 2003; Kolb & Pospisil, 1977; Julian Zapata, Ricardo Lopez, Paula Herrero, & Vicente Ferreira, 2012), but rather to characterize the specific decay patterns followed by the different aroma compounds and also to assess whether these patterns are general to all wines or if they are dependent on the specific matrix composition of a given wine.

Data from each wine were normalized to the level of compound found in the first sampling point, in order to make decay curves independent of the concentration. As the internal standard also decays with time, changes in instrumental sensitivity were corrected by normalizing the areas by those obtained for ethanol, whose levels remained stable during the experiment. Data were then processed by 2-way ANOVA (Table 3) to assess the significance of the factors wine, time (injection number) and of their interaction. Results make it possible to classify the 34 aroma compounds which could be

monitored in the four wines during the five consecutive injections into four broad categories:

1. Compounds whose concentrations in the headspaces remain unchanged

2. Compounds whose concentration in the headspace follows irregular wine-dependent trends

3. Compounds whose concentration in the headspaces decay. This category can be further subdivided in:

a. Those whose decay functions are non-wine-dependent

b. Those whose decay functions are wine-dependent

Table 3. Two-way ANOVA carried out with data from the continuous sampling of the headspaces of 4 different wines.

Compound	Wine (p)	Injection number (p)	Interaction (p)
Constant headspace concentration			
Sulfur dioxide	0.395	0.743	0.871
Acetoin	0.752	0.524	0.986
Furfural	0.925	0.293	0.981
Ethyl lactate	0.898	0.471	0.892
Diethyl succinate	0.427	0.500	0.881
Acetic acid	0.382	0.245	0.994
Butyrolactone	0.676	0.212	0.996
2-Phenylethyl acetate	0.437	0.360	0.931
β-damascenone	0.130	0.975	0.927
Isobutyl alcohol	0.791	0.634	0.677
Isoamyl alcohol	0.928	0.406	0.873
2-Phenylethanol	0.746	0.659	0.954
4-Ethylguaiacol	0.978	0.630	0.950
4-Ethylphenol	0.865	0.367	0.903
trans-Whiskeylactone	0.902	0.197	0.922

Compound	Wine (p)	Injection number (p)	Interaction (p)
Wine-dependent non-decay trends			
Acetaldehyde	**0.000**	0.394	**0.051**
2&3-Methylbutanal	**0.004**	0.868	0.273
Diacetyl	**0.036**	0.181	0.936
Isobutyraldehyde	**0.001**	**0.003**	**0.011**
Benzaldehyde	**0.001**	0.437	0.547
Simple decay trends			
Ethyl acetate	0.688	**0.000**	0.721
Propyl acetate	0.354	**0.000**	0.687
Ethyl propanoate	0.866	**0.000**	0.811
Ethyl butyrate	0.509	**0.000**	0.856
Ethyl isobutyrate	0.214	**0.000**	0.819
Isoamyl acetate	**0.097**	**0.000**	0.598
Ethyl-2-methylbutyrate	**0.062**	**0.000**	0.524
Ethyl-3-methylbutyrate	**0.087**	**0.000**	0.564
Ethyl hexanoate	0.334	**0.000**	0.766
Ethyl octanoate	0.210	**0.000**	0.751
Linalool	-	**0.000**	-
Wine-dependent decay trends			
Dimethyl sulfide	**0.050**	**0.000**	0.934
Methanethiol	**0.048**	**0.002**	**0.007**
Ethyl decanoate	**0.020**	**0.000**	0.586

p values below 0.1 are marked in bold.

The four categories in which compounds can be classified are presented in Table 3, while Fig. 1 a to d show five evolution patterns representing illustrative examples.

The first category of compounds whose levels in the headspace remain constant with time includes 15 polar or moderately non-polar and not very volatile compounds, as detailed in Table 3. The case of isobutyl alcohol is shown in Fig. 1a as an example. Compounds in this category are fusel alcohols, volatile phenols, volatile acids,

hydroxy esters, aromatic esters, diesters, whiskeylactone, β-damascenone and sulfur dioxide.

The second category includes aldehydes and diacetyl. Levels in the headspaces of these compounds evolved differently with time in each wine, which should be most likely attributed to the different levels of sulfites and of other sulfite binders present in the wines. In the case of acetaldehyde, shown in Fig. 1b as an example, it can be seen that in samples YOUN1 and AGED1, the content in the wine headspaces increased with time, while in samples AGED2 and AGED3, levels decreased with time.

Nearly a half of the compounds (14 out of 34) followed decreasing trends and are classified in the two last categories, which include non-polar compounds and some polar but very volatile compounds such as dimethyl sulfide and methanethiol. Within the ethyl ester homologous series, the rates at which levels decrease with time increase with molecular size; while the levels of ethyl acetate decay just a 30%, levels of ethyl octanoate and decanoate dropped around 80%. A remarkable observation is that polarity is useful for predicting the decay rate only within a homologous series, since molecular size, which strongly affects volatility, is also relevant. For instance, DMS and methanethiol are lost very quickly even though they have higher polarity than ethyl butyrate.

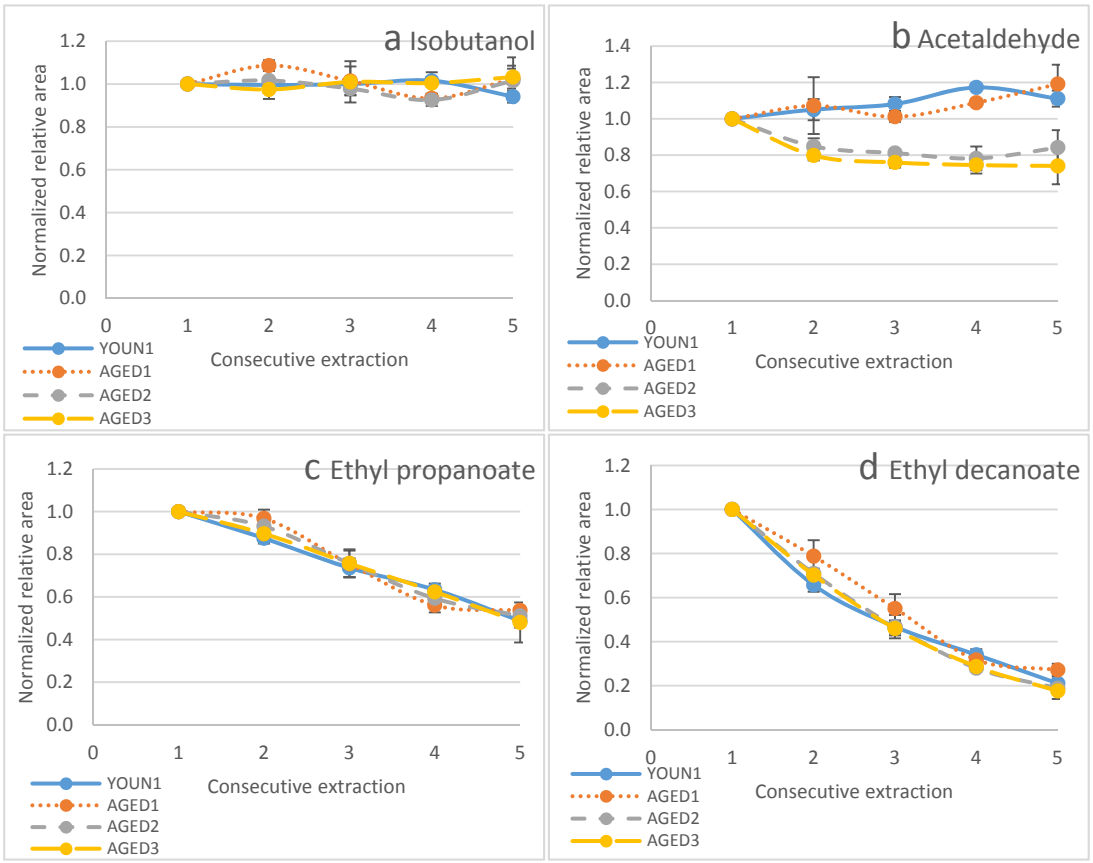

Fig. 1. Evolution patterns of headspace composition after five consecutive extractions for (a) isobutanol, (b) acetaldehyde, (c) ethyl propanoate, and (d) ethyl decanoate.

In most cases, decay trends are not affected by differences in the wine matrix so that over time the levels of those aroma compounds decrease at the same rate in any wine. An illustrative example is shown for the case of ethyl propanoate in Fig. 1c. In some few cases (isoamyl acetate, and 2 and 3-methyl butyrate), however, there is a slight effect, close to statistical significance, of the wine matrix. Moreover, in the particular case of ethyl decanoate, dimethyl sulfide and methanethiol, the effect of the wine matrix reaches significance

so that these compounds are classified into the fourth category. The particular case of ethyl decanoate is shown in Fig. 1d. This compound (the same trend observed in isoamyl acetate and 2 and 3-methyl butyrate) is slightly less retained in the youngest wine, and seem to be more retained in one particular aged red wine. The pattern observed in methanethiol and dimethysulfide, is rather the contrary, with the youngest wine showing maxima retention for both compounds. This could be related to the specific levels of metal cations in this wine, which were not measured in the present experiment.

The theory of multiple extractions was applied to those compounds following a clear decay (Ezquerro, Pons, & Tena, 2003; Kolb & Pospisil, 1977). According to this theory, if the proportion of compound extracted in each extraction remains constant, and that proportion is represented as a series of areas logarithmically transformed versus the ordinal number of the extraction minus 1, the outcome of this representation is a straight line following the equation:

$$LnAi = (i-1)\ Ln\beta + Ln\ A1 \qquad (1)$$

where i denotes the ith extraction and Ai refers to the area obtained in the ith extraction. The slope of this straight line is by convention named Lnβ and it can be demonstrated that Lnβ, in fact, reflects the proportion of compound extracted in each one of the extractions performed in a given sample. A −0.4 value, for instance, means that 40% of the compound is transferred to the headspace in each extraction. The closer to −1 is Lnβ, the higher the proportion of compound transferred to the headspace (Zapata, Lopez, Herrero, &

Ferreira, 2012).

Average Lnβ values for the compounds mentioned above are shown in Table 4. These values are in general agreement with those calculated elsewhere (Julian Zapata, Ricardo Lopez, Paula Herrero, & Vicente Ferreira, 2012) even though the instrumental setup and the purpose of the experiment were completely different. Data in the table are arranged in decreasing order of Lnβ. The least volatile is ethyl acetate for which 10% is transferred to the headspace in each extraction cycle, and the most volatile is dimethyl sulfide, for which 69% is transferred to the headspace. This implies that wine is depleted from this extremely volatile compound very soon, in agreement with previous results (Franco-Luesma, Saenz-Navajas, Valentin, Ballester, Rodrigues, & Ferreira, 2016).

Table 4. Average ln β values and results obtained in the F-test to assess significant differences in Ln β for those compounds following clear decays.

Compound	Ln β [a]	$S^2_{between\ wines}$	$S^2_{within\ wine}$	F
Ethyl acetate	-0.10	0.0000	0.0001	0.54
Ethyl propanoate	-0.18	0.0004	0.0004	1.91
Propyl acetate	-0.18	0.0001	0.0004	0.31
Ethyl butyrate	-0.22	0.0000	0.0005	0.18
Isoamyl acetate	-0.25	0.0005	0.0005	1.84
Ethyl hexanoate	-0.27	0.0002	0.0005	0.81
Ethyl isobutyrate	-0.30	0.0002	0.0003	1.35
Ethyl 3-methylbutyrate	-0.30	0.0003	0.0004	1.40
Ethyl 2-methylbutyrate	-0.31	0.0005	0.0005	2.32
Ethyl octanoate	-0.35	0.0005	0.0005	1.86
Methanethiol	-0.36	0.0025	0.0025	2.07
Ethyl decanoate	-0.38	0.0048	0.0005	**20.75**
Dimethyl sulfide	-0.69	0.0055	0.0017	**6.50**

Values in bold are significant at $p < 0.05$. [a] Ln β value calculated as the average of each of the Ln β values (n=2) obtained from each wine (n=4). $S^2_{between\ wines}$ was calculated with the 4 Ln β values (3 degrees of freedom) and $S^2_{between\ wines}$ was calculated from regression analysis (24 degrees of freedom).

As Lnβ values are slopes obtained by regression analysis, the S value provided by the regression model for the slope is an estimation of its uncertainty. The square roots of the average variances obtained for each compound in the four wines is the average within wine uncertainty, and is given in Table 4. Assuming additivity of variances, the variance of the four Lnβ values obtained for each compound in the four wines can be decomposed into within and between wines variability attending to the model:

$$S_{tot} = S_{betweenwines} + S_{withinwine} \qquad (2)$$

This makes it possible to obtain an estimation of the "between wines" variability (given in Table 4) and also to apply an F test to assess its significance. The results of this test shown in Table 4, where it can be observed that attending to this criterion, only the dimethyl sulfide and ethyl decanoate Lnβ values differ significantly between wines. It should be noted, however, that in the case of methanethiol the F quotient is abnormally low because of the huge within wine variability, which should be attributed to its extremely low levels.

3.2. The potential sensory relevance of these changes

It should be considered that the qualitative characteristics of aroma perceptions are essentially linked to the profile of odor volatiles reaching the olfactory receptors located in the nose (Stevenson & Wilson, 2007; Wright, Lutmerding, Dudareva, & Smith, 2005). Although it is outside the scope of the present chapter to make a precise assessment on this question, the data presented here indicate that the aroma profiles suffer major changes during the time that the wine is in the glass. As has been previously highlighted,

the levels of half of the aroma compounds remained constant with time, while levels of the most volatiles such as DMS, ethyl decanoate or methanethiol quickly dropped to zero. The levels of ethyl esters steadily decreased at rates related to their molecular size, which implies that the profile of volatiles emanated from the wine continuously change which should affect the quality of the odor perceived. Additionally, data indicate that the levels of most aldehydes, many of which have relevant sensory properties, followed matrix-dependent trends as do also dimethyl sulfide, ethyl decanoate, methanethiol and surely other mercaptans. This implies that in all these cases data of concentration in the liquid phase is not enough to accurately interpret the role played by the aroma compound in the product. An estimation of the specific volatility of the odorant in such specific wine should also be provided.

4. Conclusions

The proposed DHS-TD-GC-MS method makes it possible to assess how the composition of the vapors changes with time. Attending to the pattern of change, aroma compounds have been classified into four categories. Polar and not very volatile compounds (half of the total) are present in the headspaces at levels related to their concentration and do not change during time. On the contrary, non-polar and highly volatile compounds can decay very fast. Additionally, the levels and trends followed by aldehydes, dicarbonyls, methanethiol, DMS or ethyl decanoate are significantly affected by the matrix. This indicates that in these cases the data of concentration in the liquid phase should be accompanied by an

estimation of their volatility in such specific wine in order to make a reliable interpretation of their sensory role. Results confirm that wine headspace continuously changes over time, which should cause relevant changes in the odor qualities perceived.

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CHAPTER 3

The Release of Volatile Compounds from Wine: Different Effects of Physical Conditions, Wines and Time

CHAPTER 3

The release of volatile compounds from wine: different effects of physical conditions, wines and time

1. Introduction

The aroma profile of wine is a temporary retention/release balance of volatile compounds under non-equilibrium conditions in a wine glass, which is dependent on the constituents and concentrations in the headspace. However, during the given tasting period, the mass transfer of volatile compound does not only depend on its initial physicochemical characteristics, but also the interaction with matrix components (Polaskova, Herszage, & Ebeler, 2008; Villamor & Ross, 2013). Therefore, any modifications towards both vapor and liquid phases could generate the nuance of olfactory perception of a particular wine, which makes the wine tasting a more dynamic and complicated process.

Notably, some physical treatments simultaneously occur during wine tasting, such as glass stirring, gas purging due to smell, and the period of exposure to ambient conditions (Franco-Luesma, Saenz-Navajas, Valentin, Ballester, Rodrigues, & Ferreira, 2016; Hirson, Heymann, & Ebeler, 2012; Tsachaki, Linforth, & Taylor, 2009). Under the conditions of real wine tasting, stirring could homogenize the liquid and favor the mass transfer on the liquid boundary layer due to convection (Cayot, Dury-Brun, Karbowiak, Savary, & Voilley, 2008; Taylor, 2002). The nasal flow could dilute the headspace, consequently resulting in the change of headspace composition (Escudero, San-Juan, Franco-Luesma, Cacho, & Ferreira, 2014).

The Marangoni effect due to the different extent of ethanol evaporation regarding exposure time can also affect the volatiles' mass transfer rate (Villamor & Ross, 2013; Wollan, Pham, & Wilkinson, 2016).

For volatile compounds, the nature of their release may initially differ due to their physicochemical properties in an ideal solution (Athès, Peña y Lillo, Bernard, Pérez-Correa, & Souchon, 2004; Taylor, 1998). In a hydroalcoholic solution, the mass transfer of volatile compounds may be affected by ethanol content via modification of volatiles' solubility (Conner, Paterson, & Piggott, 1994; Ickes & Cadwallader, 2017). Notably, the solvating effect of ethanol has been reported to be able to cause salting out or retention phenomenon of particular volatile compounds corresponding to the hydrophobicity of volatile compounds (Aznar, Tsachaki, Linforth, Ferreira, & Taylor, 2004; Boothroyd, Linforth, & Cook, 2012). As a complicated multi-component system, wine contains hundreds of compounds with wide differences in chemical characteristics, the interactions among wine components may occur through covalent bonds, hydrophobic bonds or hydrogen bonds due to the chemical structure, functional group, molecular size or other physicochemical properties of volatile compounds (Villamor & Ross, 2013). For instance, volatile compounds with aromatic ring could combine with phenolic compounds more strongly by forming specific π-π stacking (Jung, de Ropp, & Ebeler, 2000). Otherwise, the bond strength also showed wine-dependent differences, such as the bound proportion of carbonyl compounds could be altered by the concentration of sulfur dioxide in wines (Bueno, Carrascón, & Ferreira, 2016). However, the mechanism of aroma release of wine is still not well-

defined, not only because of the aforementioned aspects of interactions but also due to the existence of wine components' evolution over the tasting-period. These phenomena can dynamically affect the release rate of volatile compounds causing the evolution of headspace composition and generating temporal aroma profiles (Lytra, Tempere, Marchand, de Revel, & Barbe, 2016). The present chapter purposes on the one hand to estimate the contributions of the main physical parameters, and on the other hand to further broaden the knowledge of the evolution of aroma release from distinct wine matrices over time.

2. Materials and methods

2.1. Reagents and chemicals

Ethanol was supplied by Merck (Darmstadt, Germany). The internal standards (methyl 2-methylbutyrate, 2,6-dichloroanisole) with purity above 97% were obtained from Sigma-Aldrich (Madrid, Spain).

2.2. Wine samples

A Spanish commercial Crianza wine (La Rioja, 2010, 13% vol) was analyzed to study the effect of chemo-physical parameters. The same red wine and a commercial white wine (Calatayud, 2014, 14% vol) from Spain were analyzed at a series of time points to compare the pattern of headspace change at their initial wine concentrations.

2.3. Dynamic headspace detection of volatile wine compounds

The DHS-TD-GC-MS method validated in Chapter 1 was applied to analyze the composition of headspace as described in

Chapter 1. By the method, 5 mL of the wine sample was transferred into a 20 mL standard headspace vial and then 20 µL of internal standards solution was added to reach a 200 µg/L concentration level. Thermal desorption and cryo-focusing were carried out under the same temperature conditions as validated in Chapter 1. Gas chromatography-mass spectrometry analysis was performed with a 7890 Agilent GC system coupled with a 5975C Agilent quadrupole mass spectrometer (Santa Clara, CA, USA). The temperature was programmed as described before. Ionization was carried out in electron impact mode at 70 eV. Spectra were acquired both in scan mode from 33 to 250 m/z and in selected ion monitoring for selected compounds (shown in Table 2 of Chapter 1).

2.4. Study of the release of volatile compounds from wine

In the present research, four main factors occurring during the tasting period, as mechanical shaking, oxidation, evaporation, and degasification were studied to estimate their effects on the release of wine aroma compounds. According to our first consideration, four treatments were applied in Session 1: (1) **still**, 5 mL of wine was transferred into a 20 mL SPME vial in the glove box from Jacomex (Dagneux, France) under argon atmosphere, and then the well-screwed vial was kept in the tray of the sampler for 2 hours before analysis; (2) **oxidation**, 40 mL of wine were poured into a 250 mL Pyrex container under the air atmosphere, and then the well-closed container was softly agitated over 30 min; (3) **evaporation**, 40 mL of wine were poured into a 250 mL glass beaker under air atmosphere without lid, then the beaker was softly agitated at the same velocity without splashing over 30 min; (4) **degasification**, 40

mL of wine were agitated under the same condition as evaporation but for 150 min. The wine sample of **control** was immediately analyzed after transferring from the wine bottle to the SPME vial in the oxygen-free glove box.

Afterward, the influence of oxygen was exclusively studied with the same red wine (different bottle) in Session 2: (1) 40 mL of wine was poured into 250 mL Pyrex containers under both air and argon atmosphere, then the well-closed container was agitated, samples were named as CAir and CAr, respectively; (2) 40 mL of wine was poured into 250 mL glass beaker without lid and then agitated under both air or argon atmosphere (in glove box), corresponding to sample treatment OAir or OAr. In all cases, the wine was agitated at the same velocity as Session 1 for 150 min. The control was analyzed as described before. After each treatment, 5mL of the wine sample was analyzed by the proposed DHS-TD-GC-MS method (Section 2.3) in triplicate.

The following Session 3 was carried out with a white and a red wine due to their distinct nonvolatile components. Therefore the patterns of the headspace evolution could be studied with variations in two distinct wine matrices. Considering the nonequilibrium and time-consuming characteristics of real wine tasting, each 40 mL of wine was poured into a 250 mL glass beaker without lid, and then the beaker was softly and continuously agitated at the same velocity as described in Session 1 and 2 during a series of evaporation-periods (0 min, 2 min, 10 min and 30 min. After agitation, 5 mL of wine from one beaker was collected to analyze the headspace composition by the proposed DHS-TD-GC-MS method (Section

2.3). Each beaker was analyzed at the given time and then discarded. Consequently, the headspace compositions of wine samples were obtained at t=0 min, t=2 min, t=10 min, t=30 min. Analyses were triplicated for each time point.

2.5. Statistical analysis

One-way analysis of variance (ANOVA) was carried out by XLSTAT 2016 (Addin software) in order to highlight the effect of release parameters. Correlation tests based on Pearson's r were applied to study the relationship between volatile release and physicochemical parameters. Two-way ANOVA (XLSTAT 2016) was applied to study the evolution of volatile compounds from two distinct wine matrix over a period.

3. Results and discussion

Due to the short purging time applied to the DHS method, it could take a snapshot of the headspace composition of the studied wines after each treatment, allowing the detection of volatile compounds from different chemical classes. As emphasized in the previous chapters, the development of this DHS method aimed to reflect an overall profile of wine headspace not to quantify volatile compounds in the liquid phase. Therefore all the obtained data were normalized to the response of internal standard methyl 2-methylbutyrate to achieve comparison between different samples and to avoid instrumental bias due to sensitivity change.

3.1. The evaporation of volatile compounds from wine

Among all the detected analytes in this experiment, methanethiol, dimethyl sulfide and fatty acid esters were almost completely released to the atmosphere after the treatment **evaporation** and **degasification**, which were designed as continuous shaking during 30-min and 150-min period, respectively (Table 1). It is clear from these observations that apolar (fatty acids esters) and very volatile compounds (DMS and methanethiol) are dominant in the first stages of wine tasting as they are easily transferred to the phase gas. This has as a consequence a fast depletion of the liquid phase which can be noticed in their practical disappearance from the headspace after 30 min. The same release behavior has been observed by Lytra et al. (2016) with HS-SPME analysis. Otherwise, sulfur dioxide showed a significant decrease around 40% by **evaporation** and **degasification**, whereas the group of carbonyl compounds and acids contrarily increased from 64% to 100% after long-time evaporation of **degasification** treatment, except for benzaldehyde, which decreased of 30% (shown in Table 1). A recent study has estimated that SO_2 and carbonyl compounds can be under bound forms in commercial red wines. However, oxidation could affect the equilibria and result in the release of carbonyl compounds at low levels of SO_2 in some particular wines (Bueno, Carrascón, & Ferreira, 2016). Their findings could partly explain the contrary change of SO_2 and carbonyl compounds after exposing to ambient conditions over 30 min and 150 min. Similar to carbonyl compounds, less volatile compounds 4-ethylphenol, 2-phenethyl acetate, 2-phenyl ethanol and γ-butyrolactone also released 15% to 60% more to the headspace after **degasification**

treatment (Table 1). It is worth noticing that all the mentioned compounds have cyclic structure, and three of them are aromatic compounds. Several research groups have found interactions between aroma compounds with aromatic ring and non-volatile phenolic compounds, and they attributed this phenomenon to the specific π-π stacking and the hydrogen bonds (Aronson & Ebeler, 2004; Dufour & Bayonove, 1999; Jung, de Ropp, & Ebeler, 2000). The increase of headspace concentration of these compounds may be a consequence of the solubility changes due to the solution structure modifications caused by the loss of ethanol and a large amount of apolar volatile compounds after the long period of exposure (Aznar, Tsachaki, Linforth, Ferreira, & Taylor, 2004; Muñoz-González, Rodríguez-Bencomo, Moreno-Arribas, & Pozo-Bayón, 2011). Also, the group of fusel alcohols showed no significant difference between treatments excluded isobutanol, which slightly decreased 9% after **degasification**. Regarding **oxidation** treatment, only several branched esters and dimethyl sulfide demonstrated a slight decrease from 7% to 20%, while isobutanol showed 10% increase after 30-min shaking in a closed container (Table 1). Our findings of the dramatic decrease of fatty acid esters highlight the importance of studying wine aroma release under nonequilibrium conditions, not only for the purpose of mimicking real wine tasting condition but also due to the enormous loss of the most volatile compounds in the open experimental system, which can certainly change the perception of the aroma profile of a particular wine (Franco-Luesma, Saenz-Navajas, Valentin, Ballester, Rodrigues, & Ferreira, 2016; Lytra, Tempere, Marchand, de Revel, & Barbe, 2016).

Table 1. Log P and vapor pressures of the detected volatile compounds in the headspace. The headspace changes of volatile compounds compared with control (shown in percentage) after each treatment and their defined release pattern.

Compound	Log P*	Vapor pressure (mmHg) *	Control	Still (%)	Oxidation (%)	Evaporation (%)	Degasification (%)
Liberation							
Acetoin	-0.36	2.7	0 b	5.4 b	4.8 b	3.6 b	41.4 a
Furfural	0.41	2.21	0 b	12.2 ab	8.4 b	4.6 b	31.3 a
Ethyl lactate	-0.18	3.75	0 b	0.1 b	19.0 ab	14.0 b	52.8 a
Diethyl succinate	1.2	0.044	0 b	11.5 b	7.6 b	6.4 b	53.7 a
Ethanoic acid	-0.17	16	0 b	-8.7 b	15.9 b	16.0 b	100.3 a
γ-Butyrolactone	-0.64	0.45	0 b	-7.2 b	5.4 b	2.6 b	41.1 a
2-Phenyl ethanol	1.36	0.087	0 b	6.5 b	8.1 b	7.9 b	48.5 a
4-Ethylphenol	2.58	0.037	0 b	5.6 b	2.6 b	2.8 b	59.5 a
2,3-pentanedione	-0.85	31.1	0 c	16.1 bc	11.0 bc	29.9 ab	43.9 a
Butyric acid	0.79	1.7	0 b	-9.0 b	8.4 b	11.4 b	97.4 a
Acetaldehyde	-0.34	900	0 b	-1.3 b	11.8 b	7.7 b	75.7 a
Diacetyl	-1.34	57	0 b	21.7 b	10.7 b	10.4 b	63.6 a
Liber+Evapor							
Benzaldehyde	1.48	0.127	0 a	-3.6 a	8.7 a	-30.0 b	-29.0 b
Sulfur dioxide	-2.2	2600	0 a	-5.5 a	3.0 a	-47.3 b	-34.0 b
Evaporation							
Ethyl acetate	0.73	93	0 a	-13.9 a	-8.1 a	-73.5 b	-99.5 c
Propyl acetate	1.24	35.9	0 a	0.0 a	-3.6 a	-81.5 b	-100 c
Ethyl butyrate	1.85	15	0 a	2.3 a	-7.2 b	-90.3 c	-100 d
Ethyl hexanoate	2.83	1.6	0 a	-1.9 ab	-5.2 b	-94.2 c	-100 d
Ethyl octanoate	3.81	0.24	0 a	-6.4 a	0.36 a	-96.9 b	-100 b
Ethyl decanoate	4.79	0.031	0 a	-18.8 a	-16.1 a	-98.3 b	-100 b
Ethyl isobutyrate	1.77	25.4	0 a	-3.6 a	-16.4 b	-96.5 c	-100 c
Isoamyl acetate	2.26	5.6	0 a	1.5 a	-7.0 b	-93.1 c	-99.7 d
Ethyl2methylbutyrate	2.26	8.03	0 a	-0.31 a	-11.6 b	-96.9 c	-100 c
Ethyl3methylbutyrate	2.26	7.98	0 a	-1.4 a	-14.4 b	-96.3 c	-100 c
Methanethiol	0.78	1500	0 ab	28.6 a	-19.0 b	-100 c	-100 c
Dimethyl sulfide	0.92	502	0 a	8.3 a	-20.8 b	-100 c	-100 c

Compound	Log P*	Vapor pressure (mmHg) *	Control	Still (%)	Oxidation (%)	Evaporation (%)	Degasification (%)
Immutable							
1-Butanol	0.88	6.7	0	11.0	12.4	3.6	-1.8
Cis-3-hexenol	1.61	0.94	0	11.1	12.2	3.6	10.1
2-Phenethyl acetate	2.3	0.068	0	5.6	6.3	-2.7	14.5
Isobuyl alcohol	0.76	11	0 a	8.5 a	11.1 a	7.2 a	-9.2 b
Isoamyl alcohol	1.16	2.4	0	-1.3	23.8	9.2	-5.4

*, Log P (octanol/water) and vapor pressure value at 25°C were calculated with EPI Suite™ software v4.1. Significant differences are calculated by ANOVA. Different letters mean significantly different according to post-hoc test.

According to the headspace composition changes mentioned above, compounds from different chemical classes were released to the headspace in different ways. For compounds from homologous series, they showed a similar change trend, although their initial concentrations varied at different concentration levels in wine. In Chapter 2, we classified volatile compounds into four categories with five evolution patterns by applying consecutive extractions during a long-time period. In that case, most compounds decayed after five extractions as a consequence of evaporation or mass loss mainly due to nitrogen purging during the Tenax extraction. Here in this chapter, we studied several treatments corresponding to the physical factor occurring during tasting. According to the changes of compounds after each treatment, the release patterns of detected compounds were categorized into four groups, immutable, liberation, evaporation and a combined group of liberation and evaporation. As shown in Table 1, the immutable group contained fusel alcohols and 2-phenethyl acetate, which remained constant over time agreeing with the result of Chapter 2. Compounds from various chemical class

were classified to the liberation group due to their increase in headspace after long-time evaporation, such as carbonyl compounds, acids and aromatic compounds. The release of fatty acid esters and volatile sulfur compounds in Table 1 were classified as evaporation because of their dramatic decrease following exposure duration to the atmosphere. Only sulfur dioxide and benzaldehyde showed a combined release behavior due to the remained level from 30-min to a much longer 150-min evaporation under the same shaking condition (Table 1). The different release behavior of volatiles also highlight the possible interactions between volatile compounds and wine matrix components, which will be further discussed in the following chapters.

3.2. The influence of physical factors occurring during wine tasting on the headspace composition

Considering wine tasting in a real wine glass, several physical factors could occur during the given period and further influence the mass transfer between two phases, i.e. wine volume, glass shape, gas dilution and oxidation in open system, alternative static incubation and liquid phase stirring, the air flow by sniffing, and the exposure duration (Escudero, San-Juan, Franco-Luesma, Cacho, & Ferreira, 2014; Zhao, Scherer, Hajiloo, & Dalton, 2004). In this chapter, we evaluated the influence of the main factors corresponding to the factors described in the method section. Individually, wine samples from **control** and **still** treatment were analyzed in well-closed and oxygen-free SPME vials with a volume ratio of 4:1 (between the gas phase and liquid phase), while the **oxidation,** CAir and CAr treatment used a well-closed glass container with the headspace of

air with a volume ratio of 7.5:1. The same ratio was used for the **evaporation**, **degasification,** OAir and OAr experiments but it must be taking into account that in these cases the containers were open.

One-way ANOVA was applied to analyze the significant changes among treatments. As shown in Table 1, the **Still** treatment produced the most similar headspace composition to the **Control**, the changes ranged from -18.8% for ethyl decanoate to 28.6% for methanethiol, but not significant in any case. However, the compound methanethiol and ethyl decanoate had the worse reproducibility according to the method validation of Chapter 1. Thus their change may be due to the variation of the method at a low concentration, especially for methanethiol. Regarding the **oxidation** treatment, several compounds from evaporation category significantly decreased around 20% compared with **control**, including dimethyl sulfide and branched esters (shown in Table 1). The result of the two-tailed t-test further confirmed their decrease (Table 2). Unexpectedly, the t-test result showed that the increase of isobutanol was significant in both still and oxidation cases (Table 2) since its content remained constant after **evaporation** in the open system.

Table 2. Results of two-tailed t-test between control and still or oxidation treatment, respectively. Significant differences are marked in bold.

	Still		Oxidation	
Compounds	t	p(t)	t	p(t)
Dimethyl sulfide	0.64	0.546	3.15	**0.020**
Ethyl isobutyrate	1.76	0.128	5.43	**0.002**
Ethyl 2-methylbutyrate	0.19	0.858	6.05	**0.001**
Ethyl 3-methylbutyrate	0.56	0.594	9.50	**0.000**
Isoamyl acetate	0.77	0.469	2.65	**0.038**
Isobutanol	2.71	**0.042**	3.09	**0.021**

In the case of **still** treatment, wine sample in a SPME vial was incubated at a constant temperature for 2 hours. This kind of long-period incubation has been commonly applied in the static headspace sampling to reach equilibration, and for nonpolar volatile compounds it could be even longer (Athès, Peña y Lillo, Bernard, Pérez-Correa, & Souchon, 2004; Kolb & Ettre, 2006). However, researchers have reported that the long incubation had no positive effect on analytes sampling for a dynamic analyzing process (Manzini, Durante, Baschieri, Cocchi, Sighinolfi, Totaro, et al., 2011; Marquez, Serratosa, Merida, Zea, & Moyano, 2014), which agreed with the minor changes obtained by **still** treatment. The sample for **oxidation** treatment was agitated in a closed container with a higher headspace volume than the SPME vial for the **still** treatment. On the one hand, the phase ratio is an important parameter that could affect the volatile release in proposed mathematical models for predicting headspace composition (Athès, Peña y Lillo, Bernard, Pérez-Correa, & Souchon, 2004; Tromelin, Ayed, Lubbers, Pages-Helary, Andriot, & Guichard, 2012). On the other hand, the continuous shaking could promote mass transfer to the headspace, as reviewed by Taylor, this

shaking effect contributes to an increase surface regeneration, but the final effect was also determined by physicochemical characteristics of volatile compounds themselves (Taylor, 2002). Therefore, the **oxidation** treatment promoted the mass transfer of the aforementioned branched esters, resulting in their slight decay after 30 min, which was far less than equilibration time. However, these subtle changes revealed a noncritical effect of oxidation considering the dramatic changes of the most volatile compound after the **evaporation** treatment.

Another concern was the liberation of less volatile compounds to the headspace, meanwhile the rare retention of more volatile compounds in the liquid phase after the long-time **degasification** treatment. Regarding their longtime exposure to the ambient conditions, it is worth mentioning the ethanol loss causing by evaporation. Although we did not check the ethanol content after each treatment, a significant decrease of ethanol content was recently reported in an uncovered wine glass after 2 hours (Wollan, Pham, & Wilkinson, 2016), which was very similar to **degasification** treatments applied in our study. As a major component in wine matrix, ethanol plays a very important role in both the stability of nonvolatile components and the volatility of volatile compounds. The ethanol evaporation could enhance the mass transfer of volatile compounds due to the Marangoni effect under nonequilibrium conditions (Tsachaki, Gady, Kalopesas, Linforth, Athes, Marin, et al., 2008). Research also has indicated the potential of ethanol to change the solubility by altering the water-ethanol structure at different content level (Ickes & Cadwallader, 2017). Specifically, ethanol effect has been considered as an important factor that could modify

the conformation of other nonvolatile matrix compounds in wine, such as protein, resulting in changing the volatile-matrix interaction and further affecting the headspace composition (Druaux, Lubbers, Charpentier, & Voilley, 1995; Voilley & Lubbers, 1998). Therefore, we hypothesize that the liberation of the less volatile compounds was promoted by the combined evaporation of the most concentrated volatile compounds and ethanol or the changes of some oxygen-sensitive matrix compounds due to air dissolving. With this consideration, exclusive treatments with long-time shaking under both air and argon atmosphere were applied as described in Session 2 of Section 2.5.

One-way ANOVA was carried out to access the difference of treatments in Session 2. The results shown in Table 3 are mostly in accordance with the data in Table 1 for Session 1. However, fatty acid esters from evaporation category were released in general in a larger proportion compared with the previous **oxidation** treatment. These esters significantly decreased in both CAr and CAir due to the longer shaking duration (30 vs 150 min). Remarkably a 65% decrease was observed for ethyl decanoate, but only subtle changes were observed for these compounds after **oxidation** treatment (shown in Fig. 1). Notably, we found no significant differences between CAr and CAir, neither OAr and OAir, although the two pairwise-treatments were carried out under both air and oxygen-free atmosphere. For the most oxygen-sensitive volatile sulfur compounds, methanethiol and dimethyl sulfide, the decreases were relatively more under air condition in both closed and open systems, but the differences were not significant (Table 3). However, the decrease of compounds in the evaporation category was much larger

in the open systems than in the closed ones (Table 3). As reported in a study of the ethanol change in different wine glasses, the evaporation of ethanol was lower in an uncovered glass (Wollan, Pham, & Wilkinson, 2016). Accordingly, the aforementioned solvating effect of ethanol could partly explain the difference under covered or uncovered conditions (Villamor & Ross, 2013). As shown in Table 3, the increase of volatiles in the liberation category was relatively larger in the open system. This finding could support our hypothesis that the effect of evaporation could alter the bond potential between nonvolatile components and this group of compounds due to the different extent of matrix change under closed and unclosed condition. Boothroyd and his coworkers reported a salting-out effect of volatiles, such as furfural, by decreasing ethanol content in whiskey models, and in the same study, they also discussed the mechanism of long-chain esters affecting the release of particular volatile compounds (Boothroyd, Linforth, & Cook, 2012). Although wine matrix is far more complex than whiskey, their findings are useful for us. Surprisingly, OAr compounds from the liberation category have a larger increase than after OAir, which was possibly due to the intermittently gas flushing for maintaining the low oxygen content in the glove chamber. So far, our obtained results demonstrate that oxygen has a limited effect on aroma release during wine tasting period, and that evaporation plays a more important role to the release behavior of compounds in both evaporation and liberation category.

Table 3. The headspace changes of volatile compounds compared with control (shown in percentage) after each treatment and their defined release pattern.

Compound	Control (%)	CAr (%)	CAir (%)	OAir (%)	OAr (%)
Liberation+evaporation					
Sulfur dioxide	0 a	-9 b	-15 b	-63 c	-57 c
Benzaldehyde	0 a	-17 a	-13 a	-45 b	-50 b
Evaporation					
Methanethiol	0 a	-41 b	-48 b	-100 c	-100 c
Dimethyl sulfide	0 a	-33 b	-36 b	-100 c	-100 c
Ethyl acetate	0 a	-6 b	-5 b	-99 c	-100 c
Ethyl butyrate	0 a	-11 b	-7 b	-100 c	-100 c
Ethyl hexanoate	0 a	-20 b	-18 b	-100 c	-100 c
Ethyl octanoate	0 a	-35 b	-40 b	-100 c	-100 c
Ethyl decanoate	0 a	-61 b	-65 b	-100 c	-100 c
Ethyl isobutyrate	0 a	-12 b	-11 b	-100 c	-100 c
Ethyl 2-methylbutyrate	0 a	-15 b	-12 b	-100 c	-100 c
Ethyl 3-methylbutyrate	0 a	-13 b	-8 b	-100 c	-100 c
Propyl acetate	0 a	-4 b	-5 b	-100 c	-100 c
Isobutyl acetate	0 a	-12 b	-10 b	-100 c	-100 c
Isoamyl acetate	0 a	-25 b	-22 b	-100 c	-100 c
Liberation					
Ethyl lactate	0 b	4 b	5 ab	16 a	16 a
Ethyl succinate	0 b	1 b	1 b	22 a	25 a
2-Phenylethanol	0 b	-5 b	-10 b	20 a	26 a
Ethanoic acid	0 ab	-12 ab	-16 b	-2 ab	18 a
Butyric acid	0 a	10 a	10 a	6 a	-3 a
Acetaldehyde	0 b	-5 b	-17 b	132 a	64 ab
Diacetyl	0 a	-5 a	-3 a	9 a	12 a
2,3-Pentanedione	0 bc	-6 c	-1 bc	15 ab	29 a
Acetoin	0 c	-2 c	-5 c	11 b	24 a
Furfural	0 bc	-16 c	-11 c	13 ab	24 a
Butyrolactone	0 ab	-11 bc	-15 c	5 a	13 a
trans-Whisky lactone	0 c	-19 cd	-21 d	19 b	50 a
4-Ethylphenol	0 b	-1 b	-3 b	25 a	34 a

Compound	Control (%)	CAr (%)	CAir (%)	OAir (%)	OAr (%)
Immutable					
Isobutanol	0 a	6 a	6 a	-10 b	-18 c
1-Butanol	0 a	2 a	1 a	-11 b	-11 b
Isoamyl alcohol	0 a	2 a	4 a	-18 b	-23 b
cis-3-Hexenol	0 ab	5 ab	8 a	-1 b	-12 c
2-Phenethyl acetate	0	-8	-7	1	3
Guaiacol	0	-1	-13	19	15

Significant differences are calculated by ANOVA. Different letters mean significantly different according to post-hoc test.

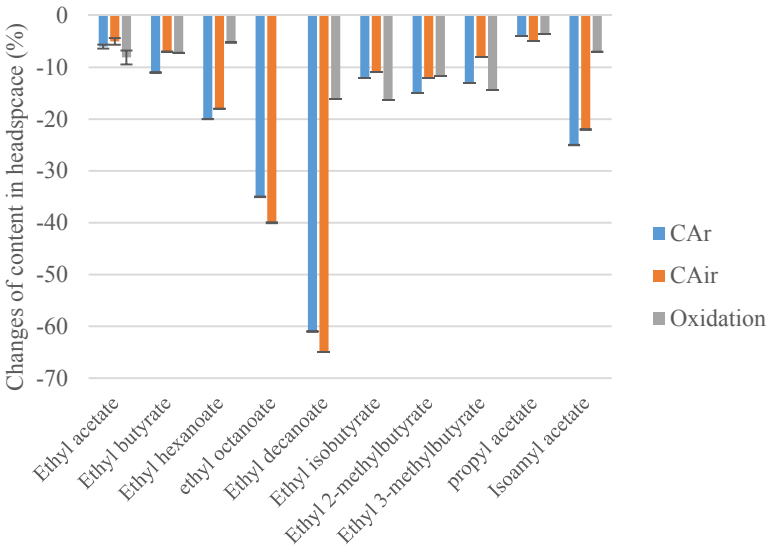

Fig. 1. Changes of headspace content of fatty acid esters after treatments in closed container. Data are normalized against the relative area of corresponding control for different sessions. Error bars indicate the standard error for triplicates.

3.3. The effect of volatile compounds' physicochemical properties

Considering the time-consuming character of real wine tasting conditions, data from the **evaporation** treatment were selected to

study the influence of compounds' physicochemical characteristics on their release behavior. The obtained relative areas were normalized against their corresponding level in control to eliminate the influence of different initial concentration. As shown in Fig. 2, the scatter plot of all the detected compounds against log P shows an unclear correlative relationship between compounds' change and log P. However, it is worth noticing the possible relative correlation between log P and compounds from different categories, especially for compounds in the evaporation category as circled in Fig. 2. The correlation tests based on Pearson's r were applied to study the relationship between compounds' physicochemical characteristics and release behavior. As shown in Table 4, strong correlations existed between compounds from evaporation and liberation categories with their corresponding log P or vapor pressure, but volatile sulfur compounds and carbonyl compounds are excluded. Within ethyl ester homologous series with straight carbon chain, the release amount was significantly correlated with both log P and vapor pressure, with correlation coefficients (r) of -0.723 and 0.909, respectively (Table 4). The amount of a given fatty acid ester that remained in the headspace was inversely correlated with its log P, that is to say, the more apolar of these esters disappeared faster than those less apolar from the same family. This correlative relationship was consistent with the reported highest release rate of ethyl decanoate in Chapter 2 and it seems logical from a solubility point of view. Notably, the scatter plot in Fig. 2 shows that the branched ester isobutyl acetate decreased more than ethyl butyrate, although the latter one has a higher log P value. The decrease of isoamyl acetate and ethyl 2(3)-methyl butyrate also suggested an unexpected

difference that the acetates decay slightly faster than ethyl esters with the same log P (Fig. 2). Our finding on different release behavior of esters is in agreement with the observation of Lytra et al. (2016), indicating that the structure of volatile compounds also plays an important role on aroma release. The positive correlation between volatility reflected by vapor pressure and headspace change of fatty acid esters also could explain the increasing release rate with the increasing effect of molecular size in Chapter 2. For compounds categorized as liberation, their release changes also significantly correlated to log P and vapor pressure (Table 4), although this was a category of compounds from various chemical classes. For fusel alcohols categorized as immutable, a negative correlation was observed with log P, but it was not significant at the 95% confidence level. However, in the cases of the most hydrophilic volatile sulfur compounds and carbonyl compounds, correlation simply with log P or vapor pressure was not obtained, suggesting a more complicated interaction with wine matrix.

Table 4. Relevant correlations of headspace changes for compounds from different categories against Log P and vapor pressure.

	Log P		vapor pressure	
	r	p	r	p
Category evaporation [a]	-0.723	0.018	0.909	0.000
Category liberation [b]	-0.465	0.000	0.917	0.000
Category immutable	-0.804	0.101	0.487	0.405

[a], compounds methanethiol and dimethyl sulfide are excluded; [b], compounds acetaldehyde and diacetyl are excluded.

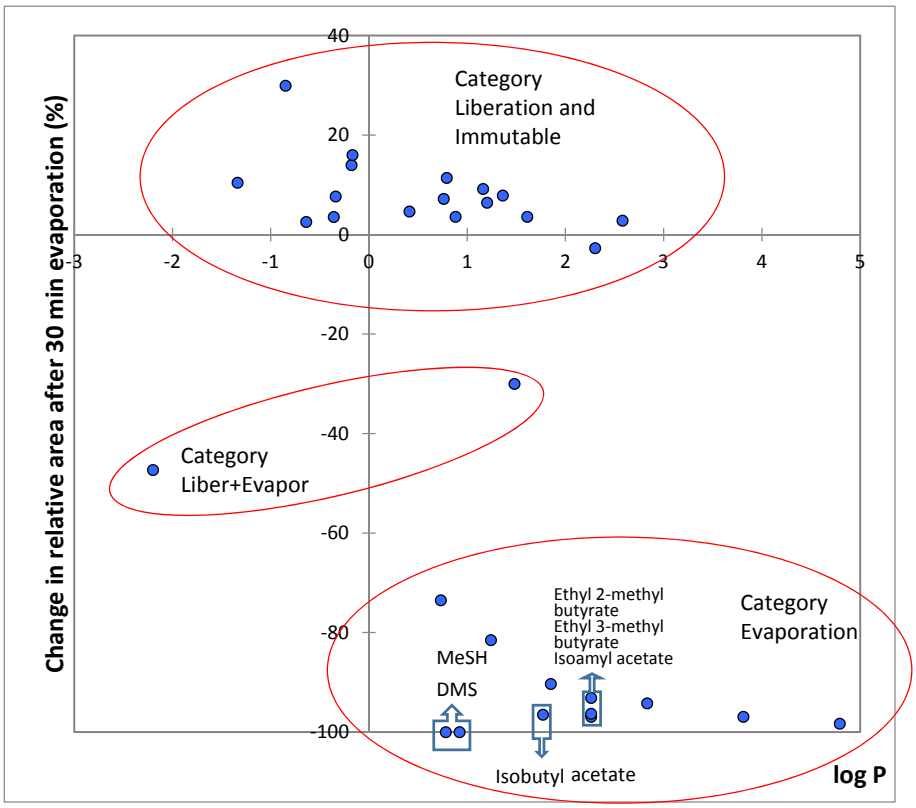

Fig. 2. Scatter plot of changes in headspace content between control and **evaporation** treatment in Session 1 against log P.

As a result, the hydrophobicity of volatile compounds could not precisely predict their change trend in the headspace over exposure time, even for the homologous esters with straight carbon chain. As we found in Chapter 2, the decay trend of ethyl decanoate showed a wine-independent character. Studies on other beverages also reported the different sensitivity of odorants to matrix composition due to their chemical structure (Ayed, Lubbers, Andriot, Merabtine, Guichard, & Tromelin, 2014; Boothroyd, Linforth, & Cook, 2012). The findings reveal the complex chemical mechanism of aroma release from wine matrix and a demand for more studies.

3.4. The evolution of headspace composition of two wines

As a preliminary step to achieve conditions more similar to real wine tasting, the experiment of Session 3 with different periods of evaporation was carried out (see Section 2.4). With these conditions, we could obtain the evolution of volatile compounds in headspace under nonequilibrium conditions over a period closer to wine tasting. A Crianza red wine and a white wine were selected due to their differences in wine components in volatile and nonvolatile compounds. A few compounds were not detected in the headspace of the white wine, such as *trans*-whiskylactone and 4-ethyl phenol due to their absence on this style of wine. Otherwise, the low concentration of methanethiol and dimethyl sulfide resulted in very high variability of instrumental response in these particular wines, thus these two compounds were excluded in the present study. The relative areas of detected analytes (against methyl 2-methylbutyrate) were calculated to reflect the headspace composition of the selected white and red wine at different time points. Then data were processed by 2-way ANOVA (Table 5) to assess the significance of the factor of wine, evaporation time and their interaction. The evolutionary trends (Fig. 3) are obtained by using the normalized relative area against the 0 min sample for each volatile compound in each wine to avoid the effect of initial concentration.

Table 5. Results of 2-way ANOVA for the headspace composition after different evaporation time of two wines. Significant difference ($p<0.05$) are marked in bold.

Compound	Wine (p)	Evaporation time (p)	Interaction (p)
Sulfur dioxide	< **0.0001**	< **0.0001**	0.372
Ethyl acetate	< **0.0001**	< **0.0001**	0.568
Ethyl butyrate	< **0.0001**	< **0.0001**	< **0.0001**
Ethyl hexanoate	< **0.0001**	< **0.0001**	< **0.0001**
Ethyl octanoate	< **0.0001**	< **0.0001**	< **0.0001**
Ethyl decanoate	< **0.0001**	< **0.0001**	< **0.0001**
Ethyl isobutyrate	**0.036**	< **0.0001**	0.923
Ethyl 2-methylbutyrate	0.164	< **0.0001**	0.302
Ethyl 3-methylbutyrate	0.410	< **0.0001**	0.595
Propyl acetate	0.237	< **0.0001**	0.322
Isobutyl acetate	0.970	< **0.0001**	**0.024**
Isoamyl acetate	< **0.0001**	< **0.0001**	< **0.0001**
Phenethyl acetate	< **0.0001**	0.340	0.465
Ethyl lactate	< **0.0001**	0.506	0.499
Diethylsuccinate	< **0.0001**	0.247	0.383
Isobutanol	< **0.0001**	**0.048**	0.239
1-Butanol	< **0.0001**	0.163	0.214
Isoamyl alcohol	< **0.0001**	0.081	0.114
2-Phenylethanol	< **0.0001**	< **0.0001**	**0.001**
Ethanoic acid	< **0.0001**	0.304	0.079
Butyric acid	< **0.0001**	0.723	0.266
Acetaldehyde	< **0.0001**	**0.000**	0.085
Diacetyl	< 0.0001	0.025	0.025
2,3-Pentanedione	< 0.0001	0.015	0.015
Acetoin	< **0.0001**	0.136	0.120
Furfural	< **0.0001**	0.050	0.229
Benzaldehyde	< **0.0001**	0.117	0.615
γ-Butyrylactone	< **0.0001**	0.408	0.408
Guaiacol	**0.040**	0.405	0.526
t-Whiskeylactone		0.054	
4-Ethylphenol		0.335	

The results in Table 5 highly agree with our previous observations in Section 3.1, while Fig. 3 illustrates the evolutional trends of particular compounds as representative examples. As shown in Fig. 3, the headspace contents of fatty acid esters categorized as evaporation were significantly affected by evaporation time following a decaying trend in both white and red wines. Regarding those compounds from the immutable category, their contents in the headspace maintained at constant levels at each time point. For other compounds regarding liberation category, the applied 30-min exposure was not enough to favor their release as discussed before. Therefore, most compounds from this category were steadily released to the headspace over the tested period. For instance, 4-ethylphenol in red wine (Fig. 3J) slightly increased the release to headspace but not significantly. However, 2-phenylethanol in the same category was significantly influenced by the time and wine factor (Table 5), showing a decrease after 2 min (Fig. 3I) more notable on the red wine. The content of most volatile compounds in the headspace showed a wine-dependent character, except four branched esters (Table 5). Otherwise, the significant difference between 0 min and the first time point (as marked in Fig. 3) suggested that the headspace composition after 2-min exposure was very close to the one of initial 0 min in both wines, only ethyl decanoate and 2-phenyl ethanol showed significant decreases. In addition, the effect of interaction between wine and time factors on particular compounds (7 esters and 2-phenylethanol, Table 5) indicated that the strength of wine impact for these compounds might alter due to the different extent of evaporation at different time points. However, it is hard to explain this phenomenon just by the difference

of volatile compound' concentration in the liquid phase or the physicochemical characteristics of volatile compound itself, since the selected wines also differed in matrix components, which could relatively affect the release behavior of volatiles by possible interactions (Taylor, 1998; Villamor & Ross, 2013). With this consideration, the representative evolutionary trends of typical volatile compounds from each category (Fig. 3) will be further discussed.

For fatty acid esters from evaporation category, Fig. 3A to D illustrate their constant decay trend over time, which is consistent with the observations in Chapter 2 and the study of Lytra et al. (2016). As discussed above, the hydrophobicity and the structure could affect release behavior among the ethyl ester homologous series. Here in the cases of ethyl butyrate and ethyl decanoate (Fig. 3A and B), the decay rate increased with the effect of increasing log P in both red and white wines. However, the similar impact of hydrophobicity was not observed for branched esters ethyl isobutyrate and ethyl 3-methylbutyrate (Fig. 3C and D), indicating compound's chemical structure influenced its release behavior. It is worth noticing that after 30min evaporation, the white wine could release proportionally much fewer esters than the red wine (as shown in Fig. 3A to D), supporting our hypothesis that wine components' modification could occur due to evaporation. Notably, the content of ethyl decanoate in red wine was slightly higher after 30 min, which was in agreement to its wine-dependent decay trend we noticed in Chapter 2. Moreover, Boothroyd and coworkers (2012) also observed a particular release trend of ethyl decanoate in whiskey models, showing that this long-chain ester was more sensitive to

matrix modification due to its physicochemical properties. Regarding the selected 2-phenethyl acetate and isoamyl alcohol from immutable category (Fig. 3E and F), the contents remained constant in the headspace without any significant change during the studied period, although an increasing trend was observed in red wine after 10-min evaporation. The evolutionary trends were more complicated for compounds with liberation potential, due to their complex interaction with wine components as reported in the literature (Bueno, Carrascón, & Ferreira, 2016; Ferreira, Franco-Luesma, Vela, López, & Hernández-Orte, 2018; Muñoz-González, Rodríguez-Bencomo, Moreno-Arribas, & Pozo-Bayón, 2011). As shown in Fig. 3G and H, pairwise evolutions of acetaldehyde and sulfur dioxide are opposite. It is reasonable to relate their behavior with changes in the proportion of bound forms of these compounds due to the evaporation of sulfur dioxide (Bueno, Carrascón, & Ferreira, 2016), especially under the applied nonequilibrium condition. However, it is more difficult to explain the fluctuant evolution of 2-phenylethanol in the red wine (Fig. 3I), specifically for its significant decrease after 2-min evaporation, although it showed the liberation potential with longer evaporation. Since the change in white wine was more consistent with our expectation, we hypothesize that this abnormal behavior in red wine is the result of the strong aroma-phenolic interactions between this compound and matrix component (Jung, de Ropp, & Ebeler, 2000), which will be further studied in the following chapter.

4. Conclusions

The application of our DHS method satisfactorily allows taking snapshots of wine headspace composition. The results obtained from this study highlight the dominant effect of continuous evaporation on aroma release during wine tasting, and thus point out the importance of studying aroma release under nonequilibrium conditions with exposure to ambient conditions. Other factors occurring during tasting could affect mass transfer of volatile compounds, such as shaking. However, oxidation due to gas dissolving only has a limited influence on the release of volatile sulfur compounds, which are oxygen-sensitive because of their particular functional group. Although we could categorize volatile compounds into different groups due to their release behavior, the mechanism of mass transfer is still unclear in such a complicated multi-component system as wine, due to the complex interactions between volatile compounds and matrix component, which are highly related to the physicochemical characteristics of a particular volatile. So far, we have validated that hydrophobicity and chemical structure could affect the release behavior of volatile compounds, especially, the release of homologous ester series is highly correlated to hydrophobicity. Otherwise, the evolutionary trend of a volatile compound is also related to its physicochemical properties and matrix component. Consequently, the headspace composition would change dynamically regarding the content of each volatile compound in the headspace over time, resulting in the temporal change of aroma profile of the wine.

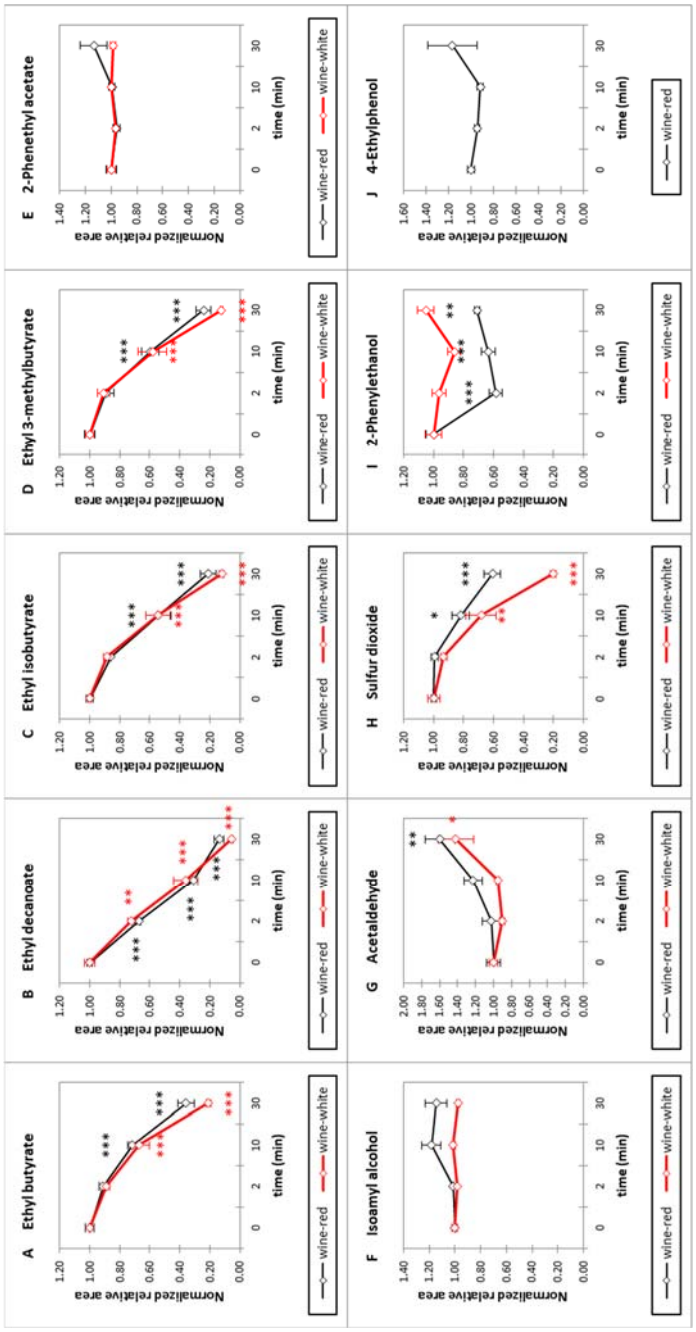

Fig. 3. Evolution of compounds over 30 min. Data are normalized against the relative area of 0 min. Significant difference between 0 min and other time points are marked: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (red for white wine, black for red wine). Error bar represents standard error for triplicates.

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CHAPTER 4

The Effect of Wine Matrix on the Initial Release of Volatile Compounds and Their Evolution in the Headspace

CHAPTER 4

The effect of wine matrix on the initial release of volatile compounds and their evolution in the headspace

1. Introduction

We have seen in previous chapters how the composition of the headspace of wine changes with time and how the change is influenced by factors like agitation, oxygenation or nonequilibrium conditions in general. Also in chapters 1 and 2, we observed that the release profile of some volatile compounds is affected not only by the factors mentioned above but also by the type of wine in which they are present. There is evidence in the literature that non-volatile wine matrix can modify the release and therefore the perception of the compounds involved in wine aroma. In this topic, many authors have work with model solutions, but only a few have work with real wines (Rodríguez-Bencomo, Muñoz-González, Andújar-Ortiz, Martín-Álvarez, Moreno-Arribas, & Pozo-Bayón, 2011; Saenz-Navajas, Campo, Cullere, Fernandez-Zurbano, Valentin, & Ferreira, 2010; Zapata, Lopez, Herrero, & Ferreira, 2012). Saenz-Navajas et al. (2010) worked interchanging the volatile and non-volatile fractions of six different wines. Shockingly, they found that a red wine matrix was able to make that a white wine aroma fraction smell like red wine and vice-versa. Rodríguez-Bencomo et al. (2011) worked with the deodorized non-volatile matrix from five different wines to which they added volatile compounds in a range of concentrations. Comparing with a control wine without non-volatile components, they observed retention effects for some compounds

that were more evident in a reconstituted sparkling wine. They also found a salting-out effect in aged-red and sweet wines for more polar or very volatile compounds. Those data were obtained with sensory analysis and GC-O (Saenz-Navajas, Campo, Cullere, Fernandez-Zurbano, Valentin, & Ferreira, 2010), or with HS-SPME-GC-MS with a very short (1 min) fiber exposure time (Jung & Ebeler, 2003). In the present chapter, we want to expand from the foundations of these works by applying the previously developed DHS-TD-GC-MS method for a better and more objective understanding of the headspace profile changes induced in the volatile profiles. The non-volatile matrix from six different wines is used to reconstitute such wines with a standard aroma solution. The headspaces of the reconstituted wines and a synthetic wine are analyzed and compared at different times, simulating a just-poured glass of wine and one that has been in the glass for 10 min. Therefore, the aim of the present study is, by using this reconstruction strategy, to assess the influence of the non-volatile matrix of wine on its aroma profile and time evolution.

2. Materials and methods

2.1. Reagents and chemicals

Ethanol and dichloromethane were supplied by Merck (Darmstadt, Germany), tartaric acid 99% was from Panreac (Barcelona, Spain). The internal standards (methyl 2-methylbutyrate, 2,6-dichloroanisole) were obtained from Sigma-Aldrich (Madrid, Spain). The standard compounds listed in Table 1 with purity above 98% in all cases were purchased from Sigma-Aldrich (Steinheim,

Germany), Fluka (Buchs, Switzerland) and Panreac (Barcelona, Spain). These aroma compounds were prepared as the aroma mixture at concentration ranges detected in Spanish commercial wines (San-Juan, Cacho, Ferreira, & Escudero, 2012) for wine reconstitutions (Table 1).

2.2. Wine samples

To obtain non-volatile wine matrices with distinct characteristics, a set of six Spanish wines with different wine-making styles were selected and all but one were commercially available. They were a 2-year-old Macabeo wine fermented in stainless steel vats; a 2-year-old Chardonnay wine fermented in an oak barrel; a one-year-old Garnacha red wine with light body from D.O. Cariñena; a five-year-old Tempranillo Reserva red wine from D.O. Ribera del Duero (24 months in oak barrel); a six-year-old Tempranillo Reserva red wine from D.O.C. La Rioja (12 months in oak barrel). A non-commercial red wine that was produced from the juice obtained by pressing grape skins and stems was specially selected due to its intense astringency. A synthetic wine was also prepared (containing 12% vol of ethanol, 5 g/L of tartaric acid, adjusted to pH 3.4 with 1 M NaOH).

Table 1. Aroma mixture concentrations in the wine reconstitution.

Compound	Log P	Concentration (mg/L)	Aroma vector
Dimethyl sulfide	0.92	0.050	Spice-woody
Ethyl acetate	0.73	28	Alcoholic-solvent
Ethyl butyrate	1.85	0.85	Fruity
Ethyl hexanoate	1.92	0.79	Fruity
Ethyl decanoate	4.79	1.0	Fruity
Linalool	2.97	0.73	Flowery
Isoamyl alcohol	1.16	239	Alcoholic-solvent
β-Phenylethanol	1.36	50	Alcoholic-solvent
Acetoin	-0.36	0.99	Lactic-acid
β-Damascenone	4.21	0.53	Fruity
Butyric acid	0.79	0.91	Lactic-acid
Hexanoic acid	1.92	3.7	Lactic-acid
trans-Whiskylactone	2.00	0.84	Spice-woody
4-Ethylphenol	2.58	0.89	Animal-leather
Vanillin	1.21	0.89	Spice-woody

2.3. The analysis of dynamic headspace compositions

The validated DHS-TD-GC-MS method was applied to analyze the composition of headspace (Chapter 1). Five mL of the sample was transferred into a 20 mL standard headspace vial and then internal standards solution was added to reach 200 µg/L concentration level. Thermal desorption and cryo-focusing were carried out as described before. Gas chromatography-mass spectrometry analysis was performed with a 7890 Agilent GC system coupled with a 5975C Agilent quadrupole mass spectrometer

(Santa Clara, CA, USA). The temperature program was the same mentioned in previous experiments. The chromatograms were collected in both full scan and SIM mode for some particular compounds (see Chapter 1).

2.4. Wine reconstitution

The nonvolatile matrices were prepared as described in the previous works of our laboratory with slight modifications (de-la-Fuente-Blanco, Saenz-Navajas, & Ferreira, 2016; Saenz-Navajas, Campo, Cullere, Fernandez-Zurbano, Valentin, & Ferreira, 2010). Briefly, 250 mL sample of each wine was poured into a 500 mL rounded flask to remove ethanol and major volatile compounds by a rotary evaporator at 24 °C for 30 min. The distilled sample was frozen at -20 °C and then was further lyophilized by a LyoQuest 85 freeze dryer system (Telstar, Tarrasa, Spain). The resulting syrup was re-dissolved in 20 mL of hydro-alcoholic solution (12% vol of ethanol). Finally, three successive liquid-liquid extractions by dichloromethane were applied to achieve complete elimination of all odor compounds. After removing the remained solvent by the rotary evaporator, the obtained liquid was re-dissolved in 250 mL of synthetic wine to its initial volume, then the blank was analyzed by the aforementioned DHS-TD-GC-MS method to estimate the volatile elimination efficiency. Each of the deodorized wines was spiked with a known amount of aroma mixture (shown in Table 1) to reconstitute six wine models that contained the same aroma compounds but distinct matrix properties. A synthetic wine model was also prepared in the same way.

2.5. The chemical composition of wine matrices

Free and total sulfur dioxide of each reconstituted wine was determined by the Rankine method recommended by OIV (International Organization of Vine and Wine) (OIV-MA-AS323, 2009). Total polyphenol index (TPI) was estimated as absorbance at 280 nm by a UV-VIS spectrophotometer UV-17000 Pharma Spec (Shimadzu, Japan) (Ribéreau-Gayon, Dubourdieu, Donèche, & Lonvaud, 2006). Total acidity was measured by titration with 0.1 M NaOH. pH was determined with a pH meter. The non-volatile matrices were weighted after lyophilization. Copper, iron, manganese and zinc were analyzed by Inductively Coupled Plasma-Mass Spectrometry following the protocol described by Vela et al. (2017).

2.6. The effect of wine matrix on aroma release evolution

To study the interactions between volatile compounds and wine matrices during wine tasting period, two evaporation strategies were applied to each reconstituted model wine. Forty mL of each model wine sample was transferred into a 250 mL uncovered glass beaker, and then 5 mL of wine sample was collected from one of the beakers after pouring or after 10 min-period shaking. The aforementioned DHS method was applied, thus obtaining the headspace composition of the initial status (t=0 min) and under tasting (t=10 min). The analyses were triplicated for each model wine.

2.7. Data analysis

XLSTAT (Addinsoft) software was used for the statistical analysis. One-way ANOVA was performed to assess the influence of

the different wine matrices. Principal component analysis (PCA) was carried out to examine the relationship between wine matrix composition and volatile compounds in the headspace.

3. Results and discussion

In the present chapter, the interactions between volatile compounds and nonvolatile matrix were studied by standardizing the aroma composition of very distinct wines. With this purpose, volatile compounds in Table 1 were selected to reflect broad differences in physicochemical characteristics, and also they could represent typical aroma compounds from different categories as grouped in the previous chapter and various aroma vectors as recently reviewed by Ferreira et al. (in press). This "standard" aroma solution was added to the nonvolatile dearomatized matrices isolated from several wines, varying in typicality due to their different enological styles. The physicochemical characteristics of each real wine matrix were measured and presented in Table 2. These results showed remarkable differences in characteristics of nonvolatile composition among the studied wine models, which are supposed to affect release behavior of volatile compounds. Although ethanol concentration is variable in wine, during this experiment it was kept the same for all the reconstituted wine models and the synthetic wine to avoid the widely reported volatility variation of aroma compounds due to ethanol effect (Aznar, Tsachaki, Linforth, Ferreira, & Taylor, 2004; Boothroyd, Linforth, & Cook, 2012; Diako, McMahon, Mattinson, Evans, & Ross, 2016; Wollan, Pham, & Wilkinson, 2016).

In Chapter 3, we applied continuous shaking treatments with

different exposure time to study the headspace evolution of a red and a white wine. The data revealed that 2-min exposure produced a headspace snapshot very close to the initial state, while the apolar esters in wines were almost depleted after 30 min. For the sake of simplicity, in the present, the experiment only two points in time were taken: the initial 0-min (equivalent to just pouring the wine) and a 10-min exposure with agitation (equivalent to a situation in which the wine has been in the glass for a while).

3.1. The effect of different wine matrix composition on the initial release of volatile compounds

As it has been shown in previous chapters, time spent after wine pouring substantially modifies the headspace of many compounds. For that reason, we decided to split the data between the initial 0-min set and the 10-min set. The initial headspace responses for each compound and matrix are shown in Table 3. The results of a one-way ANOVA to assess the influence of wine matrix are also included in Table 3. Most compounds showed significant differences in their quantities released to the headspace depending on the reconstituted matrix. Only ethyl decanoate was not significant ($p=0.068$), likely due to higher than average variability on its determination.

Dimethyl sulfide release showed marked differences between wines. Commercial young wines (YR and YW) were the matrices where dimethyl sulfide release was lower, while reserva wines (CU and PE) and pressed wine (PR) matrices had the higher release (Fig. 1). It has been proved that sulfur compounds, especially H_2S and mercaptans, can form reversible cation-complexed forms (Franco-Luesma & Ferreira, 2014). Although dimethyl sulfide was not

complexed in that report, we can observe in this experiment that there is a correlation between the content of Cu on the matrix and the release of the added dimethyl sulfide to the headspace (Pearson coefficient = -0.816, p=0.025). YR and YW that have the highest content of Cu showed the lowest release of DMS to the headspace, pointing to an influence of Cu content on the release of DMS. There it was not significant correlation with the other cations analyzed in the matrices (Fe, Zn, Mn).

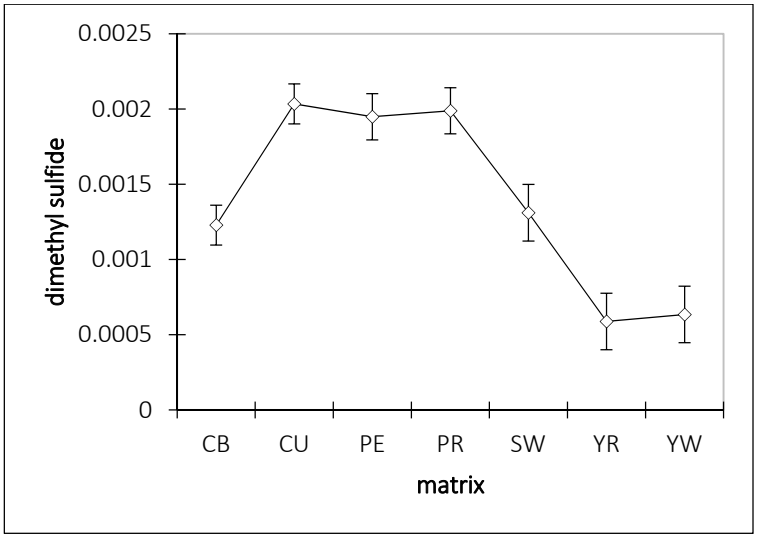

Fig. 1. Average relative areas for dimethyl sulfide at t=0 min.

The four ethyl esters added with the standard aroma solution showed a similar release trend within their group. Given their apolar nature and fast release from wine, it is difficult to observe significant differences between matrices for these compounds. Rodriguez-Bencomo et al. (2011) only observed differences in release for this chemical family in a reconstituted sparkling wine, while a white, young-red, aged-red and sweet wines did not differ from the

synthetic wine. We did not observe an increase in the headspace of ethyl esters as described by Saenz-Navajas et al. (2010). In our study, only PE appeared differentiated from the other matrices. In all cases, PE wine matrix produced the lowest release of the ethyl esters to the headspace, this was especially notable in the case ethyl acetate which a reduction of ca. 50% compared to the synthetic wine. It was not possible to find and explanation for this behavior based on the data from the chemical composition of the matrices.

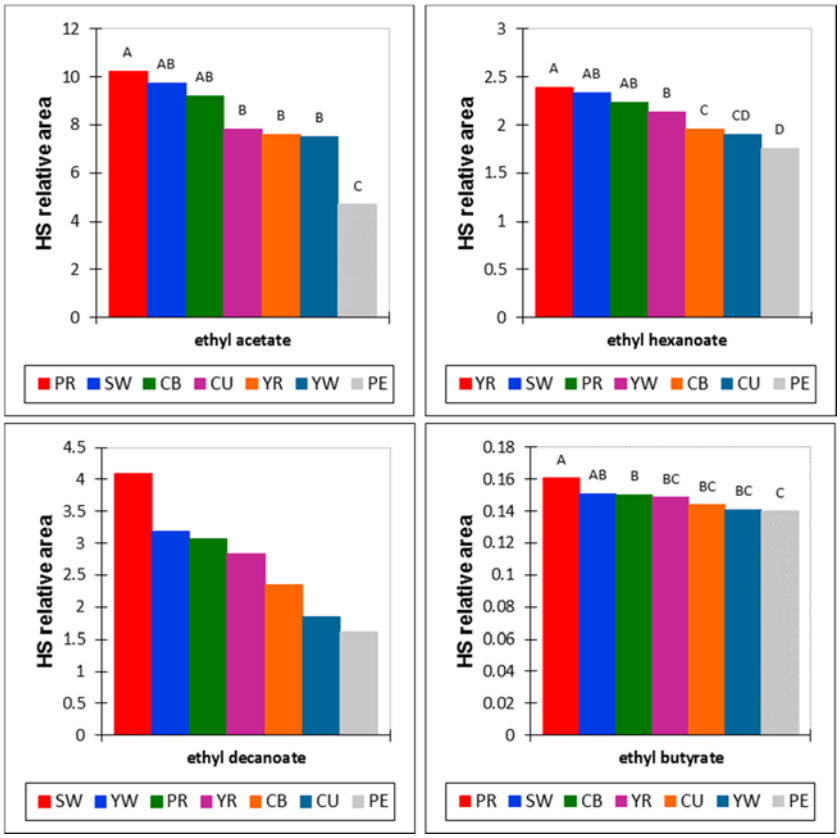

Fig. 2. Average relative areas in the HS for ethyl esters at t=0 min. A, B, C, D: Different letters indicate that the mean is significantly different among samples at $p < 0.05$ by Fisher's test after a significant one-way ANOVA.

Carbonyl compounds are highly reactive molecules that are known to interact with many molecules present in wines like amino acids, proteins or SO_2 among others (de Azevedo, Reis, Motta, Rocha, Silva, & de Andrade, 2007). Therefore, it was expected to observe marked differences across matrices. Such was the case for acetoin, where specially young-red (YR) wine showed a 2-fold increase in release compared to the synthetic wine (Fig. 3). While a percentage of the added carbonyl could be expected to be bonded to the free SO_2 in the matrices (Bueno, Carrascón, & Ferreira, 2016), it is, however, difficult to explain the large difference of YR with the synthetic wine given the absence of SO_2 in such solution. pH differences could account for a potential shifting of SO_2 equilibrium and different adduct's formation ratios, but it is unlikely given the short range of pHs in the matrices (from 3.10 to 3.72, see Table 2). It is possible that the differences of release between the synthetic wine and some of the reconstituted matrices could be due to a salting-out effect and not only to the presence of SO_2.

β-Damascenone also showed significant differences across matrices. In this case, aged-reds (CU, PE), press (PR) and barrel fermented white (CB) reconstituted wines released quantities of β-damascenone lower than the synthetic wine. Similar to the case of acetoin, β-damascenone is able to form adducts with SO_2 (Daniel, Elsey, Capone, Perkins, & Sefton, 2004). However, in our experiment it seems there is no correlation between the lower release of this compound and the presence of free SO_2, this is in agreement with the absence of SO_2 bound damascenone reported by Bueno et al. (2016). It can not be ruled out, that the binding of damascenone may be caused by other nucleophiles different from SO_2.

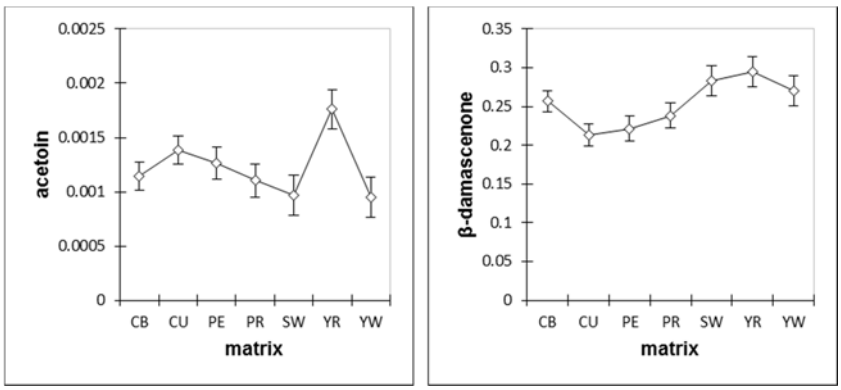

Fig. 3. HS average relative areas for carbonyl compounds at t=0 min.

Butyric and hexanoic acids were the two compounds where differences in release appeared more dramatic. They showed a similar profile across the reconstituted wines (see Fig. 4) with white (YW, CB) and young-red (YR) wines with higher concentrations of acids above their headspace. Only neutral forms of the acids can be released to the headspace. Therefore it seems reasonable to think that pH should modify the release of the acids through their correspondent acid-base equilibrium. A similar effect was observed by Saenz-Navajas et al. (2010) in white wines for volatile fatty acids. Although the not significant correlation was found between pH or total acidity with acids release, the wine with higher headspace concentration of butyric and hexanoic acids was the one with the lowest pH and highest total acidity (YR).

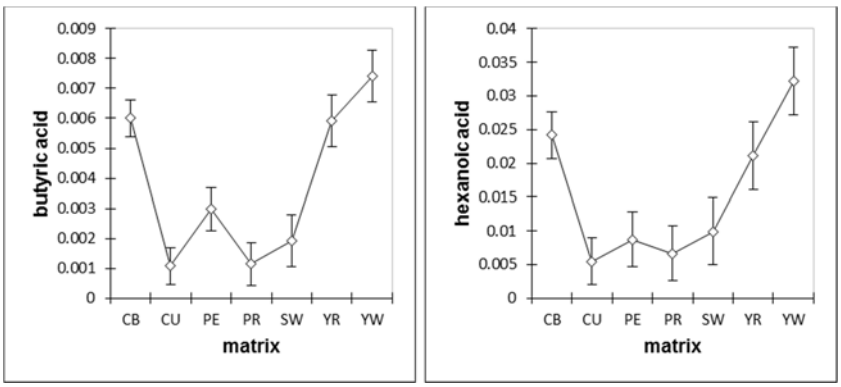

Fig. 4. HS average relative areas for acids at t=0 min.

The variation on the release of less apolar and heavier compounds, including linalool, isoamyl alcohol, 2-phenylethanol, t-whiskylactone, 4-ethylphenol and vanillin, is shown in Fig. 5. All compounds but isoamyl alcohol showed a pretty similar behavior: the retention of the compounds in the matrices was not very different from the synthetic wine. It is clear that this group of compounds was not affected as much as previous groups by the non-volatile components of the matrices. Only the young red wine (YR) showed a salting-out effect compared with the rest of matrices. A previous publication comparing the release of these compounds from different wine matrices found similar results for alcohols, although in the case of linalool it was reported an increase in retention that was not visible in our matrices (Rodríguez-Bencomo, Muñoz-González, Andújar-Ortiz, Martín-Álvarez, Moreno-Arribas, & Pozo-Bayón, 2011).

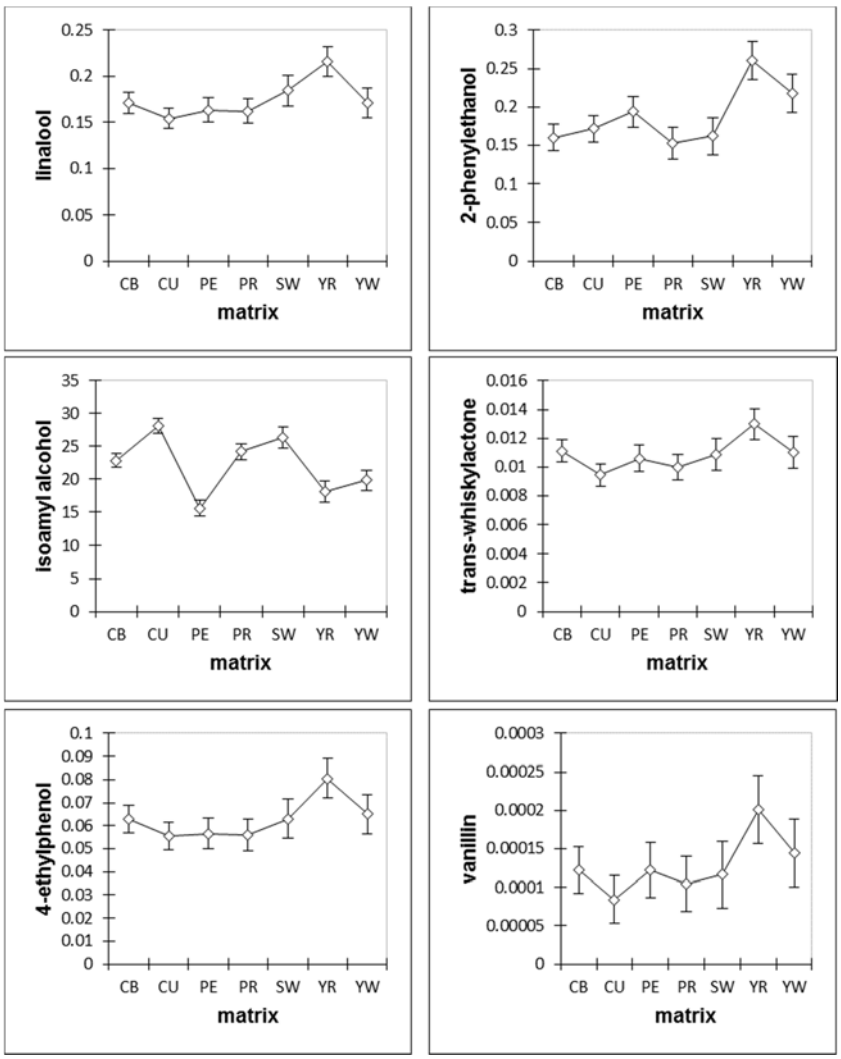

Fig. 5. HS average relative areas for polar and heavier compounds at t=0 min.

The study of the influence of non-volatile composition on headspace profile has shown strong evidence of differential behaviors, both increasing (salting-out) or decreasing the release of the volatiles. Even in a situation of no time for equilibration (t=0 min), it is possible to observe that different chemical families of volatiles are influenced depending on their physicochemical

properties and non-volatile wine composition.

3.2. The effect of different wine matrix composition on the release of volatile compounds after 10 min

We have seen in previous chapters that there is an evolution in the release profile above the headspace of wine, and that the evolution is influenced by factors like time, agitation and oxygen exposure. Also, we have seen in section 3.1 that wine matrix composition modifies the release of most of the volatile compounds. In the present section, we will analyze how these variations evolve after wines stay for 10 min in an open container. The headspace responses for each compound and matrix and the results of a one-way ANOVA to assess the influence of wine matrix are shown in Table 4. After 10 min of agitation, most compounds presented significant differences across matrices, although this time the number of nonsignificant compounds was larger than at t=0 min. Only ethyl decanoate at t=0 min, but β-damascenone, t-whiskylactone, 4-ethylphenol and vanillin at t=10 min were not affected by wine matrix.

As in the case of release at t=0 min, commercial young wines (YR and YW) were the matrices with lower headspace response for dimethyl sulfide (Fig. 6). Also, these were the only matrices which showed significant differences with the synthetic wine matrix (SW). It is not surprising given that after 10 min approximately 80% of the dimethyl sulfide had been lost (Table 4), as a consequence the response was close to the detection limit of the method. The correlation with Cu content was not significant in this case, likely due to the low response, but again the matrices with larger amounts

of Cu (Table 2) released less DMS to the headspace. None of the other metals analyzed were correlated with dimethyl sulfide release. As can be seen in Table 4, the decrease in dimethyl sulfide was fairly homogeneous across matrices, with variations ranging from -69% to -85% of the original compound in the headspace after 10 min.

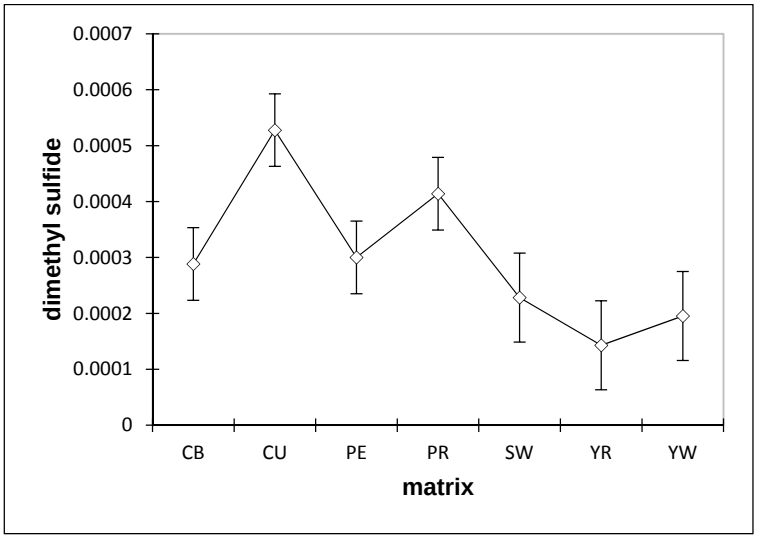

Fig. 6. Average relative areas for dimethyl sulfide at t=10 min.

Table 2. Chemical components of dearomatized wine matrices.

Wine matrix	Abbr.	Vintage (year)	Non-volatile residue (g)	Total SO2 (mg/L)	Free SO2 (mg/L)	pH	Total acidity (tartaric acid g/L)	TPI	Cu (µg/L)	Fe (µg/L)	Zn (µg/L)	Mn (µg/L)
Synthetic wine	SW	---	---	---	---	3.40	5.00	---	---	---	---	---
Young red wine	YR	1	9.15	30.40	22.93	3.55	4.80	56.50	542.10	1575.80	226.40	521.40
Young white wine	YW	2	6.45	129.60	31.05	3.10	5.97	10.34	517.40	1324.50	731.60	452.10
White wine (barrel fermentation)	CB	2	8.40	99.36	38.57	3.46	4.88	14.36	36.90	842.50	932.60	1096.10
Press wine	PR	1	9.87	17.60	8.63	3.67	4.99	77.20	34.20	551.20	670.20	908.60
Reserva Rioja	CU	6	9.90	40.00	22.15	3.47	4.58	51.90	35.50	1111.20	273.50	457.40
Reserva Ribera Duero	PE	5	10.7	25.60	6.83	3.72	4.91	64.00	98.20	703.30	252.10	603.30

Table 3. Volatile response in the headspace at t=0 min in each reconstituted wine matrix. Values expressed as average relative areas together with the standard deviation of the mean (s/√3). Codes are SW: synthetic wine, YR: young red, YW: young white, CB: barrel fermented white, PR: press wine, CU: Rioja reserva, PE: Ribera reserva.

Compound	SW	YW	CB	YR	PR	CU	PE
Dimethyl sulfide***	0.0013±0.0001b	0.0006±0.0001c	0.0012±0.0001c	0.0006±0.0001b	0.0020±0.0001a	0.0020±0.0001a	0.0019±0.0001a
Ethyl acetate**	9.74±0.68ab	7.52±1.88b	9.55±0.43b	7.62±1.66ab	10.24±0.18a	7.82±0.13b	4.68±0.25c
Ethyl butyrate**	0.151±0.001ab	0.141±0.004bc	0.150±0.004bc	0.149±0.008b	0.161±0.003a	0.144±0.002bc	0.140±0.002c
Ethyl hexanoate***	2.33±0.03ab	2.14±0.20a	1.96±0.04b	2.40±0.05c	2.24±0.04ab	1.90±0.01cd	1.76±0.01d
Ethyl decanoate	4.09±0.07	3.19±1.87	2.46±0.13	2.83±1.00	3.08±0.03	1.84±0.10	1.61±0.08
Acetoin***	0.0010±0.0001d	0.0009±0.0001d	0.0011±0.0001cd	0.0018±0.0001a	0.0011±0.0001cd	0.0014±0.0001b	0.0013±0.0001bc
β-Damascenone***	0.283±0.002a	0.271±0.028a	0.258±0.006ab	0.295±0.002bc	0.238±0.002cd	0.213±0.004e	0.222±0.002de
t-Whiskylactone**	0.0109±0.0001b	0.0110±0.0009a	0.0111±0.0003b	0.0130±0.0014b	0.0100±0.0001bc	0.0095±0.0001c	0.0106±0.0001bc
Linalool**	0.184±0.001b	0.171±0.008a	0.171±0.004bc	0.216±0.023bc	0.162±0.002c	0.154±0.003c	0.163±0.002c
Butyric acid***	0.0019±0.0001cd	0.0074±0.0001a	0.0060±0.0004b	0.0059±0.0001b	0.0011±0.0001d	0.0011±0.0002d	0.0030±0.0002c
Hexanoic acid***	0.0099±0.0003c	0.0322±0.0074a	0.0242±0.0010b	0.0211±0.0022b	0.0067±0.0005c	0.0055±0.0007c	0.0088±0.0004c
Isoamyl alcohol***	26.4±0.1a	19.9±0.8c	22.6±0.6c	18.1±0.4b	24.2±0.2b	28.1±0.8a	15.6±0.2d
2-Phenylethanol***	0.162±0.001d	0.218±0.014a	0.158±0.006b	0.261±0.031d	0.153±0.003d	0.171±0.007cd	0.194±0.002bc
4-Ethylphenol**	0.0630±0.0004b	0.0650±0.0048a	0.0625±0.0021b	0.0805±0.0120b	0.0561±0.0005b	0.0555±0.0005b	0.0567±0.0011b
Vanillin*	0.00012±0.00002bc	0.00014±0.00002a	0.00012±0.00001ab	0.00020±0.00005bc	0.00010±0.00001bc	0.00008±0.00001c	0.00012±0.00001bc

*Significant differences, ANOVA ($p \leq 0.05$); **Significant differences, ANOVA ($p \leq 0.005$); ***Significant differences, ANOVA ($p \leq 0.001$); a, b, c, d, e: Different letters indicate mean is significantly different among samples at $p < 0.05$ by Fisher's test after a significant one-way ANOVA.

Table 4. Volatile response in the headspace at t=10 min in each reconstituted wine matrix. Values expressed as average relative areas together with the standard deviation of the mean (s/√3). Codes are SW: synthetic wine, YR: young red, YW: young white, CB: barrel fermented white, PR: press wine, CU: Rioja reserva, PE: Ribera reserve.

Compound	SW	YR	YW	CB	PR	CU	PE
Dimethyl sulfide***	0.00023±0.00005cde	0.00014±0.00001e	0.00020±0.00003de	0.00029±0.00004cd	0.00041±0.00001b	0.00053±0.00004a	0.00030±0.00001c
Ethyl acetate*	3.57±0.27abc	4.85±0.64a	3.41±0.02bc	3.03±0.18c	4.20±0.17ab	4.10±0.62ab	2.71±0.08c
Ethyl butyrate***	0.0795±0.0012bc	0.0891±0.0006a	0.0855±0.0029ab	0.0781±0.0018c	0.0915±0.0004a	0.0863±0.0039ab	0.0725±0.0008c
Ethyl hexanoate***	1.13±0.06abc	1.13±0.02abc	1.11±0.02bc	1.04±0.00cd	1.23±0.02a	1.21±0.06ab	0.94±0.01d
Ethyl decanoate***	1.21±0.11cd	1.09±0.04d	1.22±0.10cd	1.28±0.02bc	1.41±0.02b	1.59±0.06a	0.72±0.04e
Acetoin***	0.0013±0.0002bc	0.0019±0.0001a	0.0011±0.0002bc	0.0011±0.0001bc	0.0010±0.0001c	0.0013±0.0000b	0.0012±0.0001bc
β-Damascenone	0.277±0.014	0.271±0.005	0.254±0.015	0.262±0.006	0.245±0.001	0.218±0.001	0.253±0.030
t-Whiskylactone	0.0128±0.0025	0.0109±0.0000	0.0107±0.0007	0.0105±0.0002	0.0104±0.0000	0.0095±0.0002	0.0097±0.0001
Linalool*	0.190±0.022a	0.177±0.003ab	0.161±0.011bc	0.165±0.003bc	0.161±0.001bc	0.147±0.002c	0.156±0.001bc
Butyric acid***	0.0016±0.0001d	0.0033±0.0005c	0.0045±0.0005b	0.0075±0.0006a	0.0002±0.0001e	0.0018±0.0005d	0.0023±0.0001cd
Hexanoic acid***	0.0068±0.0002e	0.0146±0.0015c	0.0195±0.0010b	0.0260±0.0012a	0.0057±0.0002e	0.0011±0.0006f	0.0082±0.0004d
Isoamyl alcohol***	25.0±0.1b	15.7±0.2d	19.0±0.8c	25.3±1.2b	21.1±0.2c	29.5±0.4a	26.0±0.8b
2-Phenylethanol**	0.184±0.002bcd	0.217±0.006a	0.201±0.013ab	0.167±0.010cde	0.159±0.003e	0.166±0.003de	0.189±0.000bc
4-Ethylphenol	0.0759±0.0147	0.0645±0.0002	0.0626±0.0047	0.0601±0.0006	0.0578±0.0006	0.0548±0.0005	0.0559±0.0003
Vanillin	0.00008±0.00004	0.00010±0.0000	0.00010±0.00001	0.00014±0.00004	0.00010±0.00001	0.00008±0.00002	0.00012±0.00001

*Significant differences, ANOVA ($p \leq 0.05$); **Significant differences, ANOVA ($p \leq 0.005$); ***Significant differences, ANOVA ($p \leq 0.001$); a, b, c, d, e, f: Different letters indicate mean is significantly different among samples at $p < 0.05$ by Fisher's test after a significant one-way ANOVA.

The ethyl esters headspace response was similar to that observed at t=0 min. *Reserva* wine from D.O. Ribera (PE) again provided the matrix with higher retention (less release) of ethyl esters (Table 3). When compared with the synthetic wine (SW) a salting out effect of the matrices was not evident. As reported by other authors (Mitropoulou, Hatzidimitriou, & Paraskevopoulou, 2011), tannin concentration has a weak influence on the release of ethyl esters. Such is the case in the wines studied here because there was no correlation between total polyphenol index (Table 2) and the response in the headspace. As expected from previous experiments, all ethyl esters showed a decrease in the response in the headspace after 10 min (Table 4) due to a significant depletion in the amount of compound in the liquid phase.

Among the compounds with carbonyl groups, only acetoin exhibited significant differences across matrices. Actually, the trend observed for acetoin at t=10 min (Fig. 7) was pretty similar to that observed a t=0 min (Fig. 3). Furthermore, it can be seen in Table 4 that except for the synthetic wine, the variations in headspace response were minimal and not significant between the two different times. These data are in agreement with the conclusions reached in chapter 2, where acetoin maintained a constant headspace concentration not affected by successive extractions. However, it should be considered that although acetoin is constant in the headspace, there is an influence of the matrix because, as at t=0 min, YR matrix showed a salting-out effect with a larger release to the headspace than the rest of wines.

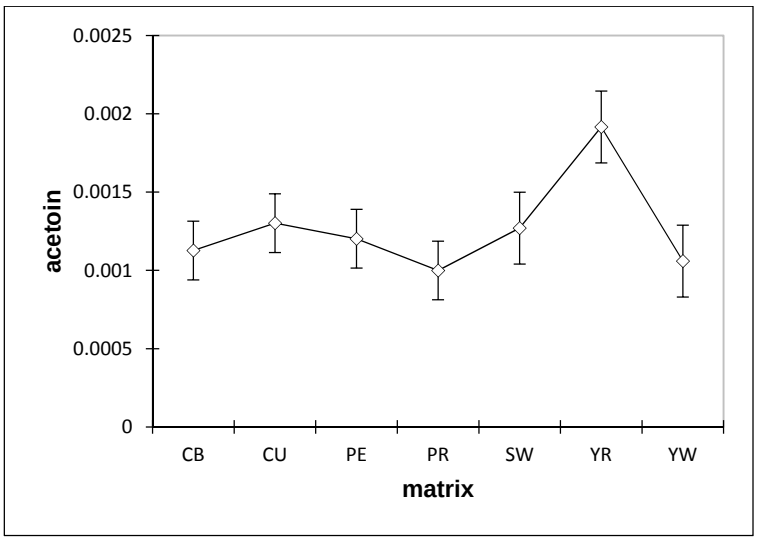

Fig. 7. HS average relative areas for acetoin at t=0 min.

Likewise, butyric and hexanoic acids exhibited the same differences among matrices observed at t=10 min than those at t=0 min (Fig. 8 and 4, respectively). The white wines matrices (YW, CB) presented a salting-out effect that could be explained by a lower pH and higher total acidity (Table 2) in the case of YW, but not in the case of CB. Rodriguez-Bencomo et al. (2011) found a similar effect for their white wines with octanoic acid in an experiment carried out with HS-SPME, although they did not find an explanation for this behavior. Regarding the changes between t=10 and t=0 min in the headspace response, it was not found a similar pattern. In most cases there it was a decreased of the acids in the headspace at t=10 min(Table 4), but there were several matrices with increases(CB and CU). Although quantitatively large, these increases were not significant from a statistical point of view.

In the group of heavier or more polar compounds, linalool, 2-phenylethanol and isoamyl alcohol exhibited different release profiles depending on the wine matrix. Linalool showed a similar profile at t=10 min to the one found at t=0 min (Fig.s 5 and 9). Synthetic wine matrix showed less retention power. Although significant, the difference of release was not very high in this case. Comparing within matrices a slight decrease in the headspace response was noted in all wines except the synthetic one.

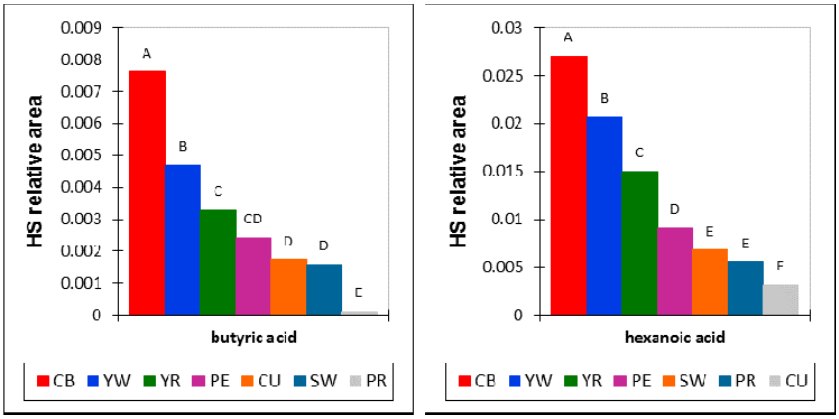

Fig. 8. HS average relative areas for acids at t=10 min.

Isoamyl alcohol response in the headspace was also similar at t=10 min, except for wine PE. There it was a very large increase at t=10 min (Fig. 9) in the headspace for this compound. This is very surprising given the previous findings of the constant headspace concentration of this compound. As in t=0 min, the young wines (YR, YW) presented a larger retention effect for isoamyl alcohol. At this point, we have no chemical explanation for this effect, but it is really an important finding from the point aroma. It has been proved that isoamyl alcohol (and also isobutanol) can suppress the fruity, lactic

and woody notes of wines (de-la-Fuente-Blanco, Saenz-Navajas, & Ferreira, 2016). If some wines like YR and YW can decrease almost a 50% the headspace concentration of these alcohols, it could change the perception of their aroma completely compared to what we would expect from just a chemical analysis of the liquid phase.

Finally, 2-phenylethanol was the last of the compounds that exhibited significant responses in the headspace depending on the reconstituted matrix. Fig. 5 and 9, for t=0 and t=10 min, respectively, of 2-phenylethanol are almost identical, as it was expected for a compound with constant headspace concentration (see chapter 2). Rodríguez-Bencomo et al. (2011) found a retention effect of 2-phenylethanol in sparkling wine, while Mitropoulou et al. (2011) found salting out effect only at concentrations of skin tannins of 10 g/L but not for the same concentration of seed tannins. In our measurements, 2-phenylethanol presented salting out effect for YR matrix and a retention effect for PR matrix (Fig. 9). In theory, given the origin of PR wine, it should have had a higher skin and seed tannin concentration. Unfortunately, the TPI analysis cannot differentiate between the two types of polyphenols and we could not assess if the registered salting out effect were related to the concentration of the tannins.

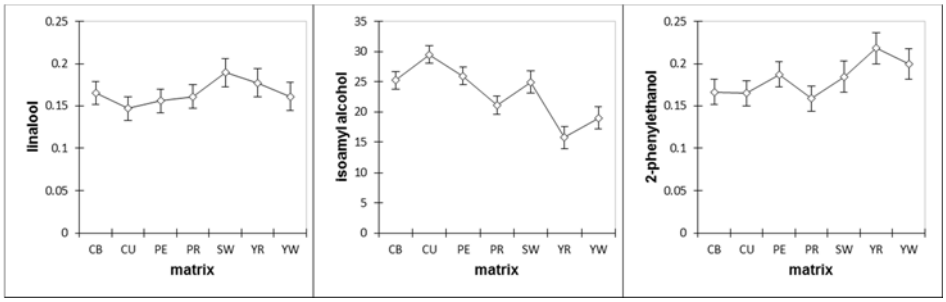

Fig. 9. HS average relative areas for linalool, isoamyl alcohol and 2-phenylethanol at t=10 min

3.3. Principal component analysis

We have seen in the previous sections that there are multiple behaviors on the headspace response depending not only on the matrix but also on the particular compound or family of compounds. A better understanding of these interactions could be obtained with a global study of the data using principal component analysis (PCA). For an easier interpretation, the data set was divided in headspace responses at t=0 and t=10 min before the PCA, which also included the compositional parameters of the reconstituted matrices and synthetic wine (Table 2).

For the t=0 min data set, the first three principal components were studied. The first principal component (PC1) explained 28.4% of the variance in the data. PC1 was highly correlated with dimethyl sulfide (-0.692), linalool (0.906), β-damascenone (0.783), t-whiskylactone (0.908), 4-ethylphenol (0.946), and the total residue (-0.819) and Mn content (-0,753). PC2 explained 25.7% of the variance, and it was correlated with butyric acid (0.784), hexanoic acid (0.854), and the compositional parameters total SO_2 (0.884), free SO_2 (0.804) and pH (-0.639). PC3 accounted for 17.9% of the

variance and was correlated with ethyl acetate (0.908), butyrate (0.870) and hexanoate (0.635), negatively with isoamyl alcohol (-0.621), and also with Cu (0.642) and Fe (0.695). Fig. 10 shows the distribution of the reconstituted wines in the space defined by PC1 and PC2. As could be expected, SW matrix was differentiated from the other matrices, likely due to its compositional data. Whites wines were in the top part of the chart, due to their larger release of acids associated with PC2, while "Reserva" wines PR and CU, where together in the bottom-left part of the chart showing a similar general behavior of release of volatiles. Fig. 11 is a representation of the matrix in the space defined by PC1 and PC3. In this case, also SW is clearly differentiated from the rest of matrices, while young red wine matrix (YR) is also characterized by PC3 due to a combination of higher content in Cu and Fe, more release of ethyl esters and less release of isoamyl alcohol.

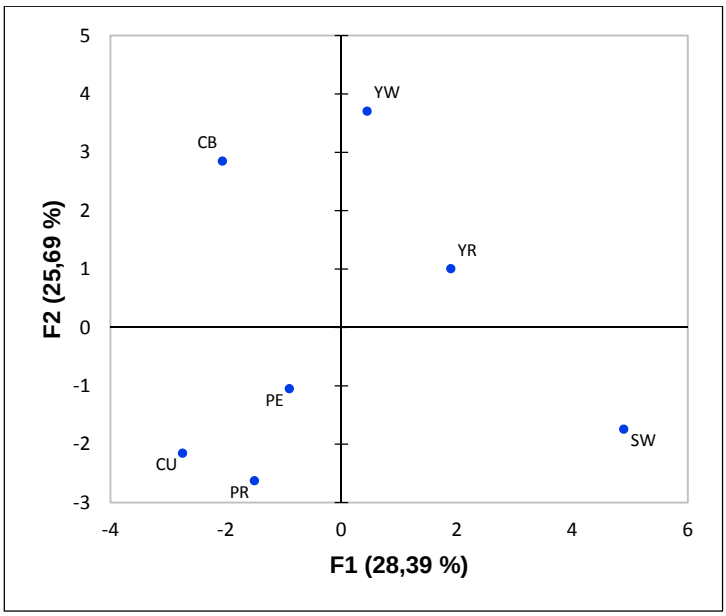

Fig. 10. Chart of the reconstituted matrices in the dimensional space defined by the first two principal components for t=0 min (in brackets: percentage of variance explained by each component).

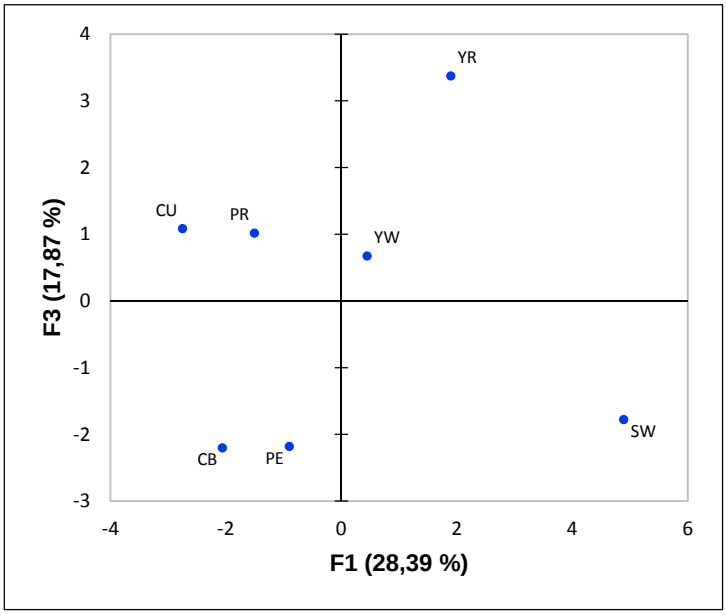

Fig. 11. Chart of the reconstituted matrices in the dimensional space defined by the first and third principal components for t=0 min (in brackets: percentage of variance explained by each component).

The first three PCs were selected for the t=10 min data set, with many similarities in the most correlated variables for each PC. PC1 explained 28.8% of the variance and it was correlated with dimethyl sulfide (-0.937), linalool (0.763), β-damascenone (0.861), 2-phenylethanol (0.708), trans-whiskylactone (0.726), 4-ethylphenol (0.781) and negatively with the compositional parameters total residue (-0,684) and TPI (0.711). PC2 explained 26.1% of the variance and was correlated with butyric (0.777) and hexanoic (0.754) acids, vanillin (0.767), total SO_2 (0.860), free SO_2 (0.856) and Mn (0.846). PC3 explained 18.0% of the variance and it was correlated with ethyl acetate (0.878), butyrate (0.859) and hexanoate (0.605), negatively with isoamyl alcohol (-0.649), and with Cu (0.697) and Fe (0.741). The synthetic wine was separated in Fig. 12 due to the lowest factor score in PC2 due to a completely different compositional data. White wines also appeared isolated in the chart, but in this case in the top-right corner of the distribution with higher factor scores for PC1 and PC2. Fig. 13 shows the space defined by PC1 and PC3, where YR wine exhibited a differentiated behavior along the vertical axis. PC1 was also able to differentiate between young and synthetic wines (YW, YR, SW) with similar behaviors regarding less volatile compounds like β-damascenone, 2-phenylethanol, t-whiskylactone and 4-ethylphenol.

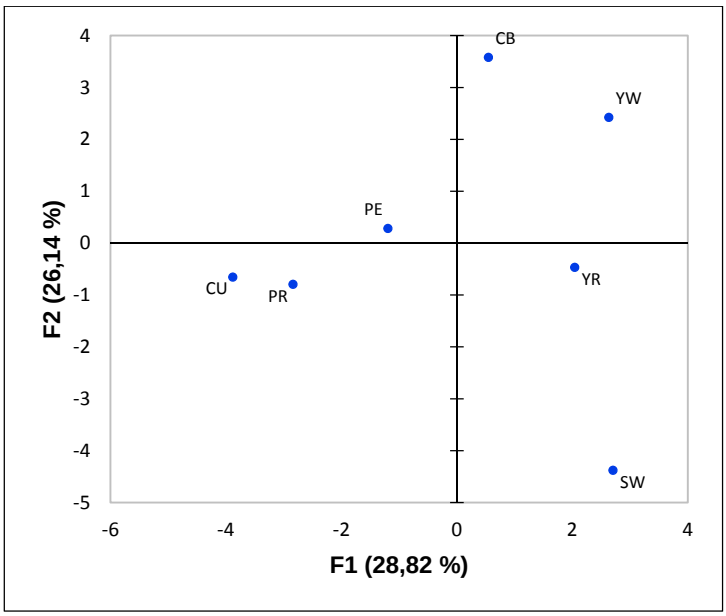

Fig. 12. Chart of the reconstituted matrices in the dimensional space defined by the first two principal components for t=10 min (in brackets: percentage of variance explained by each component)

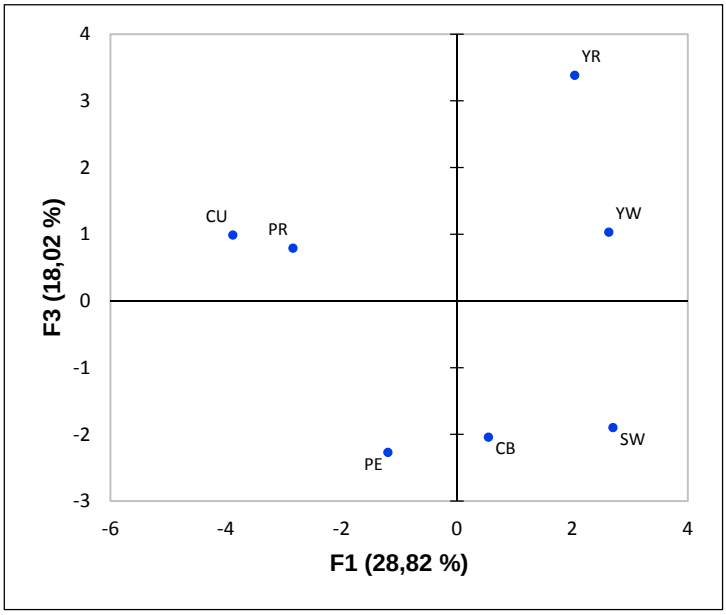

Fig. 13. Chart of the reconstituted matrices in the dimensional space defined by the first and third principal components for t=10 min (in brackets: percentage of variance explained by each component).

4. Conclusions

The experiments carried out in this chapter has proved that the same volatile composition in the liquid phase of very different non-volatile wine matrices produces a headspace profile above the wines that can be significantly different. As shown by the data, the interactions between the non-volatile matrix components and volatile compounds produce both retention and salting-out effects depending on the wine matrix. The magnitude of the changes observed in the headspaces profiles can undoubtedly influence the perception of wine aroma and can explain why the same aroma composition can produce different aroma perceptions.

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CHAPTER 5

The Effect of Several Polysaccharides and Polyphenols on the Initial Release of Volatile Compounds and Their Evolution in the Headspace

CHAPTER 5

The effect of several polysaccharides and polyphenols on the initial release of volatile compounds and their evolution in the headspace

1. Introduction

The aroma typicality of a particular wine is owing to its complicated constituents, not only the volatile compounds that could be directly perceived, but also the nonvolatile compounds in the matrix that can exert a powerful effect on the release of odorants. The nature of the interaction between volatile compounds and wine matrix has been evidenced as results of chemical bindings, such as hydrophobic interaction, hydrogen bonds or covalent bonds. Therefore, the capacity of binding differs not only depending on the physicochemical characteristics of volatile compounds, but also on the chemical properties of nonvolatile components (Muñoz-González, Rodríguez-Bencomo, Moreno-Arribas, & Pozo-Bayón, 2011; Pozo-Bayón & Reineccius, 2009; Villamor & Ross, 2013; Voilley & Lubbers, 1998). Over the dynamic tasting process, the headspace composition is dynamically changing depending on evaporation and other conditional alterations as we have observed in previous chapters, accordingly generating temporal aroma profiles of wine over tasting (Lytra, Tempere, Marchand, de Revel, & Barbe, 2016; Saenz-Navajas, Campo, Cullere, Fernandez-Zurbano, Valentin, & Ferreira, 2010).

Wine matrix is mainly constituted by proteins, polyphenols, polysaccharides and carbohydrates coming from grapes and yeasts

(Ribéreau-Gayon, Dubourdieu, Donèche, & Lonvaud, 2006). Most of them will be eliminated by fining treatments to achieve the stability and balance of flavor. With this purpose, commercial additives are widely used in wine industry, such as bentonite, plant protein, natural polymers, mannoproteins from yeast and tannin extracts from oak (Marchal & Jeandet, 2009). In the present study, commercial samples of gum arabic, carboxymethylcelluloses (CMC), oak tannins and yeast mannoproteins were used to model the synthetic wine matrix behavior with one of this additives. The extent of volatile-macromolecule interaction will be studied in these simplified wine models.

Headspace sampling techniques have been used to estimate the extent of odorant-matrix interactions by analyzing the concentrations of the analytes in the headspace above the solution under both static and dynamic conditions (Athès, Peña y Lillo, Bernard, Pérez-Correa, & Souchon, 2004; Rodríguez-Bencomo, Muñoz-González, Andújar-Ortiz, Martín-Álvarez, Moreno-Arribas, & Pozo-Bayón, 2011; Taylor, Tsachaki, Lopez, Morris, Ferreira, & Wolf, 2010). In this chapter, the previously validated method based on dynamic headspace sampling will be applied to study the volatile-macromolecule interactions and the headspace evolution under dynamic conditions.

2. Materials and methods

2.1. Reagents and chemicals

Ethanol was supplied by Merck (Darmstadt, Germany), tartaric acid 99% was from Panreac (Barcelona, Spain). The internal

standards (methyl 2-methylbutyrate, 2,6-dichloroanisole) were obtained from Sigma-Aldrich (Madrid, Spain). The standard compounds listed in Table 1 with purities above 98% in all cases were purchased from Sigma-Aldrich (Steinheim, Germany), Fluka (Buchs, Switzerland) and Panreac (Barcelona, Spain). These aroma compounds were prepared as the aroma mixture at concentration ranges detected in Spanish commercial wines (San Juan, Cacho, Ferreira, & Escudero, 2012) to model synthetic wines (Table 1).

2.2. Preparation of synthetic wines with macromolecules

Four commercial macromolecules that are commonly used before bottling in the wine industry were studied to estimate their effects on volatile release. Arabic gum, carboxymethyl cellulose (CMC) and oak tannins were purchased from Enartis (Italy), and mannoproteins were provided by Fermentis (France). A hydroalcoholic solution was used (containing 12% vol of ethanol, 5 g/L of tartaric acid, adjusted to pH 3.4 with 1M NaOH) to dissolve all the mentioned macromolecule products, and to further prepare synthetic wines with 2 mL/L for gum arabic (contain minimum content 20.5 %, Citrogum), 2 mL/L for CMC (composed of 5% E466 CMC, Cellogum L), 3 g/hL for untoasted oak tannins (Tan Style) and 0.4 g/L for mannoproteins (Springcell Manno), respectively. A control wine without macromolecule addition was also prepared. Afterward, the described aroma mixture was added to each synthetic wine to obtain the concentrations shown in Table 1.

Table 1. Concentrations of volatile compounds used for synthetic wine models.

Compound	log P	Concentration (mg/L)	Aroma vector
Dimethyl sulfide	0.92	0.050	Spice-woody
Ethyl acetate	0.73	28	Alcoholic-solvent
Ethyl butyrate	1.85	0.85	Fruity
Ethyl hexanoate	1.92	0.79	Fruity
Ethyl decanoate	4.79	1.0	Fruity
Linalool	2.97	0.73	Flowery
Isoamyl alcohol	1.16	239	Alcoholic-solvent
2-Phenylethanol	1.36	50	Alcoholic-solvent
Acetoin	-0.36	0.99	Lactic-acid
β-Damascenone	4.21	0.53	Fruity
Butyric acid	0.79	0.91	Lactic-acid
Hexanoic acid	1.92	3.7	Lactic-acid
trans-Whiskylactone	2.00	0.84	Spice-woody
4-Ethylphenol	2.58	0.89	Animal-leather
Vanillin	1.21	0.89	Spice-woody

2.3. The analysis of dynamic headspace compositions

The validated DHS-TD-GC-MS method was applied to analyze the composition of headspace according to Chapter 1. Briefly, 5 mL of the sample was transferred into a 20 mL standard headspace vial and then 20 μL of internal standards solution was also added. The thermal desorption was performed by TDU-CIS system (Gerstel, Denmark) and was programmed as described before. Gas chromatography-mass spectrometry analysis was performed with a 7890 Agilent GC system coupled with a 5975C Agilent quadrupole mass spectrometer (Santa Clara, CA, USA). The temperature program was: initial oven temperature 35°C held for 3 min, then raised to 220°C at 10°C/min, and 7 min of final hold time. Ionization

was carried out in electron impact mode at 70 eV. Spectra were recorded both in scan mode from 33 to 250 m/z and in selected ion monitoring.

2.4. The effect of macromolecules on aroma release and the evolution over time

Forty mL of each synthetic wine containing different macromolecules and same aroma mixture was transferred into a 250 mL uncovered glass beaker, and then 5mL of wine sample was collected from one beaker after pouring or after 10 min-period shaking. The DHS method above was applied to analyze samples, thus obtaining the headspace composition of the initial status (t=0 min) and after 10 min evaporation. The analyses were triplicated for each synthetic wine model.

2.5. Data analysis

XLSTAT (Addinsoft) was applied to access the statistical analysis, the headspace content of each volatile compound in each synthetic wine models was compared with the corresponding value in control to study the retention/salting out effect. One-way ANOVA was applied to compare the differences of volatile-macromolecule binding extent. A t-test was used to evaluate the evolution of headspace for each wine models. The significance level was set at the confidence interval of 95% throughout this study.

3. Results and discussion

In the present chapter, the interactions between volatile compounds and macromolecules were studied. With this purpose,

volatile compounds in Table 1 were selected to reflect large differences in physicochemical characteristics, also for the reason that they could represent typical aroma compounds from different categories as we grouped in the previous chapter and from various aroma vectors as recently reviewed by Ferreira et al. (in press). Also, the studied macromolecules are generally applied in the wine industry for stabilizing wine color and balancing wine body before bottling, which would artificially constitute the whole wine nonvolatile matrix (Ribéreau-Gayon, Dubourdieu, Donèche, & Lonvaud, 2006). Ethanol concentration was kept the same for all the synthetic wine models to avoid volatility variations due to ethanol effect (Aznar, Tsachaki, Linforth, Ferreira, & Taylor, 2004; Boothroyd, Linforth, & Cook, 2012; Diako, McMahon, Mattinson, Evans, & Ross, 2016; Wollan, Pham, & Wilkinson, 2016). The headspace contents of analytes were presented as relative areas against methyl 2-methylbutyrate to avoid the bias of instrumental response (Table 2).

3.1. The effect of macromolecules on the volatility of aroma compounds

To better understand the retention/salting-out effect of macromolecules, the comparison between the headspace content of each volatile compound between control and each synthetic wine model was applied. Table 3 shows the result expressed as the percentage. Accordingly, the negative or positive values reflect whether the interaction effect was retention or salting-out with that particular macromolecule.

Mannoproteins produced by yeast are one of the most abundant

polysaccharides in wines, with valuable properties for wines. Therefore, they are frequently added to wine as commercial products. We choose one of these commercial products to quantify its influence in wine aroma. The wine model with mannoproteins showed a particularly weak retention effect for ethyl butyrate, hexanoate and decanoate. These results are in agreement with a previous study by Dufour and Bayonove (1999a) where they found that ethyl hexanoate volatility was not affected by mannoproteins in the range of 5-20 g/L. However, other studies have found quite the opposite with a marked retention effect of ethyl hexanoate with mannoproteins (Chalier, Angot, Delteil, Doco, & Gunata, 2007; S Lubbers, Charpentier, Feuillat, & Voilley, 1994). The explanation of these phenomena should be found in the diverse composition of mannoproteins, with variable contents of lipids and proteins depending on the extraction and purification procedures carried out. However, less hydrophobic compounds like major alcohols or heavier compounds such us β-damascenone, t-whiskylactone, 4-ethylphenol and vanillin were affected with a marked retention effect. Although the literature does not provide information about the interaction of these compounds with mannoproteins, two similar compounds hexanol and β-ionone showed analogous binding effects to those observed here (Chalier, Angot, Delteil, Doco, & Gunata, 2007; Dufour & Bayonove, 1999a; S. Lubbers, Voilley, Feuillat, & Charpentier, 1994). From a technological point of view, and given the commercial nature of the product selected in our experiment, it is evident that this particular product does not affect ester content in the headspace, but could decrease the release of other important aroma compounds, changing the sensory perception of the wine

effectively

Like mannoproteins, gum arabic is a polysaccharide, but in this case, its origin is from plant material rather than from yeast. This gum is obtained from trees and contains not only polysaccharides (mainly made of arabinose and galactose), but also glycoproteins. Gum arabic is used frequently in wine for tartaric stabilization purposes, reducing the need for cold stabilization. The influence of headspace of gum arabic on the model wine at 0 min is shown in table 3. Release from gum arabic model was similar to that observed with mannoproteins. A strong retention effect was evident for less hydrophobic compounds, while more apolar compounds like ethyl esters showed less or no decrease in volatility at all. As far as we know, there is no scientific bibliography on the specific interaction between wine aroma compounds and gum arabic. However, some publications have studied the effect of arabinogalactan-proteins (whether coming from gum arabic or grape material).

Table 2. Average relative area and standard deviation of each volatile compound in the headspace of each synthetic wine model at different time points.

	0 min					10 min			
Compound	Control	Gum arabic	Tannins	Mannoprotein	CMC *	Control	Gum arabic	Tannins	Mannoprotein
Dimethyl sulfide	0.00910 ± 0.0002	0.00810 ± 0.0003	0.00440 ± 0.0006	0.00830 ± 0.0011	0.00490 ± 0.0011	0.00480 ± 0.0003	0.00320 ± 0.0007	0.00160 ± 0.0005	0.00370 ± 0.0015
Ethyl acetate	3.57 ± 0.126	3.30 ± 0.172	3.32 ± 0.0550	3.02 ± 0.128	2.85 ± 0.227	3.34 ± 0.0993	2.73 ± 0.315	2.94 ± 0.414	2.73 ± 0.0424
Ethyl butyrate	0.142 ± 0.0033	0.145 ± 0.0054	0.152 ± 0.0029	0.147 ± 0.0025	0.150 ± 0.0014	0.122 ± 0.0054	0.123 ± 0.0008	0.124 ± 0.0079	0.126 ± 0.0118
Isoamyl alcohol	27.8 ± 1.91	14.3 ± 0.543	14.1 ± 0.573	17.8 ± 1.07	13.0 ± 0.171	26.5 ± 1.05	13.5 ± 0.219	14.6 ± 4.354	16.2 ± 1.03
Ethyl hexanoate	1.98 ± 0.0315	2.09 ± 0.0307	1.90 ± 0.0337	1.97 ± 0.0272	1.9215 ± 0.0031	1.72 ± 0.0918	1.80 ± 0.0445	1.60 ± 0.0952	1.67 ± 0.211
Acetoin	0.00130 ± 0.0001	0.000800 ± 0.0001	0.00100 ± 0.0000	0.00120 ± 0.0001	0.000900 ± 0.0001	0.00160 ± 0.0001	0.000800 ± 0.0000	0.00100 ± 0.0002	0.00110 ± 0.000
Linalool	0.331 ± 0.0160	0.184 ± 0.0030	0.188 ± 0.0075	0.222 ± 0.0073	0.179 ± 0.0020	0.309 ± 0.0180	0.171 ± 0.0024	0.192 ± 0.0449	0.205 ± 0.0141
Butyric acid	0.0030 ± 0.0002	0.0015 ± 0.0001	0.00260 ± 0.0004	0.0026 ± 0.0001	0.0019 ± 0.0001	0.00330 ± 0.0010	0.00210 ± 0.0002	0.00270 ± 0.0019	0.00240 ± 0.0007
Ethyl decanoate	3.23 ± 0.485	2.86 ± 0.0495	2.36 ± 0.103	3.27 ± 0.111	2.58 ± 0.0874	2.39 ± 0.215	2.25 ± 0.150	2.05 ± 0.170	2.50 ± 0.408
Hexanoic acid	0.0109 ± 0.0005	0.00820 ± 0.0005	0.0108 ± 0.0007	0.0136 ± 0.0011	0.0104 ± 0.0007	0.0118 ± 0.0028	0.00800 ± 0.0016	0.0110 ± 0.0039	0.00980 ± 0.0039
β-Damascenone	0.386 ± 0.0255	0.237 ± 0.0052	0.260 ± 0.0134	0.321 ± 0.0116	0.250 ± 0.0031	0.387 ± 0.0173	0.222 ± 0.0061	0.269 ± 0.0594	0.294 ± 0.0206
t-Whiskylactone	0.0572 ± 0.0032	0.0319 ± 0.0014	0.0335 ± 0.0024	0.0412 ± 0.0018	0.0321 ± 0.0011	0.0559 ± 0.0027	0.0293 ± 0.0005	0.0366 ± 0.008	0.0382 ± 0.0022
2-Phenylethanol	0.456 ± 0.0194	0.236 ± 0.0150	0.264 ± 0.0241	0.324 ± 0.0240	0.238 ± 0.0149	0.422 ± 0.0261	0.224 ± 0.0078	0.286 ± 0.0684	0.309 ± 0.0143
4-Ethyl phenol	0.111 ± 0.0054	0.0603 ± 0.0030	0.0642 ± 0.0050	0.0782 ± 0.0032	0.0591 ± 0.0024	0.107 ± 0.0053	0.0556 ± 0.0003	0.0685 ± 0.0158	0.0739 ± 0.0020
Vanillin	0.0006 ± 0.0001	0.0002 ± 0.0000	0.0005 ± 0.0001	0.0003 ± 0.0000	0.0002 ± 0.0000	0.0005 ± 0.0002	0.0002 ± 0.0001	0.0005 ± 0.0003	0.0003 ± 0.0000

*The results of volatile compounds in the wine model with CMC at 10min are not presented due to the lack of repetitions.

Firstly, Dufour and Bayonove (1999a) studied the effect of arabinogalactan-protein fractions in the release of several volatile compounds. They found that high levels of these macromolecules were needed to decrease the volatility of ethyl hexanoate. However, at low arabinogalactan-protein concentrations, in the range of to those used in our experiment, there it was equivalent weak salting out effect on ethyl hexanoate. Also similarly, they found a marked decrease in the volatility of 1-hexanol at all concentrations, that result agrees with the depressive effect observed in our more polar compounds. More recently, Mitropoulou et al. (2011) have studied the impact of arabinogalactan polysaccharides on the headspace concentration of volatile compounds in model wines. For low concentrations of arabinogalactan (up to 1 g/L) they found weak salting out effect for ethyl esters, 2-phenylethanol and linalool, and practically no change in the release of 2-methyl-1-butanol and octanoic acid. From the data in the literature and reported here, it can be deduced that at low concentrations of arabinogalactans and arabinogalactan-proteins the volatility of ethyl esters is not affected or slightly increases. However, at the same concentrations, more polar compounds will reduce their presence in the headspace compared with a control wine, causing a change in the volatile profile.

Carboxymethyl cellulose (CMC) is a heterogeneous mixture of polysaccharides of various molecular sizes and modifications (Hoogendam, De Keizer, Cohen Stuart, Bijsterbosch, Smit, Van Dijk, et al., 1998). Since its approval by the OIV in 2009, it has been used in wine to help with tartrate stabilization in white wines. As with the

previous two polysaccharides, the release from a model wine with a typical commercial dosage of the CMC (0.01%) was measured. The results, presented in Table 3 for 0 min release, showed a strong retention effect for most compounds. Only ethyl butyrate, ethyl hexanoate and hexanoic acid were weakly or not affected by the addition of CMC. At high concentrations, CMC increases the viscosity of liquid solutions causing a decrease in the release of volatile compounds of all polarities (Roberts, Elmore, Langley, & Bakker, 1996). At lower levels of addition of CMC (1%), De Roos (2000) also reported lower release rates from water for a range of volatile compounds. The maximum level of CMC addition in wine allowed by the E.U. (0.01%) is well below those quantities. However, even at the very low levels used in our experiment, it is evident from Table 3 that a modification in the profile of release should be expected. Whether this modification is caused by a change of viscosity of the model wine or by specific binding interactions of CMC with the aroma compounds is a question that remains to be investigated.

The commercial tannins used for this experiment were composed of hydrolyzable ellagic tannins extracted from the untoasted oak. The effect of the tannins in the model wine was for most compounds a decrease in their volatility at 0 min (Table 3). Only ethyl butyrate showed a weak salting-out effect of 7%. According to the commercial information, this tannin product could be applied to correct reductive off-odors, suggesting its potential to bind volatile sulfur compounds, which could explain the high retention of dimethyl sulfide in this particular wine model. The literature on the interaction of odorant with polyphenols is abundant

and it has been reviewed elsewhere (Muñoz-González, Rodríguez-Bencomo, Moreno-Arribas, & Pozo-Bayón, 2011; Polaskova, Herszage, & Ebeler, 2008). Different phenomena can cause the interactions with this molecules. On the one hand, the hydrophobicity-driven interaction between volatile compounds and polyphenols could enhance the solubility of some aroma compounds and then reduce their volatility (Dufour & Bayonove, 1999b). In our case, this is evident in all ranges of polarities from ethyl decanoate to acetoin (Table 3). On the other hand, the formation of π-π stacking between the gallic ring of polyphenols and the aromatic ring of volatile compounds could further strengthen the stability of hydrogen bonds resulting in a high retention effect (Jung, de Ropp, & Ebeler, 2000). This could be the case of 2-phenylethanol and 4-ethylphenol in our experiment.

The nature of volatile-macromolecule interactions have been widely agreed as a result of chemical bindings, including hydrophobic interaction, hydrogen bonds and covalent bonds (Muñoz-González, Rodríguez-Bencomo, Moreno-Arribas, & Pozo-Bayón, 2011; Pozo-Bayón & Reineccius, 2009; Villamor & Ross, 2013; Voilley & Lubbers, 1998). Among all the mentioned interactions, the hydrophobicity of volatile compounds was especially emphasized (Dufour & Bayonove, 1999a, 1999b; S. Lubbers, Voilley, Feuillat, & Charpentier, 1994; Piombino, Moio, & Genovese, 2018; Robinson, Ebeler, Heymann, Boss, Solomon, & Trengove, 2009). However, our observations showed that hydrophobicity is not the only mechanism involved because the retention effect of macromolecules did not completely correlate to the log P. In the cases of homologous esters in Table 3, the

macromolecules showed a very limited impact on their volatility. In general, ethyl acetate showed a higher retention effect than butyrate and hexanoate (and similar to decanoate with CMC), when the opposite should be expected based on its smaller log P value. However, ethyl acetate seemed to be affected more by the structural modification of the hydroalcoholic solution due to the addition of macromolecules, likely limiting its access to the gas-liquid interface. Ethyl butyrate and ethyl hexanoate even showed slight salting out effect in wine models with tannins and gum arabic, revealing the possible competition between these compounds and macromolecules to bind water-ethanol molecules as reported in another food system (Jouquand, Aguni, Malhiac, & Grisel, 2008). It is possible that the macromolecules may organize the water and ethanol molecules between them, giving less free water to interact with volatile compounds. The volatility of ethyl decanoate presented the highest reductions by macromolecules in the series except for mannoprotein, showing the existence of mutual interaction with macromolecules and a more matrix-dependent character. This observed phenomenon is in agreement with the findings that esters with longer carbon chain could show greater retention effect of polyphenols (Aronson & Ebeler, 2004). Although ethyl decanoate was the most hydrophobic volatile in Table 3, the other compounds from different chemical classes with lower log P values presented higher reductions in release behavior. The only exceptions were the two acids and vanillin in the wine model with tannins (Table 3). This finding points to an impact of volatile-macromolecule interactions that is far more complicated than just a hydrophobicity-driven mechanism. The observed complex matrix effects could also be

attributed to the functional groups and the chemical structures of the particular volatile compounds (Ayed, Lubbers, Andriot, Merabtine, Guichard, & Tromelin, 2014). Interestingly, no significant differences in retention behavior were observed for alcohols, trans-whiskylactone and 4-ethylphenol in the given wine models with tannins and polysaccharides (Table 3), indicating the reduction extent of their volatility was independent on the added macromolecules. The same observation was reported in different reconstituted wines (Rodríguez-Bencomo, Muñoz-González, Andújar-Ortiz, Martín-Álvarez, Moreno-Arribas, & Pozo-Bayón, 2011). For dimethyl sulfide and acids, the reduction extent highly depended on the type of macromolecules. Although the detection of dimethyl sulfide's odor threshold with different wine matrix revealed the clear matrix-dependent characteristics of its release (Lytra, Tempere, Zhang, Marchand, de Revel, & Barbe, 2014), very few literature have reported the interaction between volatile sulfur compounds and macromolecules, as well as acids.

In summary, the main effect observed in Table 3 was a reduction in the release of the volatile compounds at 0 min. Ethyl butyrate was an exception showing slight salting out effect in all the analyzed wine models. It is logical to relate the reduction with the alteration of viscosity in the polysaccharide-containing synthetic wines, which could form a protective colloid and consequently affect the diffusion of volatile compounds and further modify the partitioning between two phases (Anne, Yacine, & Isabelle, 2010; Taylor, 2002; Terta, Blekas, & Paraskevopoulou, 2006). The different chemical properties of the three polysaccharides used in this experiment (such as chemical structure and molecular weight) could differentially

affect the interaction with particular aroma compounds (Dufour & Bayonove, 1999a; Jouquand, Aguni, Malhiac, & Grisel, 2008; S. Lubbers, Voilley, Feuillat, & Charpentier, 1994) and explain some of the observed differences.

Table 3. Headspace content at 0 min. Change of volatile compounds in each synthetic wine model expressed as the difference between the average relative areas of the model wines and the control wine, divided by the average relative area of the control wine(in percentage).

Compound	Control	Gum arabic	Mannoprotein	CMC	Tannins
Dimethyl sulfide	0% a	-11% a	-8% a	**-46%** b	**-52%** b
Ethyl acetate	0% a	-8% ab	**-16%** bc	**-20%** c	-7% ab
Ethyl butyrate	0% b	2% ab	3% ab	6% ab	**7%** a
Ethyl hexanoate	0% b	**5%** a	-1% bc	-3% bc	**-4%** c
Ethyl decanoate	0% a	-12% ab	1% a	**-20%** b	**-27%** b
Linalool	0% a	**-45%** c	**-33%** b	**-46%** c	**-43%** c
Isoamyl alcohol	0% a	**-49%** c	**-36%** b	**-53%** c	**-49%** c
2-Phenylethanol	0% a	**-48%** c	**-29%** b	**-48%** c	**-42%** c
Butyric acid	0% a	**-49%** b	-15% b	**-38%** b	-15% b
Hexanoic acid	0% b	**-25%** c	**24%** a	-5% b	-2% b
Acetoin	0% a	**-37%** b	-4% a	**-25%** b	**-18%** ab
β-Damascenone	0% a	**-39%** c	**-17%** b	**-35%** c	**-33%** c
t-Whiskylactone	0% a	**-44%** c	**-28%** b	**-44%** c	**-41%** c
4-Ethyl phenol	0% a	**-46%** c	**-30%** b	**-47%** c	**-42%** c
Vanillin	0% a	**-76%** b	**-57%** b	**-61%** b	-14% a

Significant changes in headspace content of volatile compound between control wine and other wine models are indicated in bold ($p \leq 0.05$). Different letters mean significant differences in headspace content among all the wine models according to one-way ANOVA.

3.2. The effect of macromolecules on the evolution of aroma compounds in the headspace

In the previous chapters, we have observed the evolution of volatile compounds in the headspace over time. Moreover, the

temporal change trend of a particular volatile compound was relatively influenced by its physicochemical characteristics and its binding behavior with the wine matrix. In the present chapter, the evolution of volatile compounds belonging to different chemical classes was studied in different synthetic wine models whose matrix components were simplified by adding single types of macromolecules. The headspace change of each volatile compound over time was calculated by comparing the relative area at both time points with each synthetic wine model (Table 4). Data for CMC wine model were excluded in the table due to the lack of repetitions of the headspace contents after 10 min evaporation caused by a technical error in the instrument. A t-test was applied to obtain the statistical significance of the headspace evolution in each synthetic wine models (shown in Table 4).

Table 4. The headspace evolution in different wine models. Values are the average relative area of each model wine at 10 min minus the average relative area of the same wine at 0 min, divided by the relative area of the same wine at 0 min (expressed in percentage).

	Control			Gum arabic			Tannins			Mannoprotein		
Compound	Change	t	p(t)	Change	t	p(t)	Change	t	p(t)	Change	t	p(t)
Dimethyl sulfide	**-47%**	17.7	**<0.001**	**-60%**	11.45	**<0.001**	**-64%**	6.05	**<0.01**	**-56%**	4.47	**0.01**
Ethyl acetate	**-7%**	2.55	**0.03**	**-17%**	2.72	**0.03**	-11%	1.56	0.1	**-9%**	3.66	**0.01**
Ethyl butyrate	**-13%**	4.86	**<0.01**	**-15%**	6.78	**<0.01**	**-19%**	5.81	**<0.01**	**-15%**	3.11	**0.02**
Ethyl hexanoate	**-13%**	4.66	**<0.01**	**-14%**	9.23	**<0.001**	**-16%**	5.1	**<0.01**	**-15%**	2.43	**0.04**
Ethyl decanoate	**-26%**	2.73	**0.03**	**-21%**	6.62	**<0.01**	**-13%**	2.69	**0.03**	**-24%**	3.18	**0.02**
Linalool	-7%	1.64	0.09	**-7%**	6.09	**<0.01**	2%	0.15	0.44	-8%	1.86	0.07
Isoamyl alcohol	-5%	1.08	0.17	**-6%**	2.49	**0.03**	4%	0.2	0.43	-9%	1.83	0.07
2-Phenylethanol	-7%	1.8	0.07	-5%	1.12	0.16	8%	0.52	0.31	-4%	0.9	0.21
Butyric acid	11%	0.55	0.3	**39%**	4.45	**0.01**	7%	0.17	0.44	-8%	0.51	0.32
Hexanoic acid	8%	0.53	0.31	-3%	0.23	0.42	2%	0.09	0.46	-28%	1.59	0.09
Acetoin	**25%**	3.41	**0.01**	-3%	0.45	0.34	0%	0.03	0.49	-5%	0.78	0.24
β-Damascenone	0%	0.04	0.48	**-6%**	3.19	**0.02**	3%	0.25	0.41	-8%	1.93	0.06
trans-Whiskylactone	-2%	0.53	0.31	**-8%**	3.02	**0.02**	9%	0.64	0.28	-7%	1.78	0.08
4-Ethyl phenol	-3%	0.85	0.22	**-8%**	2.67	**0.03**	7%	0.45	0.34	-5%	1.95	0.06
Vanillin	-13%	0.88	0.21	42%	1.6	0.09	-10%	0.3	0.39	8%	0.58	0.3

Significant differences in headspace content of volatile compound at 0 min and 10 min in each wine model are indicated in bold ($p \leq 0.05$).

The evolutions of the headspace content in the studied wine models over 10 min are presented in Table 4. Negative values indicate that a compound in a particular wine has decreased its concentration in the headspace at t=10 min compared to t=0 min. The differences in the headspace evolution were not constant, rather depending on the particular compound and model. The results showed that dimethyl sulfide and esters significantly decayed due to evaporation, as we have confirmed in previous chapters. However, the decay was not equivalent for this two groups of compounds. For example, dimethyl sulfide and ethyl acetate evolved relatively more slowly in the control wine than in the model wines. This is surprising given the retention effect observed at 0 min for macromolecules. However, ethyl decanoate presented a contrary trend of evolution, especially in the wine model with tannins. Considering its matrix-dependent release behavior, we have observed in the previous chapters and other research (Boothroyd, Linforth, & Cook, 2012), we hypothesized that reversible interactions existed between the volatile compounds and macromolecules, thereby causing the temporal variation in mass transfer of some particular compounds. The other two esters showed similar evolution behavior (depletion around 15%) in all wine models, presenting a weak effect of macromolecules on their headspace change. The studied alcohols, β-damascenone, t-whiskylactone and 4-ethylphenol showed a constant trend in their evolution over 10 min in all the synthetic wine models, only in the case of gum arabic, small changes of some of the compounds above were significant (Table 4). It is worth noticing that although there existed remarkable retention of these compounds by macromolecules (Table 3), their evolutionary trend seems

independent of the matrix composition. Therefore, their headspace content is maintained more or less constant during the tasting period compared to their initial response and independently of the macromolecules. This finding may explain the stable release pattern of these compounds that we observed in the previous chapter. For acetoin and acids, their evolutions were more complicated upon the addition of the macromolecules. As shown in Table 4, slight changes of acetoin were found in the wine models, while a 25% of the increase was observed in control wine, exhibiting a great dependence of their evolution on the matrix composition. The significant differences in volatility may be related to the possible changes in the structure of water-ethanol solution due to ethanol evaporation during the 10-min period, while the addition of other macromolecules suppressed this effect. Butyric and hexanoic acids did not show significant differences in their headspace evolution except for one case. Butyric acid presented striking salting out effect with gum arabic that however, did not appear in hexanoic acid (Table 4). The same effect was observed for vanillin, although in this case was not significant due to the low instrumental response and the associated increase in the variability of the determination.

4. Conclusions

The present chapter showed that some of the commercial products based on macromolecules used as enological additives in the wine industry could interact with volatile compounds resulting in a global retention effect. For some volatile compounds, the interaction extent would further influence the evolution of headspace composition over time. Moreover, the chemical characteristics of the

volatile compounds strongly impact the binding capacity, such as the hydrophobicity and chemical structure. According to our results, the studied macromolecules showed differences in binding capacity with some volatile compounds due to their different chemical properties. However, real wine matrix is far more complicated than the simplified wine models in this study, the higher order interactions between macromolecules and volatile-macromolecule interactions should be studied in the future. Otherwise, the headspace sampling techniques could only prove the existence of interactions between volatile compounds and macromolecules, but they could not provide information about the interaction mechanism at the molecular level.

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CHAPTER 6

Volatile and Sensory Evolution of Wine Headspace: A Study of Their Correlations

CHAPTER 6

Volatile and sensory evolution of wine headspace: a study of their correlations

1. Introduction

Wine has a very complex nature of aroma characters. Wine chemists have no doubt that such complexity is caused by the chemical complexity of wine, not only because there are different wines with similar composition of odor-active compounds differing in aroma nuances, but also the aroma of a wine evolves with time while it is waiting in the glass to be consumed (Ferreira & Cacho, 2009; Hirson, Heymann, & Ebeler, 2012). For the first concern, the initial volatility of aroma compounds is strongly affected by their chemical nature and the potential of interaction with nonvolatile components as discussed elsewhere (Cayot, Dury-Brun, Karbowiak, Savary, & Voilley, 2008; Polster & Schieberle, 2015; Villamor & Ross, 2013). For the second concern, we have discussed the evolution of headspace composition under different conditions and in different wines over time in previous chapters. Hirson and coworkers (2012) also found the time-dependent changes of both sensory intensity and headspace composition in a wine glass. Other research evidenced that the evolution of fruity was related to the evolution of the headspace concentration of esters and dimethyl sulfide during 30-min tasting period by using reconstituted model wine (Lytra, Tempere, Marchand, de Revel, & Barbe, 2016). However, aroma compounds in the headspace above a real wine can mask or enhance the intensity of each other, or even can interact

synergistically to produce aromas with new characteristics, causing the complexity of olfactory responses (Atanasova, Thomas-Danguin, Langlois, Nicklaus, & Etievant, 2004; Ferreira, 2012; Ferreira, Sáenz-Navajas, Campo, Herrero, de la Fuente, & Fernández-Zurbano, 2016; Tempere, Schaaper, Cuzange, de Lescar, de Revel, & Sicard, 2016). Otherwise, the maximum concentration in the headspace or nasal cavity does not correspond to the highest odor intensity (Ferreira, 2012). Therefore, without sensory evaluation, observed evolutions in the headspace should not be used to predict the aroma changes of wine over tasting.

Studies have attempted to combine conventional sensory analysis and chemical methods in order to find the relationship between sensory and instrumental data, especially to evaluate volatiles that are available as potential stimulus corresponding with specific aroma profiles (Escudero, Campo, Fariña, Cacho, & Ferreira, 2007; Guth, 1997; Hjelmeland, King, Ebeler, & Heymann, 2013; Noble & Ebeler, 2002). However, most of them used strategies that do not take the dynamics during consumption into consideration. Since tasting is a dynamic process, the temporal dimension should be introduced into such analysis. Temporal Dominance of Sensation (TDS) and recently developed Temporal-Check-All-That-Apply (TCATA) are the most frequently used strategies that could measure the perceived complexity of a given product by recording the intensities of multi-attribute within one-intake (Meillon, Urbano, & Schlich, 2009; Meyners & Castura, 2018; Schlich, 2017). They are somehow more appropriate than descriptive analysis (DA) to study the overall sensation of tastes and aromas or cross-modal interactions between them over a short period of one-intake, but not

for the case that aromas are only interested over a much longer real wine tasting period. New approaches combining the above-mentioned dynamic sensory methods and dynamic techniques as APCI-MS or PTR-MS has been applied, and it is fast enough to monitor the real-time changes of the headspace and the perception during usually a short period (Fiches, Saint Eve, Jourdren, Déléris, Brunerie, & Souchon, 2016; Taylor, Linforth, Harvey, & Blake, 2000). However, the main pitfall of the technique is that it is not sensitive enough for monitoring the volatiles at very low concentrations in wines that could, however, influence wine aroma.

The objective of this chapter is to study the relationships between the evolution of sensory characteristics as perceived by tasters and headspace composition of premium wines over a prolonged tasting period. To achieve our aim, descriptive analysis was applied to evaluate the aroma notes of each wine every 5 min during the whole tasting period, and the corresponding headspace composition at each time point was analyzed by the validated DHS method.

2. Materials and methods

2.1. Reagents and chemicals

Solvent ethanol, methanol and dichloromethane at HPLC gradient grade were supplied by Merck (Darmstadt, Germany). The internal standards (methyl 2-methylbutyrate, 2,6-dichloroanisole) Sigma-Aldrich (Madrid, Spain). The standard compounds listed in Table 1 with purity above 98% in all cases were purchased from Sigma-Aldrich (Steinheim, Germany), Fluka (Buchs, Switzerland)

and Panreac (Barcelona, Spain). The standard volatile compounds for headspace quantification were prepared as mixtures at the concentration ranges detected in real wines (San-Juan, Cacho, Ferreira, & Escudero, 2012) (Table 1). The sorbent tubes of Tenax TA (2,6-diphenylene oxide polymer) were purchased from Gerstel (Germany).

2.2. Wine samples

Two premium Spanish commercial red wines from La Rioja and Campo de Borja were selected and analyzed to study the aroma evolution by both sensory and chemical analysis. They were coded as wine R and wine V, and detailed information is also presented (Table 1). Another commercial wine from La Rioja (Viña Tondonia, 2002) was selected for the sensory session to collect aroma notes.

Table 1. Detailed information for analyzed wines.

Wine	Commercial name	Origin	Vintage	Oak ageing (month)	Alcohol content (v/v)	TPI	pH
Wine R	Gran Reserva 904	La Rioja	2005	48 (American oak)	13.5	71.58	3.50
Wine V	Veraton	Campo de Borja	2015	16 (French oak)	15.5	56.74	3.37

2.3. Sensory analysis

2.3.1. Tasting conditions

Twelve judges (4 males and 8 females) were selected for their long wine-tasting experience and well-trained skills for wine sensory evaluation. They were all researchers at LAAE, University of Zaragoza, and volunteered in this study. Forty mL of wine was served to each panel member in a closed room at controlled room

temperature (22°C), and commercial red wine glasses (22 cm in height, 10 cm in diameter) were used.

2.3.2. Sensory analysis of wines over tasting period

A glass of one premium aged wine was served to each judge. During the tasting period, judges were asked to control their own time with a chronograph. During the whole 30 min tasting session, they were asked to swirl the glass three times before each olfaction except the first olfaction and every 5 min. In this session, judges were allowed to freely cite descriptors for each olfaction according to their wine tasting experience. After the whole process, all the panel members discussed the aromatic characteristics of the wine at every time point, and finally generated an agreed descriptor list containing fresh fruit, dry fruit, woody, animal, lactic, spicy, and alcoholic. Before the formal tasting sessions, all the panel members were semi-trained to familiarize the aromatic attributes in the list by using aromatic references.

The studied premium wines were served in two different tasting sessions under the same environmental conditions. Generally, the evaluation started immediately after serving to the panelist. Each judge controlled their own tasting time and swirled the glass three times at a 5-min interval before each olfaction except the first olfaction corresponding to t=0 min. Then they chose descriptors on the agreed list and marked the perceived intensity for each descriptor in a 5-point scale (from “not detectable” to “strong dominant”) after each olfaction. The first session for wine R lasted 30 min. Afterward, the second session for wine V was compressed to 20 min considering the fatigue of panelists.

2.4. Analytical analysis

2.4.1. Wine sample preparation

Wine samples for analytical analysis were prepared under the same conditions as the aforementioned sensory evaluation, thus to assess the corresponding chemical evolution of the wine served to the panel. Forty mL of wine was poured into wine glasses and then swirled third times every 5 min except the sample of 0 min. For wine R, the wine in one glass was collected at 0 min, 5 min, 15 min and 30 min after swirling for the following analysis of headspace composition. While for wine R, wine sample was collected after each swirling, consequently obtaining wine samples at 0 min, 5 min, 10 min, 15 min and 20 min along the tasting period. Volatile compounds in the liquid phase were analyzed for the wine only after the first and last swirling for both wines.

2.4.2. Analysis of headspace composition evolution

The headspace composition was monitored by the developed DHS-TD-GC-MS method (see Chapter 1). According to the method protocol, each 5 mL of sample and 200 μg/L of internal standards were added into a standard headspace vial for a short incubation and then were extracted by Tenax TA tube. Gas-chromatography-mass spectrometry analysis was performed with a 7890 Agilent GC system coupled with a 5975C Agilent quadrupole mass spectrometer (Santa Clara, CA, USA). The method was programmed as validated in the previous research. The analysis was duplicated for each wine sample and each time point.

To obtain the mass of compounds released into the headspace, 2

µL of a solution of each selected standard compound in methanol (Table 2) were spiked onto the fritted end of a pre-conditioned Tenax TA tube. Then the tubes were loaded onto the instrumental tray, following the same analytical program as described above. Each mixture was analyzed in duplicate.

Table 2. Mixed standards in methanol for the quantification of headspace components.

Compound	Ions (m/z)	Concentration (ppm)	Injected mass (ng)	Average absolute area	RSD %
Isoamyl acetate	87	164.0	327.9	456309.0	9%
Isoamyl alcohol	70	4026	8053	26111427.5	13%
Ethyl lactate	45	9438	18880	122472935.0	6%
Ethyl acetate	88	29330	58670	11657194.5	12%
Isobutanol	74	96.13	192.3	97240.3	6%
Propyl acetate	61	19.40	38.79	175931.7	5%
Acetoin	88	22.79	45.57	60265.3	12%
Ethyl isobutyrate	116	14.63	29.25	92198.7	4%
Ethyl 2-methylbutyrate	102	25.33	50.66	265509.0	9%
Ethyl 3-methylbutyrate	88	32.01	64.02	387749.3	7%
Ethyl butyrate	88	76.84	153.7	562049.7	7%
Ethyl hexanoate	99	100.5	201.0	1012741.3	9%
Diethyl succinate	129	125.9	251.8	2786485.7	4%
Ethyl octanoate	127	107.2	214.3	700079.7	6%
β-Phenylethanol	122	43.24	86.48	666342.3	4%
γ-Butyrolactone	86	64.25	128.5	22135.0	1%
Linalool	121	8.86	17.71	1873.5	11%
Guaiacol	109	9.77	19.55	39351.0	1%
4-Ethylphenol	107	6.50	13.00	47084.5	0%
2-Phenethyl acetate	104	116.1	232.3	587136.5	4%
4-Ethylguaiacol	137	55.70	111.4	269525.5	1%
t-Whiskylactone	99	9.90	19.80	14827.0	5%
β-Damascenone	121	6.57	13.14	14036.5	4%
Ethyl decanoate	155	27.66	55.32	238723.3	4%

Volatile sulfur compounds in the headspace were analyzed by the method developed in our laboratory with modification (Franco-Luesma & Ferreira, 2014). Briefly, 10 mL of brine was added to a 20 mL standard headspace vial in the oxygen-free glove chamber. Then the vial was well capped and 200 µL of wine and 20 µL of internal standards were added to the vial. Finally, the prepared sample was analyzed immediately by SPME-GC-SCD in duplicate.

2.4.3. Analysis of compounds in the liquid phase

Major volatile compounds in wine were quantified by using the method published by Ortega and colleagues (2001). According to the method, 3 mL of wine sample mixed with internal standards (2-butanol, 4-methyl-2-pentanol, 4-hydroxy-4-methyl-2-pentanone, and 2-octanol) was extracted by dichloromethane following a liquid-liquid microextraction strategy. Then the extract was analyzed by GC with a FID detector. The area of each analyte was normalized by its corresponding IS and then was quantified by external calibration plot.

The quantitative analysis of minor and trace compounds in wine was carried out by a solid phase extraction method proposed by our laboratory (López, Aznar, Cacho, & Ferreira, 2002). In accordance with the method, standard SPE cartridges were filled with LiChrolut EN resins and then the resins were pre-conditioned. Fifty mL of wine sample mixed with a surrogates solution were slowly passed through the SPE cartridge, followed by a washing step. The resins were then dried by passing air under negative pressure over 10 min. The analytes were eluted by 1.6 mL of a dichloromethane-methanol solution and a mixed solution of internal standards (about 10 µg of

2-octanol and 4-hydroxy-4-methyl-2-pentanone) were added. The extracts were directly analyzed by GC (Varian 450 GC) with ion trap mass spectrometry (Varian Saturn 2200 ion trap MS) detection.

Total polyphenol index (TPI) was estimated as absorbance at 280 nm by a UV-VIS spectrophotometer UV-17000 Pharma Spec (Shimadzu, Japan) (Ribéreau-Gayon, Dubourdieu, Donèche, & Lonvaud, 2006). pH and ethanol content were also measured by pH meter and GC-FID, respectively.

2.5. Statistical analysis and data treatment

XLSTAT software (Addinsoft, version 2016) was applied to access the comparison of samples from different time points in both sensory and chemical aspects by ANOVA. To test the correlation between sensory and chemical data. The statistically significant level was 10% and 5% for sensory and chemical data, respectively.

3. Results and discussion

3.1. Evolution of intensity of aroma notes in wines during tasting

Descriptive analysis of the two premium wines by 12 semi-trained panelists revealed that the perceived intensity of several aroma notes changed significantly in the glass during the given tasting period. As shown in Fig. 1 and Fig. 2, the evolution of aroma profiles in the two selected wines were complex. While no dramatic change was observed during the tasting session, there are several notes in which an apparent change is shown. For wine R, the fresh fruit, dry fruit, woody, spicy and animal (Fig. 1A) maintained a relatively higher intensity level than the other notes in Fig. 1B at

each time point. Although it is remarkable that the fresh fruit decreased half of the intensity after 20 min and the reduction note was estimated as a strong note at 0 min and later quickly decreased to almost 0. It is worth noticing that the trend of the intense notes in Fig. 1A started to change at 15 min, the dry fruit and animal decreased, while woody and spicy increased. However, only the evolution of fresh fruit, reduction and alcoholic were significant over time according to the result of a 2-way ANOVA (Fig. 1C, D and E). In the case of fresh fruit (Fig. 1C), the overall decay trend was in agreement with previous observations by Lytra et al. (2016). However, these authors also found increases of fresh fruity notes at the beginning of the tasting period, although none of them were statistically significant. The reduction smell in wine is mainly caused by volatile sulfur compounds (VSCs), such as H_2S, MeSH and DMS (Franco-Luesma, Saenz-Navajas, Valentin, Ballester, Rodrigues, & Ferreira, 2016; Mestres, Busto, & Guasch, 2000). In wine R, its intensity dramatically decreased after 5 min (Fig. 1D), the most likely explanation was the fast evaporation of the corresponding volatile sulfur compounds from the liquid phase. Otherwise, the suppression of fresh fruit may be caused by VSCs at relatively low concentrations (Franco-Luesma, Saenz-Navajas, Valentin, Ballester, Rodrigues, & Ferreira, 2016). Also, the alcoholic note in wine R fluctuated at low intensity (Fig. 1E), it is interesting that none of the panelists cited this aroma note at 5 min.

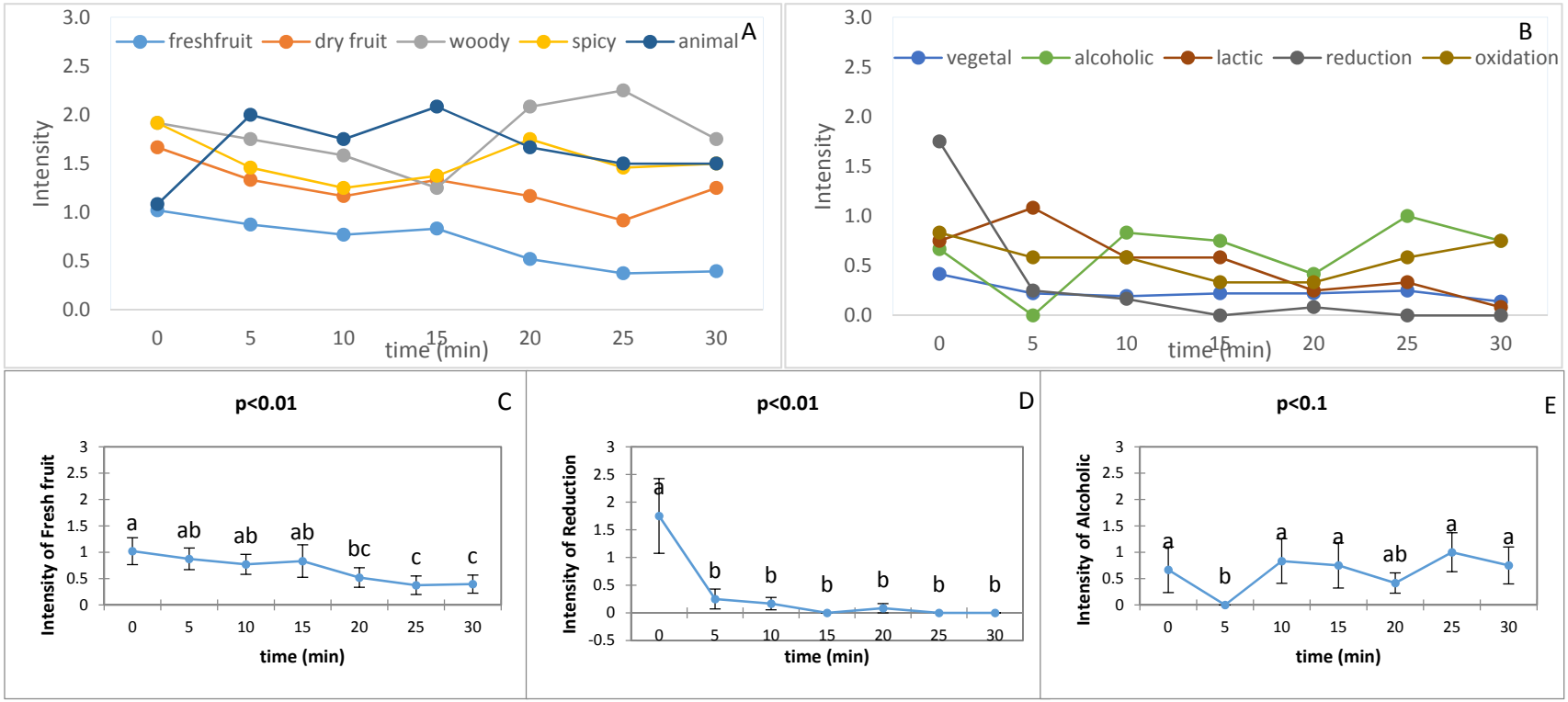

Fig. 1. Changes in the intensity of aroma notes in **Wine R** during the 30-min sensory evaluation session. Aroma notes with significant changes are shown according to 2-way ANOVA results (different letters indicate mean is significantly different among times at $p < 0.1$ by Fisher's test). Error bars are standard error of each note at each olfaction.

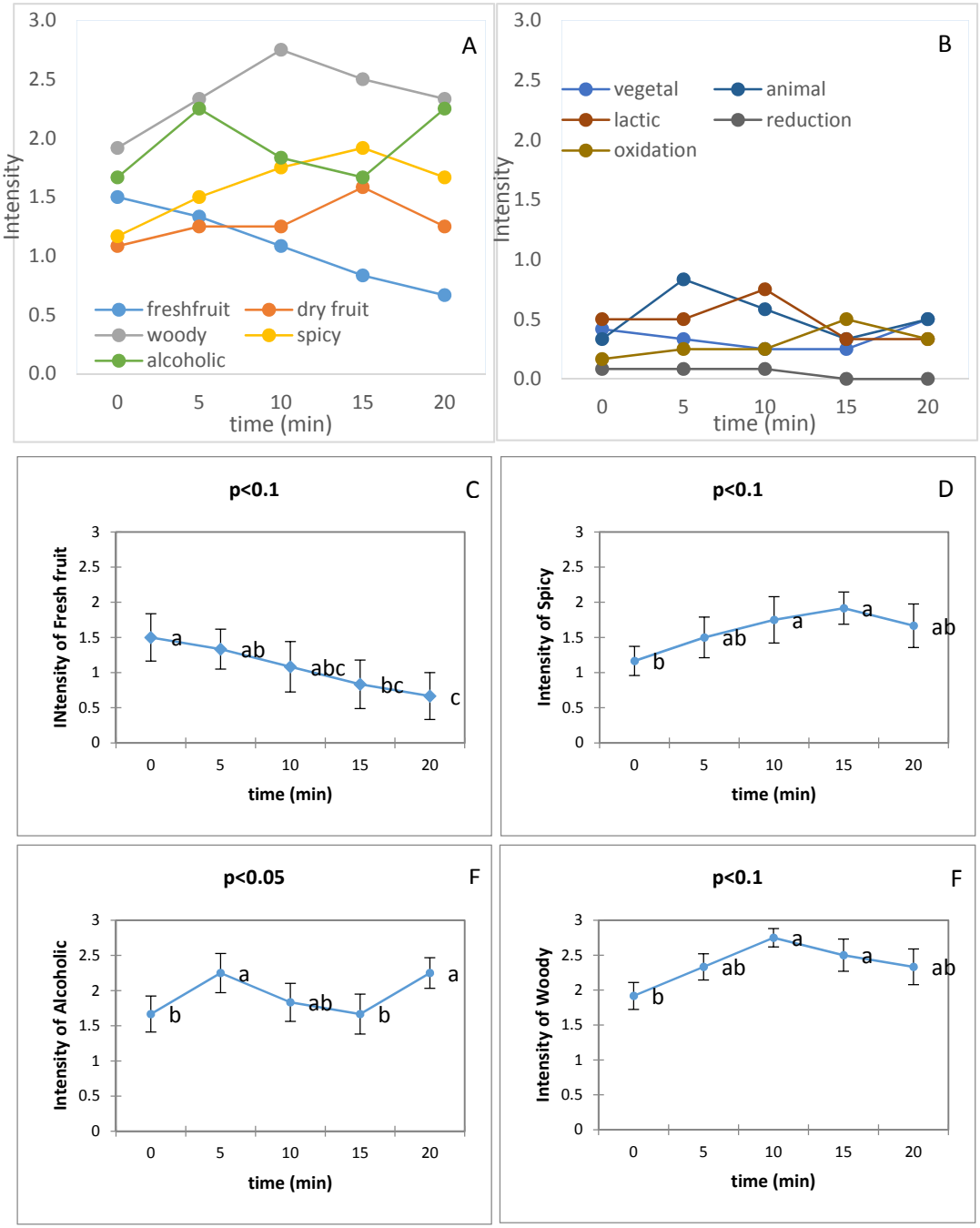

Fig. 2. Changes in the intensity of aroma notes in **Wine V** during the 20-min sensory evaluation session. Aroma notes with significant changes are shown according to 2-way ANOVA results (different letters indicate mean is significantly different among times at $p < 0.1$ by Fisher's test). Error bars are standard error of each note at each olfaction.

Although the tasting period was compressed to 20 min for wine V, the intensity of several aroma notes still presented an evident time-dependent behavior. As shown in Fig. 2A, the average intensities of woody and alcoholic remained identical (>1.5) through the session. Besides, dry fruit and spicy showed a constant increase trend until 15 min, while the fresh fruit behavior was similar to wine R, showing a decay trend throughout the whole session. However, the other aroma notes shown in Fig. 2B were less perceived, showing low intensity (<1) over the whole tasting period. The evolution of the aroma notes which significantly changed was presented from Fig. 2C to F according to the results of the 2-way ANOVA. As mentioned before, the fresh fruit of wine V showed the same decay trend as wine R (Fig. 2C), and the intensity of alcoholic also fluctuated but at a higher intensity level (>1.5) (Fig. 2E). Specifically, spicy and woody showed an increasing trend and their intensities remained significantly higher than the first olfaction from 10 min and then slightly decreased at 20 min (Fig. 2D and F), which were opposite to their corresponding intensities for wine R (Fig. 1A).

3.2. Change of headspace composition above wine glass

The volatile compounds of the two wines were analyzed at different time points for both liquid phase and headspace by the methods above. The results shown in Table 3 are in high agreement with our previous observations. In both wines, the decay of fatty acid esters in the vapor phase was from 17% to 44% after each tasting session due to evaporation. However, the decreases were less than we observed in previous chapters since a gentler shaking strategy was applied in this study. It is interesting to notice that their

concentrations in the liquid phase correspondingly decreased in most cases, except ethyl hexanoate and ethyl decanoate whose concentration increased after the tasting period (Table 3), indicating that the release of long-chain esters might be more sensitive to the changes of wine matrix due to their relatively high hydrophobicity (Boothroyd, Linforth, & Cook, 2012). For fusel alcohols, aldehydes, acids and other studied compounds, their contents in the vapor phase remained constant over time, except 2-phenylethyl acetate for wine V that slightly decreased and hexanoic acid and 4-ethylphenol for wine R that increased. However, the wine V contained very few 4-ethylphenol and 4-ethylguiacol resulting in high variability in the headspace content determination caused by a close concentration to the limit of detection. Another significant point to take into account is that, despite its volatility, the ethanol content of the wines remained constant over time without significant changes (data not shown).

Table 3. The concent change in liquid phase (represent by concentration) and vapor phase (represent by area) for two wines at the beginning and the end of sensory sessions, respectively.

	Concentration in liquid phase (mg/L)		Changes with time *		Concentration in liquid phase (mg/L)		Changes with time *	
Compound	Wine R-0 min	Wine R-30 min	Liquid phase	Vapor phase	Wine V-0 min	Wine V-20 min	Liquid phase	Vapor phase
Ethyl acetate	151	119	-21%	**-17%**	113	100	-11%	**-17%**
Ethyl butyrate	0.13	0.09	-32%	**-25%**	0.13	0.11	-15%	**-27%**
Ethyl hexanoate	0.17	0.20	14%	**-23%**	0.29	0.34	19%	**-30%**
Ethyl octanoate	0.16	0.11	-28%	**-24%**	0.16	0.15	-7%	**-36%**
Ethyl decanoate	0.076	0.086	13%	**-30%**	0.65	0.72	11%	**-44%**
Ethyl isobutyrate	0.21	0.14	-31%	**-29%**	0.20	0.15	-23%	**-31%**
Ethyl 2-methylbutyrate	0.044	0.028	-36%	**-28%**	0.042	0.035	-17%	**-29%**
Ethyl isovalerate	0.077	0.048	-38%	**-29%**	0.082	0.065	-22%	**-33%**
Isoamyl acetate	0.20	0.14	-31%	**-25%**	0.20	0.20	0%	**-28%**
Ethyl lactate	256	257	0%	3%	105	90.1	-14%	-12%
Diethyl succinate	41.7	42.1	1%	3%	14.9	10.8	-27%	-9%
2-Phenylethyl acetate	0.0102	0.0106	4%	-6%	0.0113	0.0116	2%	**-23%**
Acetaldehyde	1.04	1.19	14%	-18%	0.699	0.551	-21%	19%
Acetoin	15.2	14.0	-11%	12%	31.2	26.8	-14%	-15%
Diacetyl	0.20	0.22	11%	3%	0.29	0.50	76%	-1%
β-Damascenone	0.00054	0.00059	9%	-1%	0.0015	0.0015	3%	-
Isobutanol	32.1	31.3	-3%	-2%	43.3	35.3	-18%	-7%
Isoamyl alcohol	216	200	-7%	-4%	255	235	-8%	-14%
β-Phenylethanol	45.6	44.5	-2%	-5%	48.1	42.9	-11%	-11%
Ethanoic acid	933	804	-14%	17%	852	686	-19%	-17%
Butyric acid	1.39	1.27	-9%	8%	0.137	0.128	-7%	-31%
Hexanoic acid	2.38	2.44	2%	**24%**	1.73	1.62	-7%	-22%
Guaiacol	0.0134	0.0142	5%	1%	0.02	0.02	35%	-37%
4-Ethylguaiacol	0.109	0.113	4%	7%	0.0045	0.0044	-1%	-35%
4-Ethylphenol	1.09	1.13	4%	**7%**	0.01	0.01	-1%	-21%
t-Whiskylactone	0.095	0.100	5%	31%	0.14	0.14	3%	-1%
γ-Butyrolactone	14.9	13.7	-8%	12%	26.4	20.4	-23%	-11%

*Changes in the liquid phase and vapor phase are calculated for each wine by normalizing the concentration in the liquid phase and vapor phase of the sample from the last olfaction of each sensory session against the corresponding data of the first one, respectively. Significant differences in headspace content between samples of each wine are indicated in bold by ANOVA ($p \leq 0.05$).

The mass in the headspace for selected volatile compounds were determined by spiking known amounts of their standards (Table 2) onto the fritted end of a Tenax TA tube. Their mass in the vapor phase for each olfaction was thus calculated against the corresponding area/mass of the spiked standards. However, the mass of the more volatile compounds might be overestimated due to their fast

evaporation when waiting for the thermal desorption. Our first consideration was to calculate the odor active value (OAV) for each volatile compounds at each olfaction point to give the potential importance of the volatile compounds. However, odor thresholds in the air were not measured in our study. Looking into the previous literature, very limited compounds were available, but most of them were detected well before 2000 by different methodologies. The differences for the same compound were in the range of several orders of magnitude, which made questionable the reliability of the reported data. Therefore, the calculated mass was directly used in this study instead of the OAVs in air. Eight compounds from different chemical classes were selected to show the evolution over the whole tasting for each wine (Fig. 3). Generally, the evolution trends of volatile compounds were in accordance with Chapter 3, but the changes were less dramatic due to the gentler shaking during the sensory sessions. The mass in the vapor phase of esters such as ethyl decanoate followed the known decay trend, although statistical significance was not found until the last time points (Fig. 3). Ethyl isobutyrate for wine R was slightly different, with a mild increase after 5 min and then the general decay. Esters are widely reported as aroma contributor to fruity notes (Ferreira, Sáenz-Navajas, Campo, Herrero, de la Fuente, & Fernández-Zurbano, 2016; Lytra, Tempere, Zhang, Marchand, de REVEL, & Barbe, 2014). Their decay trend in the vapor phase in our study was in agreement with the intensity decrease of fresh fruit in both wines (Fig. 1C and 2C). Meanwhile, the alcoholic note is related to ethanol and fusel alcohols in wines which are the major and essential volatile compounds for wines and the so-called wine aroma buffer (de-la-Fuente-Blanco, Saenz-

Navajas, & Ferreira, 2016; Ferreira, de la Fuente, & Sáenz-Navajas). As shown in Fig.3, the content of isoamyl alcohol remained constant in the headspace over time in both wines, as well as ethanol (data not shown). The intensity and steadiness of the alcoholic note (Fig. 1E and 2E) of both wines may be related to their constant concentration in the headspace. The rest of the compounds in Fig. 3 were stable over the tasting period, which was in agreement with our previous observations. Some of these compounds were frequently studied for their ability to combine with nonvolatile compounds in wines (Muñoz-González, Rodríguez-Bencomo, Moreno-Arribas, & Pozo-Bayón, 2011; Pozo-Bayón & Reineccius, 2009). Among them, 4-ethylphenol and whiskylactone were related to animal and woody, respectively. The wine V contained much less 4-ethylphenol than wine R, which might result in the intensity variations of animal note for two wines (Fig. 1A and Fig. 2B). Moreover, both of them were reported as suppressors to fruity notes, as well as fusel alcohols (Atanasova, Thomas-Danguin, Langlois, Nicklaus, & Etievant, 2004; Cameleyre, Lytra, Tempere, & Barbe, 2015; de-la-Fuente-Blanco, Fernández-Zurbano, Valentin, Ferreira, & Sáenz-Navajas, 2017; de-la-Fuente-Blanco, Saenz-Navajas, & Ferreira, 2016). However, the perceptive interactions between volatile compounds are complicated, especially during the real wine tasting and cannot be assessed only by the study of the individual compounds.

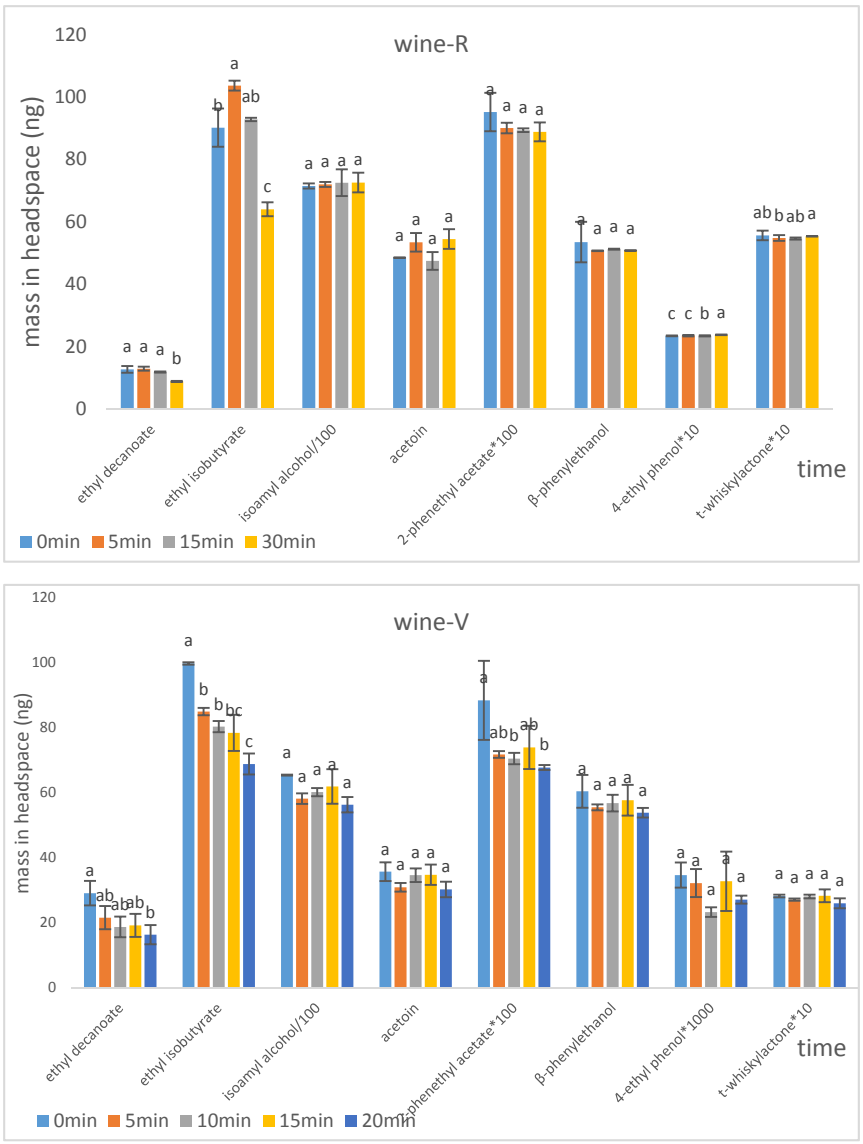

Fig. 3. The mass changes of volatile compounds in the headspace over time. Error bar indicates standard error. Different letters mean significant differences according to post-hoc test.

3.3. Correlation between volatile compounds and aroma notes

Pearson's correlation test was applied to study the relationship between volatile compounds and aroma notes by using the corresponding mass and intensity at different time in both wines. As

shown in Table 4, many significant correlations were found between volatile compounds and 6 of the studied aroma notes. However, after the visual inspection of the correlation graphs, it was obvious that some of them (marked with asterisks in Table 4) were artifactual correlations caused by the large disparity in the concentration of some compounds between both wines. Although these correlations reflected the ability of our tasters and analytical methods to discriminate between the differences in intensity and concentration, they did not allow to study the dynamic evolution of the sensory profile. Therefore they will not be discussed here. After this preliminary screening there still remained interesting significant correlations.

Fresh fruit was positively correlated with dimethyl sulfide and several esters, the correlation graphs of dimethyl sulfide and ethyl octanoate are shown in Fig. 4A and B. Although isolated dimethyl sulfide can be considered as an off-flavor due to its characteristic odor, in combination with other compounds it has been previously reported as an enhancer of fruity notes in wine (Escudero, Campo, Fariña, Cacho, & Ferreira, 2007; Segurel, Razungles, Riou, Salles, & Baumes, 2004). More recently, Lytra et al. (2016) have confirmed the active role of dimethyl sulfide in the dynamic perception of red berry and fresh fruit notes together with ethyl esters and acetates. These reports are in full agreement with the observations in table 4, where the sensory evolution during tasting in the fresh fruit note of wine V and R is correlated not only with dimethyl sulfide but also with the concentration of several ethyl esters and isoamyl acetate in the headspace above the wine glass. 2-Phenylethanol was also correlated with the fresh fruit note.

Table 4. Relevant correlations of aroma notes and volatile contents in the headspace. Significant coefficients are in bold ($p<0.05$).

Compound	Fresh fruit	Alcoholic	Animal	Woody	Lactic	Oxidation
Dimethyl sulfide	**0.800**	**0.763 ***	**-0.802**	0.613	-0.025	**-0.759 ***
Ethyl acetate	-0.367	**-0.947 ***	**0.880 ***	**-0.793 ***	0.398	**0.686 ***
Ethyl isobutyrate	0.633	-0.414	0.296	-0.386	**0.780**	-0.177
Ethyl butyrate	**0.716**	-0.275	0.112	-0.206	**0.763**	-0.275
Ethyl lactate	-0.479	**-0.922 ***	**0.873 ***	**-0.799 ***	0.231	**0.731 ***
Ethyl 2-methylbutyrate	**0.802**	-0.154	0.073	-0.128	**0.760**	-0.382
Ethyl 3-methylbutyrate	0.464	-0.611	0.441	-0.515	**0.784**	0.036
Isoamyl acetate	**0.851**	0.544	-0.632	0.495	0.256	**-0.739**
Ethyl hexanoate	**0.880**	0.167	-0.317	0.145	0.493	-0.562
Diethyl succinate	-0.449	**-0.919 ***	**0.851 ***	**-0.826**	0.173	**0.724 ***
Ethyl octanoate	**0.918**	0.315	-0.474	0.215	0.306	-0.597
2-Phenethyl acetate	-0.063	**-0.840**	0.622	**-0.828**	0.241	0.553
Ethyl decanoate	**0.833**	0.643	**-0.730**	0.440	-0.014	**-0.736**
Acetoin	-0.479	**-0.958**	**0.826**	**-0.734**	0.230	**0.738**
Isobutanol	-0.454	**-0.936**	**0.860 ***	**-0.794 ***	0.259	**0.741 ***
Isoamyl alcohol	-0.178	**-0.962**	**0.724**	**-0.759**	0.406	0.638
2-Phenylethanol	**0.709**	0.664	**-0.869**	0.587	-0.131	-0.582
Guaiacol	0.545	0.638	**-0.777**	0.612	-0.256	-0.499
4-Ethylguaiacol	-0.492	**-0.923 ***	**0.883 ***	**-0.798 ***	0.230	**0.730 ***
4-Ethylphenol	-0.492	**-0.917 ***	**0.883 ***	**-0.802 ***	0.226	**0.726 ***
γ-Butyrolactone	0.422	**0.779 ***	**-0.859**	0.665	-0.321	**-0.667**
t-Whiskylactone	0.380	**0.886 ***	**-0.736 ***	**0.695**	-0.331	**-0.813**
β-Damascenone	-0.475	**-0.919 ***	**0.883 ***	**-0.802 ***	0.251	**0.723 ***

Correlations marked with * are actually artifactual correlations due to the disparity in contents of the two wines for some particular compounds.

The results for the alcoholic note were somehow surprising. Apart from the artifactual correlations, the data analysis revealed four negative correlations with this note: 2-phenylethyl acetate, acetoin, isobutanol and isoamyl alcohol (Fig. 4C). These last two compounds are the main major alcohols in wine and also the main contributors to the buffering characteristics of wine aroma (de-la-Fuente-Blanco, Saenz-Navajas, & Ferreira, 2016). Their role in wine

aroma characteristics and quality is not clear, with controversial data reported in the scientific literature, although there are evidence that they are most likely detrimental to the perception of wine aroma (de-la-Fuente-Blanco, Sáenz-Navajas, & Ferreira, 2017). Given their odor characteristics (solvent-like, fusel), a priori hypothesis would have been to expect a positive correlation between these two compounds and the alcoholic note. However, the opposite was found. There are several potential explanations for this finding. Firstly, the alcoholic note generated by higher alcohols has been shown to be wine dependent (de-la-Fuente-Blanco, Saenz-Navajas, & Ferreira, 2016) (de-la-Fuente-Blanco et al. 2016). Secondly, the existence of strong perceptual interactions or synergistic mechanisms between the alcoholic note and other sensory notes in wine is highly probable (Gottfried, 2010; Laing, Eddy, & John Best, 1994; Le Berre, Atanasova, Langlois, Etiévant, & Thomas-Danguin, 2007). Finally, although the tasters were previously trained, the evaluation of the alcoholic note could have been confused with other nuances, like for example the alcoholic perception of ethanol and not exactly the more solvent-like note of major alcohols.

The animal note was anti-correlated with dimethyl sulfide, ethyl decanoate, 2-phenylethanol and guaiacol, and positively correlated with acetoin and isoamyl alcohol (Fig. 4D). Animal notes have been previously explained by the presence of ethylphenols and vinylphenols (Aznar, López, Cacho, & Ferreira, 2003; Chatonnet, Dubourdieu, & Boidron, 1995). In our study, 4-ethylphenol and 4-ethylphenol were two orders of magnitude more concentrated in the Rioja wine (R) than in the Campo de Borja wine (V) which clearly differentiated both wines in the perception of the animal note from a

dominant note (wine R) to a almost not detected note (wine V). This disparity produced the graph shown in figure 4E. Considering only wine R, the animal note increased after the first tasting to remain stable after 5 min (Fig. 1A), while the 4-ethylphenol content in the headspace (Fig. 3) slightly increased. The increase in the perception of the animal note could be a perceptual interaction with the simultaneous decrease observed on the fruity note (San-Juan, Ferreira, Cacho, & Escudero, 2011).

Woody note was correlated with t-whiskylactone (Fig. 4F), which should be not surprising given that t-whiskylactone is the strongest oak-wood aroma component (Ferreira et al. 2016). The variance explained by the correlation coefficient was below 70% which suggest that other compounds are probably involved in the perception of this note. The evolution of the content in the headspace of four esters including the 3 branched ethyl esters was positively correlated with the temporal change of the lactic note (Table 4). Finally, the oxidation note was positively correlated with acetoin content and negatively correlated with isoamyl acetate, ethyl decanoate, γ-butyrolactone and t-whiskylactone.

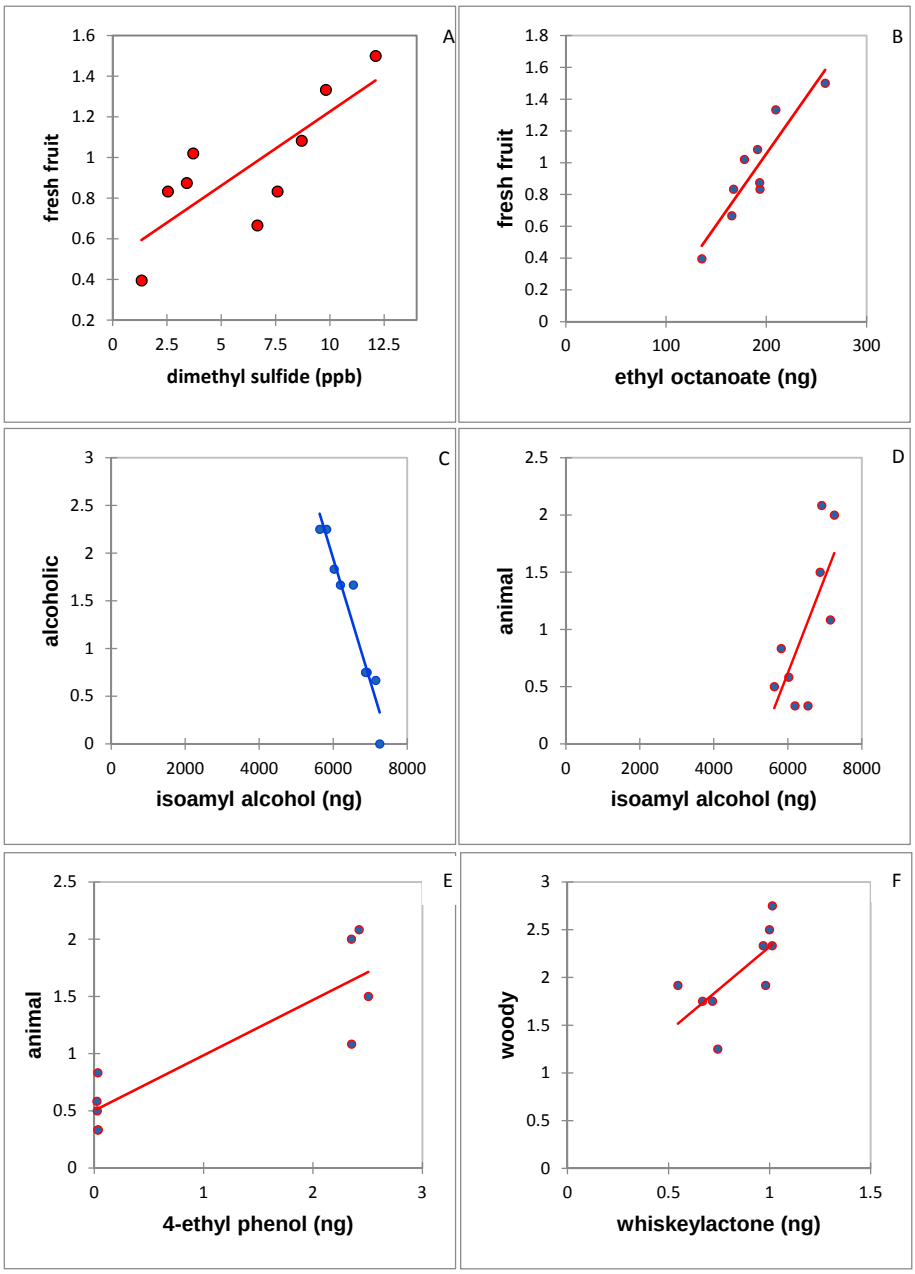

Fig. 4. Correlation graphs of the content of volatile compounds in headspace over time versus the intensity of aroma notes over time.

4. Conclusions

The present chapter simultaneously studied the changes of aroma intensity and headspace contents during real wine tasting. To our knowledge, it is the first time that a complete analysis of the headspace is combined with a sensory analysis in dynamic tasting conditions. Despite the high complexity of the tasting of complex premium wines combined with parallel analytical methods, the obtained results have provided very interesting data.

The result of the descriptive analysis showed that the intensity of some aroma notes evolved during consumption, especially for fresh fruit, alcoholic, spicy and woody. Also, the content of volatile compounds in the headspace above wine glass changed over time in different ways according to their physicochemical characters. Furthermore, the temporal evolution of aroma notes was partly correlated to the content changes in the headspace, especially the relationship between fresh fruit and esters and dimethyl sulfide. Alcoholic note was anti-correlated with major alcohols, pointing to complex perceptual interactions. Other notes like animal or woody showed variations related with compounds like 4-ethylphenol or t-whiskylactone.

The work presented in this chapter is the culmination of the previous chapters, all of them dedicated to the study of the analysis of dynamic headspace evolution in wine tasting. Thanks to the analytical method developed in the first chapter to study wine headspace, it has been possible to confirm in real wine tasting conditions that our previous findings about the behavior of compounds released to the headspace can explain some of the

sensory changes and characteristics of wine aroma profile. The work presented here opens the door to new possibilities in the field of the study of the dynamic changes in wine aroma. Future challenges include, among others, the development of improvements in the headspace analytical method to achieve quantification of ultra-trace compounds of relevance for aroma (e.g., varietal thiols), and the normalization of sensory analysis to obtain data less affected by taster variability.

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CHAPTER 7

Determination of ppq-levels of Alkylmethoxypyrazines in Wine by Stir-bar Sorptive Extraction Combined with Multidimensional Gas Chromatography-Mass Spectrometry

CHAPTER 7

Determination of ppq-levels of alkylmethoxypyrazines in wine by stir-bar sorptive extraction combined with multidimensional gas chromatography-mass spectrometry

1. Introduction

3-Alkyl-2-methoxypyrazines (MP) are a family of compounds whose presence has been amply reported in a variety of food products (Maga, 1992). These compounds are also well known for their contribution to wine aroma. The importance of MP in the aroma of wine has been widely studied since the first report of 3-isobutyl-2-methoxypyrazine (IBMP) in Cabernet Sauvignon grapes in 1975 (Bayonove, Cordonnier, & Dubois, 1975). The presence of IBMP, 3-sec-butyl-2-methoxypyrazine (SBMP) and 3-isopropyl-2-methoxypyrazine (IPMP) has been related with the green and vegetative aromas characteristic of some wines made with Cabernet Sauvignon, Sauvignon Blanc, Merlot or Cabernet Franc grapes (Allen, Lacey, Harris, & Brown, 1991; Chapman, Thorngate, Matthews, Guinard, & Ebeler, 2004; Escudero, Campo, Fariña, Cacho, & Ferreira, 2007; Preston, Block, Heymann, Soleas, Noble, & Ebeler, 2008; Roujou de Boubée, Van Leeuwen, & Dubourdieu, 2000; Sala, Busto, Guasch, & Zamora, 2005). Although there are some wine styles with notable MP levels, it has been demonstrated that these compounds exert a negative influence on the perception of wine fruitiness (Campo, Ferreira, Escudero, & Cacho, 2005; Hein, Ebeler, & Heymann, 2009). They have also been found to take part in negative vectors of quality in premium Spanish red wines

(Ferreira, Juan, Escudero, Culleré, Fernández-Zurbano, Saenz-Navajas, et al., 2009) and IBMP has even been considered as a marker for grape unripeness (Roujou de Boubée, Van Leeuwen, & Dubourdieu, 2000). The origin of these compounds is mostly endogenous as they form part of the chemicals produced in the first stages of grape development, their levels being strongly correlated with vine vigor and shade conditions (Ryan, Watkins, Smith, Allen, & Marriott, 2005; Ryona, Pan, Intrigliolo, Lakso, & Sacks, 2008). Nevertheless, the isopropyl isomer may have its origin in the infestation of the vine by the multicolored Asian lady beetle, Harmonia axyridis (Botezatu, Kotseridis, Inglis, & Pickering, 2013).

MP have extremely low sensory detection thresholds. In the case of IBMP, detection thresholds of 10 ng/L in red wine have been reported (Kotseridis, Anocibar Beloqui, Bertrand, & Doazan, 1998), although there is evidence of IBMP modifying the aroma of wine in concentrations as low as 1 ng/L (Allen, Lacey, Harris, & Brown, 1991). IPMP may be still more powerful since its thresholds can be as low as 0.3 ng/L in white wine or 2.3 ng/L in red (Pickering, Karthik, Inglis, Sears, & Ker, 2007). In addition, these compounds can act additively (Campo, Ferreira, Escudero, & Cacho, 2005), or can even interact with some oxidation compounds to intensify unpleasant aroma attributes (Coetzee, Brand, Emerton, Jacobson, Silva Ferreira, & du Toit, 2015). Thus, MPs have a significant impact on wine aroma at very low concentrations levels, and this has led to continuous improvement in their analytical determination. Early determination strategies involved laborious methods of sample preparation based on distillation and selective isolation in the cation-exchange resin (Lacey, Allen, Harris, & Brown, 1991). Methods

developed in the last decade are simpler and mainly based on headspace solid-phase microextraction (HS-SPME) (Callejón, Ubeda, Ríos-Reina, Morales, & Troncoso, 2016) or solid-phase extraction (SPE) (Culleré, Escudero, Campo, Cacho, & Ferreira, 2009; López, Gracia-Moreno, Cacho, & Ferreira, 2011).

However, it has been acutely pointed out (Schmarr, Ganß, Koschinski, Fischer, Riehle, Kinnart, et al., 2010) that both HS-SPME and SPE have limited extraction selectivity in a complex matrix such as wine. Considering the low detection limits required for MP monitoring, together with the matrix complexity, one-dimensional gas chromatographic analysis with mass spectrometric detection (GC–MS) involves a high risk of co-elution in critical cases (Schmarr, et al., 2010). For this reason, various authors have developed different two-dimensional chromatographic techniques for MP analysis in wine or wine grapes (Legrum, Slabizki, & Schmarr, 2015; Ryan, Watkins, Smith, Allen, & Marriott, 2005; Ryona, Pan, & Sacks, 2009). Despite its very high separation efficiency, GC×GC is probably not the simplest approach for a limited number of target analytes as is the case with MP. Due to its more straightforward experimental setup and easiness for data processing, heart-cut multidimensional chromatography (MDGC) has also been applied to the analysis of MP in wine by combing SPE or SPME (Botezatu, Pickering, & Kotseridis, 2014; Culleré, Escudero, Campo, Cacho, & Ferreira, 2009; Kögel, Botezatu, Hoffmann, & Pickering, 2014). More selectivity could be obtained through tandem mass spectrometry MDGC–MS/MS (Legrum, Gracia-Moreno, Lopez, Potouridis, Langen, Slabizki, et al., 2014). Ochiai et al. (2011) proposed a MDGC–MS combined with

olfactometry and with preparative fraction collection for the determination of IBMP among other off-flavors.

Among the variety of sample preparation techniques applied to wine aroma analysis, stir-bar sorptive extraction (SBSE) (Baltussen, Sandra, David, & Cramers, 1999) has several advantages that make it a good choice for the analysis of MP in wine. Compared with SPE, SBSE is more easily automated and requires no solvent; on the other hand, SBSE has a much more considerable amount of sorbent than SPME, which results in a higher sample extraction capacity and consequently better sensitivity and fewer matrix effects (David & Sandra, 2007). To the best of our knowledge, Franc et al. reported the first application of SBSE to IBMP analysis in wine (Franc, David, & de Revel, 2009). Comparing it with other aroma extraction methods, Gamero et al. (2013) found that SBSE was the most sensitive extraction method for IBMP. Very recently, SBSE has also been applied to the determination of MP in Chinese Syrah wines (Zhao, Gao, Qian, & Li, 2017). Due to the increase in the amount of volatiles extracted by SBSE compared with other techniques, the risk of interference and column overloading is also increased. Hjelmeland et al. (2016) addressed this challenge coupling SBSE with GC–MS/MS for a more selective method of MP determination.

In the present study, we also propose a method for the determination of MP in wine using SBSE, but the required additional selectivity is provided by a simpler and more affordable experimental setup based on MDGC–MS using only one chromatographic oven. The method optimization and validation for the quantitative determination of MPs at pg/L levels are presented,

together with its application to a large number of wines.

2. Materials and methods

2.1. Wine samples

A commercial 2013 vintage Crianza red wine from La Rioja was used to optimize the extraction conditions. After optimization of the experimental parameters, a synthetic wine and four commercial Spanish wines of different grape varieties (Tempranillo red wine, Cabernet Sauvignon red wine, Cabernet Sauvignon rosé wine and Sauvignon Blanc white wine) were used for the validation of the proposed method.

A total of 111 different wine samples were analyzed with the proposed method. These samples were elaborated using non-commercial, recently identified grape cultivars from the regions around the Pyrenean massif. The 56 French wine samples comprised 8 white, 24 rosé and 24 red wines, while the 55 Spanish wine samples were made up of 11 white, 9 rosé and 35 red wines. All the wines were produced in the same conditions and with the same yeast starter cultures for each category (white, rosé and red).

2.2. Reagents and standards

3-Isopropyl-2-methoxypyrazine (IPMP), 3-isobutyl-2-methoxypyrazine (IBMP), citric acid and trisodium citrate dihydrate were purchased from Sigma-Aldrich (Steinheim, Germany). Ethanol was supplied by Merck (Darmstadt, Germany) and tartaric acid was provided by Panreac (Barcelona, Spain). Water was purified in a Milli-Q system supplied by Millipore (Bedford, Germany). The

citrate buffer was prepared with 8% (v/v) of 0.5 M citric acid and 92% (v/v) of 0.5 M trisodium citrate dihydrate.

Deuterated standards of MP (3-alkyl-2-[$2H^3$] methoxypyrazines) were chosen as internal standards. The deuterated MP were synthesized in-house as described previously (Schmarr, Sang, Ganß, Koschinski, & Meusinger, 2011). The internal standards solution was prepared with the deuterated MP in ethanol.

Stir-bars coated with 126 µL polydimethylsiloxane (PDMS, 20 mm length × 1.0 mm thickness) were obtained from Gerstel (Müllheim an der Ruhr, Germany). Before the first use, each stirbar was conditioned at 300 °C under constant helium flow for 2 h.

2.3. Sample preparation

2.3.1. Optimization

A Spanish Crianza red wine was used to optimize the extraction parameters: dilution factor, pH and extraction time. Such sample was firstly spiked with deuterated and non-deuterated MP at 40 ng/L. Then, three 5 mL-volumes of the spiked sample were taken, the first one was directly extracted, the second diluted with 1 mL of Milli-Q water (1.2 dilution factor), and the third with 5 mL (2.0 dilution factor). Then, with the optimum dilution factor, the effect of pH was studied by adding 1 mL of Milli-Q water or citric acid-sodium citrate buffer to adjust the pH to 5.4. Finally, extraction times of 15, 30 and 60 min were evaluated under the optimized dilution and pH conditions. Each condition was analyzed in triplicate.

2.3.2. Proposed method

Five mL of the sample was transferred into a clean 25 mL

Erlenmeyer flask, and 1 mL of 0.5 M citric acid-sodium citrate buffer was added to the same flask to adjust the pH to 5.4. Then 50 µL of the internal standards solution were added. A conditioned stirbar was inserted into the flask using tweezers. The closed flask was placed onto a 20-position magnetic stirrer (Gerstel, Mülheim an der Ruhr, Germany), then stirred at room temperature and 750 rpm for 30 min. After extraction, the stirbar was removed from the flask, rinsed briefly with Milli-Q water and dried with a lint-free tissue. Each stirbar was then transferred into a thermal desorption tube which was placed in the autosampler tray for analysis.

2.4. Thermal desorption

The stirbar was desorbed using a thermal desorption unit (TDU) and a cryo-cooled injection system (CIS 4) with programmable temperature vaporization (PTV) inlet (Gerstel, Müllheim an der Ruhr, Germany). The stirbar was thermally desorbed in the TDU in splitless mode. The TDU temperature was programmed from 25 °C (held for 1 min) at 60 °C/min to 270 °C (held for 7 min). The transfer line of the TDU was kept at 250 °C. The initial temperature of the CIS was set at -80 °C using liquid nitrogen. The CIS was then heated to 250 °C at a rate of 12 °C/s and held for 30 min to inject the trapped compound into the capillary columns in solvent vent mode. Complete desorption of the MP under these conditions was checked by running a blank analysis of a recently used stirbar.

2.5. Multidimensional gas chromatography

The analysis was performed using an Agilent 7890A gas chromatograph equipped with a Deans switch device (Agilent

Technologies, USA) allowing the selective transfer of heart cuts from the first column to the second. The oven temperature was first held at 45 °C for 4.5 min and then increased by 6 °C/min to 220 °C. The first column was a DB-5MS column (15 m length, 250 µm i.d., 0.25 µm film thickness) (J&W Scientific, Folsom, CA, USA) combined with a flame ionization detector (FID) and the Deans switch. An uncoated, deactivated column (6.7 m length, 180 µm i.d.) from Agilent was used as a restrictor between the FID detector and the Deans switch. The carrier gas helium was delivered at a constant pressure of 36 psi. The FID was kept at 280 °C and operated with 40 mL/min hydrogen and 450 mL/min air. Under the described conditions, the MP and their corresponding deuterated standards were eluted from column 1 between 13.7 and 17.0 min, and consequently, the Deans switch system was programmed for two cuts. The first cut between 13.7 min and 14.2 min was for IPMP and IPMP-d3, and the second cut was from 16 min to 17 min for SBMP, IBMP and the deuterated standards for both compounds.

The second column was a SAPIENS-WAX MS (Teknokroma, Barcelona, Spain) (30 m length, 250 µm i.d., 1 µm film thickness) directly connected to an Agilent 5975C mass spectrometer. The pressure was kept constantly at 31 psi. A quadrupole mass detector was operated in selected ion monitoring mode (SIM) with electron ionization. The temperature of the ion source was set at 230 °C and the transfer line was kept at 240 °C. Quantifier ions were m/z 137, 138 and 124 for IPMP, SBMP and IBMP, respectively, and 140, 141, and 127 for their related deuterated standards. Qualifier ions were 152 (36%) and 124 (23%) m/z for IPMP (155 (38%) and 127 (25%) for IPMP-d3), 151 (46%) and 124 (58%) for SBMP (127 (55%) for

SBMP-d3) and 151 (18%) for IBMP (154 (16%) for IBMP-d3). Values in brackets are relative proportions of abundance (%) to base peak.

2.6. Method validation

A set of six concentrations ranging from 0.1 to 15 ng/L of the studied MP (Table 1) and 5 ng/L of the deuterated MP were spiked into both the model wine (13% vol. Ethanol, pH 3.4 and 5 g/L tartaric acid) and a Tempranillo red wine, and then analyzed by the optimized SBSE-TD-MDGC-MS method in duplicate. The matrix effect was evaluated by comparing the slopes in both matrices with a t-test. Limits of detection (LOD) were defined as the amount of MP in a spiked red wine free of MP that produces, with the proposed method, a peak with a height equivalent to three times the average standard deviation of the baseline in the surrounding area to the ion peak. The lowest concentration of the calibration curves (Table 1) was considered as the limit of quantification. The method reproducibility was calculated by spiking a commercial red wine free of the analytes with 1 ng/L of each MP, and analyzing this wine 6 times during three weeks.

To assess the method accuracy, three commercial Spanish wines with different matrices were spiked with 0.5 ng/L of IPMP, SBMP and 4 ng/L of IBMP, after which the spiked and unspiked samples were analyzed in triplicate using the proposed method. Recovery was defined as the ratio (in %) between the amount of target analytes determined in the spiked sample minus that determined in the corresponding unspiked original sample to the exact concentration added in the wine sample.

Table 1. Linearity, limits of detection and repeatability of the method.

Compound	Concentration Range (ng/L)	Slope [a]	r^2 [a]	LOD [b] (ng/L)	Repeatability [b] (RSD %)
IPMP	0.22 - 14.9	0.2507	0.9999	0.07	10
SBMP	0.13 - 15.6	0.6044	0.9999	0.02	2
IBMP	0.11 - 14.7	3.161	0.9996	0.02	11

[a], measurement was made in model wine [b], data was measured in a spiked commercial red wine.

3. Results and discussion

3.1. Extraction optimization

The goal of the present analytical method was to determine MP in wine at sub-ng/L levels. With this objective, the following SBSE parameters were optimized during the development of the method: sample dilution factor, sample pH and extraction time.

Sorptive extraction with PDMS coating is based on the partition coefficient of the MP between the hydroalcoholic wine matrix and the PDMS phase. Since this silicone material is primarily an apolar phase, it is expected that the extraction efficiency of the MP will increase when decreasing the ethanol content of the wine matrix, which can be achieved by diluting the sample. To examine the effects of the matrix dilution on the signal, 3 different dilution conditions were tested: dilution factor of 1 (no dilution), 1.2 and 2. The results are shown in Fig. 1A. It was found that dilution had a significant effect. A dilution factor of 1.2 increased the recovery of all MP. However, a larger dilution of 2 not only did not improve the signal strength but produced a significant decrease. Despite the lower solubility of the MP in the most diluted sample, the larger phase ratio

between the PDMS on the stirbar and the sample led to a worse recovery of the analytes (Baltussen, Sandra, David, & Cramers, 1999). Therefore, dilution of the 5 mL of wine with 1 mL of water was chosen as the optimum.

MP have acid-base properties that can influence their extraction efficiency (Franc, David, & de Revel, 2009; Hjelmeland, Wylie, & Ebeler, 2016; López, Gracia-Moreno, Cacho, & Ferreira, 2011). For that reason, the pH of the sample was adjusted to 5.4 by adding 1 mL of a citrate buffer and compared to the same sample with its original pH of 3.6 but diluted with 1 mL of water. When the extraction was performed (Fig. 1B), the results showed that the extraction efficiency of the MP increased significantly at higher pH. Because MP is very weak alkalis with a pKa of around 0.5 (Boutou & Chatonnet, 2007), it is likely that most of the improvement observed in the extraction was due to an increase of the ionic strength rather than a change in the state of ionization of the MP. In any case, it was decided to choose a pH of 5.4 not only because of the better extraction but also to standardize the sample pH.

Fig. 1. (A) Effect of dilution factor on MPs recovery. Df 1: no dilution, Df 1.2: 5 mL of wine plus 1 mL of water, Df 2: 5 mL of wine plus 5 mL of water. (B) Effect of pH on MPs recovery. Error bars represent the standard error of the mean.

Finally, with the optimal conditions of dilution and pH, three different extraction times between 15 and 60 min were tested. The extraction time curves (Fig. 2) illustrated an increasing trend of the signal responses for all the studied MP over time. The results showed an increase of 35%–45% for all the analytes comparing results from 15 min and 30 min extraction. Although not statistically significant,

the 60 min extraction showed an increasing trend in the recovery of the MP. As a compromise between acceptable extraction efficiency and sample preparation time, a 30 min extraction time was selected for the proposed method.

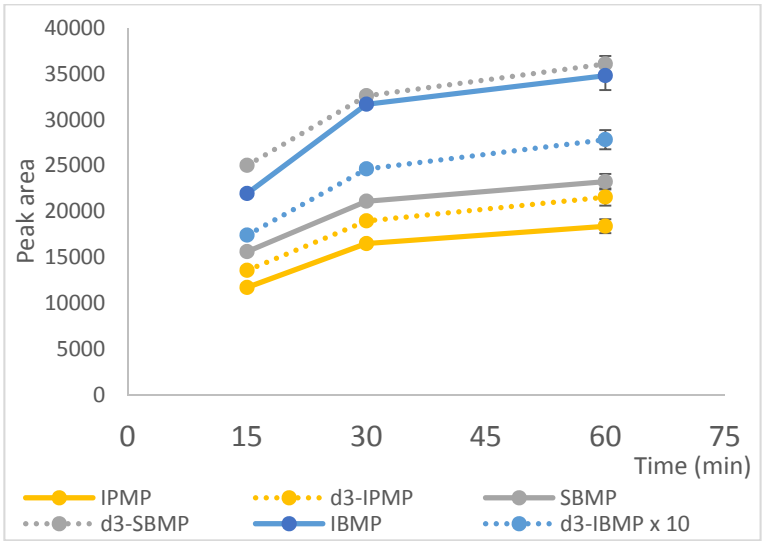

Fig. 2. Effect of extraction time on MPs recovery. Error bars represent the standard error of the mean.

3.2. Method validation

The corresponding deuterated isotopologues were used as internal standards for each MP. As shown in the method optimization, these compounds showed similar behavior to that of the targeted MP in all the procedural steps.

Method linearity, repeatability, detection and quantitation limits were assessed by spiking a model wine and a red wine free of the analytes with levels between 0.1 ng/L and 15 ng/L. In order to evaluate the absence of matrix effects, a statistical comparison of the

slopes of the calibration curves between the model and the red wines was carried out. The statistical results showed no significant differences (results not shown). Linearity covered all the range tested with determination coefficients above 0.999 in all cases (Table 1), which can be considered highly satisfactory. Method detection limits were extremely low thanks to the separation power of multidimensional chromatography (Fig. 3), to the selectivity of MS detection and the high recovery efficiency of SBSE. In fact, the method detection limits were 0.02 ng/L for SBMP and IBMP, and 0.07 for IPMP (Table 1), which, to the best of our knowledge, are the lowest published detection limits for MP in wine. These improved detection limits are possible first because in the optimized analytical strategy a large fraction of all the MP present in 5 mL of wine is transferred to the GC–MS, thanks to the large extraction capacity of the SBSE twister. Although extraction recoveries were not calculated, theoretical calculations based on logP and extraction times (Baltussen, Sandra, David, & Cramers, 1999) suggest that the fraction extracted was in all cases above 80%. I.e., nearly all analytes present in 4.5 mL of wine are introduced into the system, which is 1–2 orders of magnitude above what can be extracted by SPME from wine or what is usually introduced in the regular injection (1–2 μL) of a concentrated SPE extract. Second, the heart-cut MDGC makes it possible to sort out the serious column overload associated with the introduction of all the material extracted by the twister. By transferring selected fractions of the overloaded separation obtained in the first dimension to the second one, perfectly resolved chromatographic peaks and very clean baselines are obtained. This cannot be attained with MS/MS approaches, which can solve the

question of the selectivity of the signal but cannot counteract the distortion of the chromatographic peaks caused by column overload.

Method reproducibility was calculated by repeated analysis of a sample spiked at 1 ng/L on six different days spanning three weeks. The results were good with RSD values around 10% in the three cases (Table 1), which can be considered satisfactory for this low concentration level and the experimental conditions.

Method accuracy was determined by a standard recovery experiment carried out on 3 different commercial wines spiked with 4 ng/L of the analytes. The results of this experiment are shown in Table 2. As shown in the table, average recoveries are in all cases close to 100% which confirms that the method is accurate and free from matrix effects. The RSD obtained in the experiment, between 2% and 7%, provides a reasonable estimate of the overall method reproducibility. These values can be considered satisfactory for the low levels of the analytes.

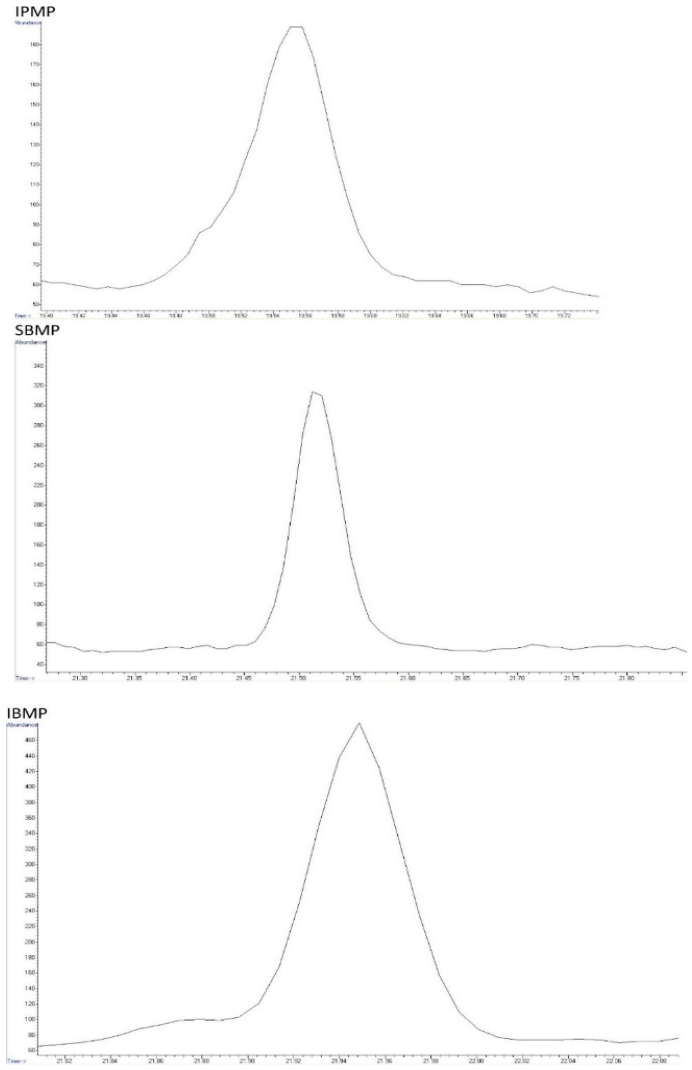

Fig. 3. SBSE-GC-MS chromatograms obtained in the analysis following the proposed procedure, of a red wine containing 0.7 ng/L IPMP (m/z 137), 0.3 ng/L SBMP (spiked) (m/z 138) and 1 ng/L IBMP (m/z 124).

Table 2. Recovery and reproducibility of the proposed method.

Wine	IPMP				SBMP				IBMP			
	Spiked concentration (ng/L)	Calculated concentration (ng/L)	Spiked recovery (%)	Reproducibility (RSD %)	Spiked concentration (ng/L)	Calculated concentration (ng/L)	Spiked recovery (%)	Reproducibility (RSD %)	Spiked concentration (ng/L)	Calculated concentration (ng/L)	Spiked recovery (%)	Reproducibility (RSD %)
Sauvignon blanc white	0.49	0.53	108	5.9	0.52	0.52	100	6.7	3.60	4.10	114	5.0
Cabernet sauvignon rosé	0.51	0.59	116	7.0	0.54	0.49	110	6.2	3.82	5.59	115	3.9
Cabernet sauvignon red	0.50	0.55	110	7.5	0.52	0.44	85	7.9	3.87	8.80	115	3.4

Table 3. Average, maximum and minimum concentrations (ng/L) of the analytes found in the wine samples from recently identified cultivars. SBMP contents were always below the detection limit. Average values were calculated as the arithmetic mean and considering a concentration of 0 ng/L for those samples below the detection limit of the method.

Type of wine	**Country of origin**	**Number of samples**	**IPMP concentration**			**IBMP concentration**		
			Average	**Minimum**	**Maximum**	**Average**	**Minimum**	**Maximum**
White	Spain	11	0.059	0.011[a]	0.41	0.11	<DL	0.29
White	France	8	0.071	<DL	0.24	0.87	0.07[a]	3.16
Rosé	Spain	9	0.052	<DL	0.15[a]	0.17	<DL	0.49
Rosé	France	24	0.034	<DL	0.16[a]	0.76	<DL	4.86
Red	Spain	35	0.047	<DL	0.21[a]	0.41	0.09[a]	1.17
Red	France	24	0.102	<DL	0.46	2.82	<DL	41.2

<DL: below the detection limit. [a] Below quantitation limit.

3.3. Wine analysis

The method was applied to the determination of the three compounds in a set of 111 experimental wines produced during 2016 with non-commercial, recently identified grape cultivars from the regions around the Pyrenean massif. The results are shown in Table 3. It should be noted that despite the very low detection limit of the method, SBMP was not even detected in the samples and therefore is not mentioned in Table 3. Our results make it possible to state that this compound is not a natural aroma compound of these wines. Regarding IPMP, this compound was found in nearly all the wines below the corresponding odor thresholds (estimated as 0.3 ng/L in white wine and 2.3 ng/L in red wine (Pickering, Karthik, Inglis, Sears, & Ker, 2007). It was found above its sensory threshold in only one Spanish white wine. Considering the values obtained in the set of wine samples, lower concentration standards for the calibration curve and a lower internal standard concentration would be more advisable for IPMP determination. IBMP was the most abundant MP in this set of samples, especially in French wines. In each wine category, the average concentration of IBMP was always higher in French than in Spanish wines. These differences can be associated to the higher humidity and more frequent rainfalls in the French regions which usually produce a higher vine vigor, associated with a larger production of IBMP (Ryona, Pan, Intrigliolo, Lakso, & Sacks, 2008). Despite the higher content of IBMP in the French wines, in only two of them were the levels above the odor threshold of 10 ng/L: the wine elaborated with Gros cabernet grapes with 11.8 ng/L and the wine produced with Bequignol grapes with 41.2 ng/L. These

results suggest that, leaving aside these two cases, IBMP is not a key odorant in this set of wines. However, it should not be concluded from these results that this compound does not play any role in the aromatic perception, since even at sub-threshold levels it could exert a suppression effect on wine aroma, as suggested by Gas Chromatography-Olfactometry (Ferreira, et al., 2009). Specific sensory testing will have to be carried out to assess this.

4. Conclusions

A semi-automated method to analyze MP in wine has been developed. The proposed method utilizes a highly efficient SBSE procedure combined with the selectivity provided by multidimensional chromatography and MS detection. The validated method allows the determination of MP at the ppq-level while using only a small volume of sample and with adequate accuracy. The usefulness of the method has been proved by analyzing 111 French and Spanish wine samples, finding in most cases levels below the threshold, suggesting that MPs do not play a relevant role in the aroma of the wines from the regions around the Pyrenean massif.

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CONCLUSIONS

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The first part of the work presented in this thesis has been dedicated to the study of the analysis of dynamic headspace evolution in wine tasting. Thanks to the analytical method developed in the first chapter to study wine headspace, it has been possible to confirm in real wine tasting conditions that our previous findings of the release behavior of volatile compounds to the headspace can explain some of the sensory changes and characteristics of the wine aroma profile. However, the obtained knowledge about the headspace evolutions is not from enough to explain the complexity of the temporal changes in aroma nuances during wine tasting. Future challenges include, among others, the development of improvements in the headspace analytical method to achieve quantification of ultra-trace compounds of relevance for aroma (e.g., varietal thiols), and the normalization of sensory analysis to obtain data less affected by taster variability. The second part of this work has shown the advantage of the state-of-art approaches in quantitation impact odorants in wines at sub-ng/L levels. The conclusions for each chapter are specific in the following part:

Chapter 1: The proposed DHS-TD-GC-MS method provides quantitative data of up to 40 different relevant aroma compounds in the vapors emanating from wine. The method has shown satisfactory validation parameters and can be used to assess the content of up to these aroma-related compounds in the headspaces emanating from wine in a relatively fast and reliable way, making possible a real evaluation of the headspace composition.

Chapter 2: The proposed DHS-TD-GC-MS method has been applied to assess how the composition of the vapors changes with time. Attending to the patterns of change, aroma compounds have been classified into four categories. Polar and not very volatile compounds (half of the total) were present in the headspaces at levels related to their concentration and did not change during time. On the contrary, non-polar and highly volatile compounds could decay very fast. Additionally, the levels and trends followed by aldehydes, dicarbonyls, methanethiol, dimethyl sulfide or ethyl decanoate were significantly affected by the matrix. This indicates that in these cases the data of concentration in the liquid phase should be accompanied by an estimation of their volatility in such specific wine in order to make a reliable interpretation of their sensory role. Results have confirmed that wine headspace continuously changes over time, which would cause relevant changes in the odor qualities perceived.

Chapter 3: The results obtained from this study highlight the dominant effect of continuous evaporation on aroma release during wine tasting, and thus point out the importance of studying aroma release under non-equilibrium conditions with exposure to ambient conditions. Other factors occurring during tasting could affect the mass transfer of volatile compounds, such as shaking. However, unexpectedly the oxidation due to air dissolving into wine only has a limited influence on the profile of volatile sulfur compounds, which are oxygen-sensitive because of their particular functional group. Although we can categorize volatile compounds into different groups regarding their release behavior, the mechanism of mass transfer is still unclear in such a complicated multi-component system as wine, due to the complex interactions between volatile

compounds and matrix components, which are highly related to the physicochemical characteristics of a particular volatile compound. So far, we have validated that hydrophobicity and chemical structure could affect the release behavior of volatile compounds, especially, the release of homologous ester series is highly correlated to hydrophobicity. Otherwise, the evolutionary trend of a volatile compound is also related to its physicochemical properties and matrix component.

Chapter 4: The experiments carried out in this chapter has proved that the same volatile composition in the liquid phase of very different non-volatile wine matrices produces a headspace profile above the wines that can be significantly different. The results show that the interactions between the non-volatile matrix components and volatile compounds produce both retention and salting-out effects depending on the wine matrix. The magnitude of the changes observed in the headspace profiles can undoubtedly influence the perception of wine aroma and can explain why the same aroma composition can produce different aroma perceptions.

Chapter 5: The findings of the present chapter show that some commercial products of polysaccharides and polyphenols used as oenological additives could interact with volatile compounds resulting in a global retention effect. For some volatile compounds, the interaction extent would further influence the evolution of headspace composition over time. Moreover, the chemical characteristics of the volatile compounds strongly impact the binding capacity, such as the hydrophobicity and chemical structure. According to our results, the studied macromolecules have shown

differences in binding capacity with some volatile compounds due to their different chemical properties. However, real wine matrix is far more complicated than the simplified wine models in this study, the higher order interactions between macromolecules and volatile-macromolecule interactions should be studied in the future. Otherwise, the headspace sampling techniques could only prove the existence of interactions between volatile compounds and macromolecules, but they could not provide information about the interaction mechanism at the molecular level.

Chapter 6: The present chapter simultaneously has studied the changes of sensory perception and headspace contents during real wine tasting. In spite of the high difficulty of the tasting of complex premium wines combined with parallel analytical methods, the obtained results have provided valuable data.

The result of the descriptive analysis has indicated that the intensity of some aroma notes evolved during consumption, especially for fresh fruit, alcoholic, spicy and woody. Also, the content of volatile compounds in the headspace above wine glass changed over time in different ways according to their physicochemical characters. Furthermore, the temporal evolution of aroma notes is partly correlated to the content changes in the headspace, especially the relationship between fresh fruit and esters and dimethyl sulfide. Surprisingly, the alcoholic note was anti-correlated with major alcohols, pointing to complex perceptual interactions. Other notes like animal or woody showed variations related with compounds like 4-ethylphenol or t-whiskylactone.

Chapter 7: A semi-automated method to analyze

alkylmethoxypyrazines in wine has been developed. The proposed method utilizes a highly efficient SBSE procedure combined with the selectivity provided by multidimensional chromatography and MS detection. The validated method allows the determination of alkylmethoxypyrazines at the ppq-level while using only a small volume of sample and with adequate accuracy. The usefulness of the method has been proved by analyzing 111 French and Spanish wine samples, finding in most cases levels below the threshold, suggesting that alkylmethoxypyrazines do not play a relevant role in the aroma of the wines from the regions around the Pyrenean massif, except for a few sauvignon-related varieties.

CONCLUSIONES

CONCLUSIONES

La primera parte del trabajo de tesis doctoral presentado se ha dedicado al estudio de la evolución del espacio de cabeza que se produce durante la cata del vino. Gracias al método analítico desarrollado en el primer capítulo para estudiar el espacio de cabeza del vino, ha sido posible confirmar en condiciones reales de cata que nuestros hallazgos previos sobre el comportamiento de los compuestos liberados al espacio de cabeza pueden explicar químicamente algunos de los cambios y características del perfil aromático. Sin embargo, el conocimiento obtenido en este campo no es suficiente para explicar por completo la complejidad de los cambios temporales de las notas sensoriales durante la cata. Los desafíos futuros incluyen, entre otros, la implementación de mejoras en el método analítico del espacio de cabeza para alcanzar niveles de cuantificación en el rango de las ultraatrazas. De esta forma sería posible incluir en nuestros modelos compuestos que aun en niveles de ng/L o menores son relevantes para el aroma del vino, por ejemplo, los tioles varietales. Otros puntos mejorables son la mejora de la normalización en el análisis sensorial para poder minimizar la influencia de la variabilidad de los catadores en los datos sensoriales. La segunda parte de este trabajo ha mostrado las ventajas de usar estrategias analíticas avanzadas que mejoran con la eliminación de la inyección líquida utilizando en su lugar la SBSE, para conseguir determinar componentes impacto en el vino en niveles de sub-ng/L. Las conclusiones para cada capítulo se especifican a continuación:

Capítulo 1: El método DHS-TD-GC-MS propuesto proporciona datos cuantitativos de hasta 40 compuestos relevantes en el espacio de cabeza que emana del vino. El método ha mostrado unos parámetros de validación satisfactorios y puede usarse para evaluar el contenido de estos compuestos del aroma de manera relativamente rápida y fiable cubriendo un amplio rango de concentraciones. Además, y todavía más importante, produciendo una "instantánea" de la composición real del espacio de cabeza.

Capítulo 2: El método DHS-TD-GC-MS propuesto se ha aplicado a la evaluación de los cambios temporales en la composición de los vapores emanados del vino. Atendiendo a los patrones de cambio temporal, los compuestos del aroma se han clasificado en cuatro categorías. Los compuestos polares y no muy volátiles (la mitad del total) aparecen en el espacio de cabeza en concentraciones relacionadas con su concentración en la fase líquida y no cambian con el tiempo. Por el contrario, los compuestos apolares y altamente volátiles decaen muy rápidamente, es decir desaparecen muy rápido del espacio de cabeza. Adicionalmente, los niveles y perfiles seguidos por los aldehídos, dicarbonilos, metanotiol, sulfuro de dimetilo o decanoato de etilo se ven afectados significativamente por la matriz. Esto hace patente que en estos casos los datos de concentración en la fase líquida deberían acompañarse de una estimación de su volatilidad en cada vino específico para poder hacer una estimación más precisa de su papel sensorial. Los resultados han confirmado que el espacio de cabeza del vino cambia continuamente con el tiempo, lo que a su vez causaría cambios relevantes en el tipo de olor percibido.

Capítulo 3: Los resultados obtenidos a partir de este estudio destacan el efecto dominante que la evaporación continua tiene en la liberación del aroma durante la cata del vino, y por tanto, apuntan a la importancia de estudiar la liberación del aroma bajo condiciones de no-equilibrio y exposición a los factores ambientales. Otros factores que suceden durante la cata, principalmente la agitación, pueden afectar a la transferencia de masa de los compuestos volátiles como muestran los resultados de este capítulo. Sin embargo, contra lo que cabía esperar, la oxidación debida a la exposición del vino y sus volátiles al aire tiene una influencia limitada en el perfil de los compuestos azufrados volátiles, los cuales son muy sensibles al oxígeno debido a la presencia de dicho elemento. Aunque podemos categorizar los compuestos volátiles en diferentes grupos de acuerdo con su perfil de liberación, el mecanismo que dirige dicha transferencia de masa no está todavía aclarado debido a las complejas interacciones entre los volátiles y los componentes de la matriz. Sin embargo, podemos asegurar que están altamente relacionados con las características físico-químicas de cada volátil particular. Hasta el momento, hemos comprobado que la hidrofobicidad y la estructura química pueden afectar al comportamiento de liberación. Especialmente, la liberación de ésteres etílicos en series homólogas aparece altamente correlacionado con la hidrofobicidad.

Capítulo 4: Los experimentos llevados a cabo en este capítulo han demostrado que la misma composición en la fase líquida, pero en diferentes matrices no volátiles produce un perfil de espacio de cabeza sobre el vino que puede diferenciarse significativamente. Los resultados muestran que los efectos causados por los componentes

no volátiles de la matriz pueden llevar a fenómenos tanto de retención como de "salting-out" dependiendo de la matriz vínica. La magnitud de los cambios observados en los perfiles del espacio de cabeza puede, sin duda, influir en la percepción del aroma del vino y puede explicar porqué la misma composición aromática puede producir distintas percepciones sensoriales.

Capítulo 5: Los resultados de este capítulo muestran que algunos aditivos enológicos basados en polisacáridos o polifenoles pueden interaccionar con los compuestos volátiles resultado en un efecto global de retención del aroma. Para algunos compuestos volátiles, la extensión de la interacción es capaz de modificar la composición también durante la evolución temporal. De nuevo, las características químicas de los compuestos volátiles afectan fuertemente la capacidad de capturar de las macromoléculas estudiadas. De acuerdo con los resultados de este experimento, las macromoléculas han mostrado diferencias en la capacidad de interaccionar con los volátiles, siendo los polisacáridos los que mayor influencia han mostrado. Hay que tener en cuenta, sin embargo, que la matriz real del vino es más compleja que los modelos estudiados en este capítulo, las interacciones de mayor orden entre macromoléculas y volátiles deben ser estudiadas en el futuro para poder confirmar los resultados en entornos de vino real.

Capítulo 6: En el presente capítulo se ha estudiado de forma simultánea los cambios en la percepción sensorial y en la composición del espacio de cabeza y la fase líquida durante una cata real de vino. A pesar de la dificultad experimental de la cata de complejos vinos Premium combinada con las determinaciones

analítica en paralelo, los resultados obtenidos han proporcionado datos altamente interesantes.

Los resultados del análisis descriptivo indican que la intensidad de algunas notas aromáticas evolucionó durante el consumo, especialmente en el caso de los descriptores "fruta fresca", "alcohólico", "especiado" y "madera". También, el contenido de compuestos volátiles en el espacio de cabeza sobre la copa de vino cambió con el tiempo de formas diferentes de acuerdo sus parámetros físico-químicos. Además, se encontró que la evolución temporal de las notas aromáticas está parcialmente correlacionada con los cambios de composición en el espacio de cabeza. Especialmente destacable es la correlación entre la nota "fruta fresca" y la cantidad de ésteres y sulfuro de dimetilo. Sorprendentemente, la nota "alcohólica" apareció anticorrelacionada con los alcoholes superiores, apuntando a interacciones perceptuales más complejas. Otras notas como "animal" o "madera" mostraron variaciones relacionadas con compuestos como 4-etilfenol y t-whiskylactona.

Capítulo 7: En este capítulo se desarrolló un método semiautomático para determinar alquilmetoxipirazinas en vino. El método propuesto utiliza una extracción SBSE muy eficiente combinada con la selectividad proporcionada por la cromatografía mutidimensional y detección espectrométrica. El método validado permite la determinación de estos compuestos a nivel de ppq empleando sólo un pequeño volumen de muestra, con mínima manipulación y una precisión adecuada. La utilidad del método ha sido demostrada analizando 111 vinos españoles y franceses. Estos

análisis han mostrado que en la mayor parte de los vinos las alquilmetoxipirazinas se encuentran por debajo de su valor umbral y es poco probable que influyan de forma relevante el aroma de dichos vinos. La excepción fueron los vinos preparados con variedades relacionadas con la familia sauvignon, en dichos casos sí se encontraron cantidades claramente superiores a los valores umbral en vino.