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2 **DEVELOPMENT OF AN *IN-MOUTH* HEADSPACE SORPTIVE**

3 **EXTRACTION METHOD (HSSE) FOR ORAL AROMA**

4 **MONITORING AND APPLICATION TO WINES OF DIFFERENT**

5 **CHEMICAL COMPOSITION**

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Abstract

An *in-mouth* headspace sorptive extraction (HSSE) procedure for the *in-mouth* volatile sampling of wine aroma compounds during wine tasting has been developed. The procedure is based on the application of a PDMS (polydimethylsiloxane) twister contained inside a tailored made glass tube placed into the headspace of the mouth after rinsing and spitting-off the wine, which is then followed by gas chromatography mass spectrometry analysis (GCMS). Various parameters that might affect the performance of the method (extraction time, aroma concentration) were firstly investigated. Despite the short selected *in-mouth* extraction time (30 s); the application of the *in-mouth* HSSE procedure using real wines allowed the detection of more than 30 volatile compounds from different chemical families in the oral cavity in a single run, and which are also present in the wine at very low concentrations. Additionally, the *in-mouth* HSSE profile allowed us to distinguish between wines types (with different non-volatile and volatile composition) in a similar way to that when using data from the headspace of the wine (wine-HSSE-GCMS). The simplicity, sensitivity, good repeatability and the easy automatization of this procedure, makes this technique a reliable and feasible tool to determine the chemical and biochemical changes of these compounds in the mouth in real physiological conditions providing useful *-in vivo* analytical data to better correlate with sensory studies.

Key words: Wine, aroma release, *in-mouth* headspace sorptive extraction, *-in vivo* aroma analysis, wine consumption.

1. Introduction

The identification and quantification of wine volatile compounds and the elucidation of their sensory relevance has been the objective of a significant amount of research concerning wine aroma. Many of these studies have been included in interesting reviews on this topic (Francis & Newton, 2005; Polášková, Herszage, & Ebeler, 2008; Robinson et al., 2014). These studies have provided an important background of knowledge, but it is still insufficient in explaining the relationship between wine volatile composition and aroma perception. Additional research related to perceptual interactions among wine volatiles (synergisms, antagonisms) or about physicochemical interactions between volatile and non-volatile compounds is also necessary for a better understanding of the role of wine volatiles on aroma perception (Pozo-Bayón, Muñoz-González, & Esteban-Fernández, 2016). In addition, the changes that odorant molecules might experience in the oral cavity during wine consumption is an incipient research that is providing new and valuable findings.

During wine tasting, aroma compounds can interact with saliva proteins (Genovese, Piombino, Gambuti, & Moio, 2009; Muñoz-González et al., 2014), they can also be modified by enzymatic conversion (Pérez-Jiménez, Rocha-Alcubilla, & Pozo-Bayón, 2018) or they might be “de novo” produced by oral microbiota metabolism (Muñoz-González, Cueva, Pozo-Bayón, & Moreno-Arribas, 2015). Excluding sensory studies, most of the above mentioned analytical studies have been done using *-in vitro* conditions (generally applying static or dynamic headspace conditions) but not in a real physiological environment. However, *in-vivo* studies are required in order to better understand the effect of physiological variables on the retronasal aroma composition, which better represent the aroma profile reaching the olfactory receptors (Shepherd, 2015). Furthermore, by monitoring the *in-vivo* (*in-mouth* or *in-nose*) aroma release

during wine tasting, more representative data could be obtained for correlation with sensory perception.

The use of *-in vivo* analytical tools for wine aroma analysis is still quite scarce. The low concentration at which these compounds occur in the wine, the chemical diversity among wine volatiles, the high and variable concentration of ethanol in the wine, the great dilution of volatiles during tasting due to the exhalation flows and the high inter-individual differences in oral physiology (saliva composition, oral microbiota), represent a great challenge for any *-in vivo* analytical wine aroma monitoring methodology. Nonetheless, different attempts have been carried out using on-line (real time) and off-line (cumulative) methods, which have been previously reviewed (Muñoz-González, Rodríguez-Bencomo, Moreno-Arribas, & Pozo-Bayón, 2011).

On-line *-in vivo* analytical approaches provide real-time *-in vivo* aroma profiles combining the direct sampling of the expelled air from the nose and mass-spectrometric techniques, such as APCI (atmospheric pressure chemical ionisation)- and PTR (Proton transfer reaction)-mass spectrometry (MS). These techniques reflect the patterns of aroma release in real time during wine consumption, which could possibly help explain aroma perception. They provide well-resolved time-intensity curves of the ions of interest that can be obtained relatively quickly, since these instruments are able to do numerous repetitions per day (Spitaler et al., 2007). However, they are difficult to apply to real food and beverage matrices, such as wines characterised by complex flavour profiles since they cannot provide clear compound identification (Poinot, Arvisenet, Ledauphin, Gaillard, & Prost, 2013). The presence of a relative and variable amount of ethanol in the wine also compromises discrimination ability, quantification, and spectra interpretation (Spitaler, et al., 2007). Recently, a smart modification of an *in vivo*- PTR-ToF-MS method was proposed for wine aroma analysis (Sémon, Arvisenet, Guichard,

& Le Quéré, 2018). However, this method was carried out using synthetic wines with no matrix effect and aromatized with nine selected aroma compounds, which do not represent wine aroma complexity. Moreover, the high economical cost of the PTR-ToF-MS equipment and the requirement of trained staff make its implementation in many labs very difficult.

Off-line *-in vivo* analysis has also been used in wine aroma release studies. Muñoz-González and collaborators (Muñoz-González, Martín-Álvarez, Moreno-Arribas, & Pozo-Bayón, 2013) used a retronasal aroma trapping device (RATD) for *-in nose* aroma monitoring during wine consumption. This method was based on the extraction of volatile compounds from the nose into a Tenax trap in which volatiles were retained using a vacuum pump. The trap was further eluted using a solvent, and the breath extract concentrated and analysed by conventional GC-MS. Moreover, an intra-oral SPME technique was also developed for *in-mouth* monitoring (Esteban-Fernández, Rocha-Alcubilla, Muñoz-González, Moreno-Arribas, & Pozo-Bayón, 2016). As well as its simplicity and good performance, another advantage of this technique is that it could be used at different times after wine rinsing providing the release kinetics of wine aroma compounds during the post-ingestion phase, which gives information about the intra-oral aroma persistence depending on molecule structure and properties. Furthermore, this technique allowed the differentiation among oral aroma release profiles depending on wine matrix composition (Esteban-Fernández, Muñoz-González, Jiménez-Girón, Pérez-Jiménez, & Pozo-Bayón, 2018). Nonetheless, the desorption of the aroma loaded SPME fibers in the GC port is not automatic, thus limiting the number of analyses per day, and making experiments involving a high number of panellists quite difficult.

Another *in-mouth* aroma extraction technique already applied to wines was the buccal odour screening system (BOSS). This technique is based on the application of a twister

(stir bar coated polymer with polydimethylsiloxane) in the mouth, which is mixed with saliva (once the food has been swallowed), and further desorbed in the GC-MS using a thermal desorption unit (TDU) and a cool injection system (CIS) (Buettner & Welle, 2004). This technique has been used to differentiate the *in-mouth* aroma profiles of two different Chardonnay wines (Buettner, 2004) and more recently to check the production of volatiles from odourless precursors (Mayr et al., 2014; Parker et al., 2017). However, as far as the authors know, there is not yet a systematic study showing the adequacy of this technique for *-in vivo* wine aroma analysis.

The importance of considering the transformations experienced by aroma compounds during oral processing to explain aroma perception during wine consumption implies the necessity of improving *-in vivo* analytical approaches, considering its adequacy for different types of wine aroma molecules. Therefore, the aim of this research was on one hand, to set up an *-in vivo* headspace sorptive extraction procedure (HSSE) to monitor the aroma released in the oral cavity simulating a wine intake situation and considering its analytical performance for a large number and various types of wine volatile compounds; and on the other hand, the application of this method to determine differences in oral aroma release among ten wines with different chemical and aroma composition.

2. Material and methods:

2.1. Chemical and reagents:

The following analytical grade compounds with the highest purity available were employed in this study (CAS number in brackets): ethyl propanoate (105-37-3), butyl acetate (123-86-4), isoamyl acetate (123-92-2), ethyl pentanoate (539-82-2), hexyl acetate (142-92-7), ethyl octanoate (106-32-1), 2-phenylethyl acetate (103-45-7),

ethyl dodecanoate (106-33-2), β -pinene (19902-08-0), linalool (126-91-0), terpinen-4-ol (2438-10-0), α -terpineol (98-55-5), β -citronellol (106-22-9), nerol (106-25-2), geraniol (106-24-1), furfural (98-01-1), 5-methylfurfural (620-02-0), furfuryl alcohol (98-00-0), α -ionone (127-41-3), β -ionone (79-77-6) from Fluka (Buchs, Switzerland), ethyl butanoate (105-54-4), ethyl 2-methylbutanoate (7452-79-1), 1-butanol (71-36-3), 1-hexanol (111-27-3), trans-3-Hexen-1-ol (928-97-2), benzyl alcohol (100-51-6), cis-whisky lactone (39212-23-2) trans-whisky lactone (39212-23-2), γ -nonalactone (104-61-0), guaiacol (90-05-1), eugenol (97-53-0), 4-ethylphenol (123-07-9), 2-methoxy-4-vinylphenol (7786-61-0), 2,3-Butanedione (431-03-8), acetovanillone (1835-11-6) from Aldrich (Steinheim, Germany, and Milwaukee, WI, USA), ethyl hexanoate (123-66-0), ethyl decanoate (110-38-3), diethyl succinate (123-25-1), ethyl cinnamate (103-36-6), β -phenylethanol (60-12-8) vanillin (121-33-5) from Merck (Darmstadt, Germany), 4-ethylguaiacol (2785-89-9), methyl vanillate (3943-74-6), ethyl vanillate (617-05-0), from Lancaster (Morecambe, England), hexanoic acid (142-62-1), decanoic acid (334-48-5) from Scharlau (Barcelona, España), isobutyl acetate (110-19-0) from M&B (Dagenham, England), α -pinene (7785-26-4), limonene (5989-27-5), cis-3-hexen-1-ol (928-96-1) from Sigma-Aldrich (Misuri, USA) and β -damascenone (23726-93-4) from Firmenich (Geneva, Switzerland). In addition, 3-octanol (589-98-0) from Fluka (Buchs), methyl nonanoate (1731-84-6) from Aldrich (Steinheim) and 3,4-dimethylphenol (95-65-8) from Alfa-Aesar (Haverhill, Massachusetts, USA) were used as internal standards (IS). Absolute ethanol (for analysis; Merck, Darmstadt, Germany) (64-17-5) and Milli-Q water (Millipore, Bedford, USA) were used as solvents. NaCl (Amresco, Ohio, USA) was used to improve the aroma extraction.

For the *-in vivo* assays, the following food grade compounds supplied by Sigma-Aldrich (Steinheim) were used: isoamyl acetate (123-92-2), ethyl butanoate (105-54-4), ethyl

pentanoate (539-82-2), ethyl hexanoate (123-66-0), ethyl octanoate (106-32-1), ethyl decanoate (110-38-3), linalool (126-91-0), β -phenylethanol (60-12-8), β -ionone (79-77-6), guaiacol (90-05-1), eugenol (97-53-0), limonene (5989-27-5), cis-whisky lactone (39212-23-2), trans-whisky lactone (39212-23-2), cis-3-hexen-1-ol (928-96-1), geraniol (106-24-1), γ -nonalactone (104-61-0) and 1-hexanol (111-27-4).

2.2. Synthetic wines

For the *in-mouth* HSSE procedure, a synthetic wine (pH 3.5 and ethanol concentration 13% (v/v)) was prepared. The pH was adjusted with tartaric acid (Panreac, Barcelona, Spain). Depending on the experiment, aromatization was performed with 9 or 18 of the above mentioned food-grade aroma compounds. All being representative of the wine volatile profile and characterised for having different physicochemical properties. For aromatisation, single independent aroma stock solutions were prepared in food-grade ethanol (Alcoholes Montplet S.A., Barcelona, Spain) and from there, each aroma compound was added to the synthetic wine to obtain different concentrations depending on the experiment (0.15, 0.25, 0.5, 1 or 1.5 mg/L).

2.3. Commercial Wines

Ten Spanish commercial wines representative of different winemaking technologies were selected for this study (Table 1): two young white wines (W1 and W5), two white wines aged on lees (W6 and W8), two young rosé wines (W2 and W7), one young red wine (W3) and three oak-aged wines (W4, W9 and W10).

2.4. Wine chemical composition

A CP-505 pH meter (Elmetron, Zabrze, Poland) was used for pH measurement. Total polyphenols were determined by the Folin–Ciocalteu method and spectrophotometric

measurement at 670 nm (Singleton & Rossi, 1965). Total procyanidins were determined by the vanillin method following the procedure previously described (Monagas, Gómez-Cordovés, & Bartolomé, 2006). Neutral polysaccharides were determined by the phenol/sulfuric acid method according to a previous procedure (Segarra, Lao, López-Tamames, & De La Torre-Boronat, 1995). Free amino acids were determined by method 5 of Doi and co-workers (Doi, Shibata, & Matoba, 1981). A Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific, Massachusetts, USA) and a Specord® plus (Analytik Jena AG, Jena, Germany) spectrophotometer were used for these determinations.

2.5. HSSE procedure for wine aroma analysis

For the HSSE procedure, a previously described method (Weldegergis, Tredoux, & Crouch, 2007) with some modifications was followed. One mL of sample (wine) was placed in a 20 mL headspace vial (Gerstel) and was made to 5 mL with a hydroalcoholic solution (13 % ethanol and pH = 3.5). A 40 µL aliquot of internal standard solutions (10 mg/L 3-octanol, 2.5 mg/L methyl nonanoate and 400 mg/L 3,4-dimethylphenol) and 1.5 g of NaCl were added to each vial. A preconditioned PDMS (polydimethylsiloxane) stir bar (Twister) (20mm length x 0.5mm film thickness), supplied by Gerstel (Mülheim, Ruhr, Germany) was suspended in the headspace of the vial using a glass insert. The vials were sealed with septa caps using a hand crimper. The extraction was carried out for 3.5 hours at 36.5 °C in a water bath at controlled temperature. After sampling, the stir bar was removed, dried with a lint-free tissue and placed in the glass tube for the thermal desorption.

The quantification of 51 aroma compounds in the wines was carried out by using the corresponding calibration curves for each standard aroma compound. For that, three

stock solutions were prepared by dissolving around 20, 25 or 70 mg/L of each target compound in absolute ethanol. The first solution included the aroma compounds: α -pinene, β -pinene, limonene, linalool, α -terpineol, β -citronellol, nerol and geraniol. The second stock contained the aroma compounds: isobutyl acetate, ethyl butanoate, ethyl hexanoate, hexyl acetate, 1-hexanol, ethyl octanoate, furfural, 5-methyl furfural, γ -nonalactone, ethyl decanoate, furfuryl alcohol, 2-phenylethyl acetate, hexanoic acid, benzyl alcohol, cis-whisky lactone, trans-whisky lactone, β -phenylethanol, , decanoic acid, isoamyl acetate, 2,3-butanedione, butyl acetate, diethyl succinate, ethyl propanoate, 1-butanol and ethyl pentanoate. The third solution included the aroma compounds: ethyl 2-methylbutanoate, trans-3-hexen-1-ol, cis-3-hexen-1-ol, β -damascenone, α -ionone, guaiacol, β -ionone, γ -nonalactone, 4-ethylguaiacol, ethyl cinnamate, 4-ethylphenol, 2-methoxy-4-vinylphenol, eugenol, vanillin, methyl vanillate, ethyl vanillate, acetovanillone and ethyl dodecanoate. The calibration curves for each aroma compound were performed with seven different volumes (0.005, 0.02, 0.05, 0.1, 0.15, 0.2 or 0.25 ml) of the three stock solutions which were placed in the 20 mL headspace vial and were made to 6 mL with a hydroalcoholic solution (13 % ethanol and pH = 3.5) and spiked with the internal standard solution as explained before. The calibration samples were analysed using the same procedure as described for the wine samples. The calibration curves, Linear ranges and correlation coefficients (R^2) are provided in Table 1S.

2.6. *In-mouth HSSE aroma analysis*

The same volunteer performed all the *in-mouth* aroma analysis in order to reduce the interindividual differences associated to oral physiology, which could mask the conclusions about the suitability of the HSSE method for oral aroma monitoring. All the assays were performed in the morning. The volunteer could not eat or drink anything

one hour previous to the sampling. In addition, he brushed his teeth with the same toothpaste thorough the study. Between wine samples, he rinsed his mouth with water and with a pectin solution (1g/L). For the experiments, fifteen millilitres of the aromatised hydroalcoholic solution or wine (depending on the experiment) were taken into the oral cavity, kept for 30 seconds and then expectorated. While keeping the wine in the mouth soft rinsing movements were made. During rinsing, special care was taken to keep the lips closed, not to swallow and not to open the velum–tongue border prior to expectoration. Five seconds after expectoration, oral aroma extraction was carried out with a 20mm x 0.5 mm (length × film thickness) PDMS twister (Gerstel) that was placed into the oral cavity for different times (15, 30 or 45 s) depending on the experiment. For the *in-mouth* twister application, a tailored made twister holder device was developed (Segainvex-UAM, Madrid, Spain) (Figure 1). This was a kind of hollow glass tube (14.5 cm) with a small widening at the back called capsule (a), where the twister is included. The total length of the capsule was 27 mm and the diameter 5 mm. These dimensions prevented the movement of the twister during the extraction. The capsule was homogenously perforated with eight pores (2 - 3 mm diameter) (b) with a distance of about 2 mm between them. A removable glass bar (c) was placed inside the hollow glass tube preventing the air flow from coming in during the extraction. At the end of the tube, a plastic cap (d) closed the device. Additionally, a glass notch (e) outside the glass tube helped keep the lips closed in the same position during the whole *in-mouth* extraction. Swallowing was not allowed throughout this process. Once the extraction was finished, the twister glass holder was taken out from the oral cavity and the aroma loaded twister carefully removed from the capsule with a magnet bar, dried with a tissue and placed into the thermal desorption unit (TDU). Analyses were

performed by the same individual three times for each aroma concentration and tested extraction time.

2.7. Thermal desorption and GC-MS conditions

All the twisters (from the extractions of the headspace of the wines or from *in-mouth* aroma extraction) were thermally desorbed using a TDU in combination with a CIS-4 (cooled injection system) injector (Gerstel), in order to desorb and cryofocus the analytes prior to their transfer onto the analytical column. Splitless thermal desorption was programmed from 40°C to 240 °C (held for 5 min) at 60°C/min. Cryofocusing was performed with liquid nitrogen at -100 °C. Injection was performed with a ramp of 12 °C/min from -100 °C to 240 °C and held for 5 min.

Analyses were performed in a 6890 N GC coupled to an Agilent 5973 N mass spectrometer. Volatile compounds were separated on a DB-WAX polar capillary column (30 m x 0.25 mm i.d. x 0.50 µm film thickness) from Agilent (J&W Scientific, Folsom, CA). Helium was the carrier gas at a flow rate of 1 mL/min. The oven temperature was initially held at 40 °C, later increased at 4 °C/min to 130 °C, then increased at 8 °C/min to 240 °C and held for 5 min following previous described conditions (Weldegergis, et al., 2007)

For the MS system, the temperatures of the transfer line, quadrupole and ion source were 270, 150 and 230 °C respectively. Electron impact mass spectra were recorded at 70 eV and the ionisation current was 10 µA. The acquisitions were performed in scan (from m/z 35 to 350) and SIM modes. The identification of compounds was based on the comparison of retention times and mass spectra. Mass spectra were compared with those from NIST 2.0 database. In the case of *in-mouth* aroma extraction, since no

internal standard was used, absolute peak areas (APAs) were obtained to express the amount of *in-mouth* aroma. APAs were sufficient for this type of analysis, as the aim of the work was to set up experimental conditions and to compare the extent of *in-mouth* aroma between wine samples.

2.8. Statistical Analysis

The statistical methods used for data analysis were one way or multifactorial ANOVA to check for differences in wine chemical composition or among extraction parameters (extraction time, aroma concentration) and Tukey test for mean comparison ($p < 0.05$). Principal component analysis (PCA) was used to examine the relationship between aroma compounds and wine types using HSSE of the wines and *in-mouth* HSSE data. XLSTAT software (v.19.01) (Addinsoft, Paris, France) was used for data processing.

3. Results and discussion

3.1. *In-mouth* HSSE extraction conditions

In this work, a procedure based on the extraction of the remaining aroma in the headspace of the mouth after wine rinsing using a PDMS twister (*in-mouth* HSSE) was developed and its performance evaluated. The effect of some extraction conditions are described in the following sections.

3.1.1 Effect of the extraction time

In-mouth extraction time should be a compromise between having enough sensitivity to extract the maximum number and amount of volatiles and trying to reduce at maximum the extraction time for a faster and reliable analysis. Extraction times for at least one hour or more are generally required for most wine aroma studies using HSSE (Callejon

et al., 2010; Ubeda, Callejón, Troncoso, Peña-Neira, & Morales, 2016; Weldegergis, et al., 2007). However, for *in-mouth* extractions, much shorter times are necessary in order to have the closest picture of the wine volatiles just released in the mouth after swallowing. This is also important for the purpose of correlating *in-mouth* aroma release and perceived aroma intensity during wine intake. Therefore, to check the effect of *in-mouth* extraction time on aroma release, an aromatised synthetic wine with nine aroma compounds at two levels of concentration (0.5 or 1 mg/L) was tested three times using very short *in-mouth* extraction times (15, 30 and 45 s). These results are shown in Table 2. In this table, results from one way ANOVA and Tukey test for mean comparison ($p < 0.05$) are also included. As it can be seen, most of the aroma compounds showed a significant increase in aroma release with an increase in the extraction time (from 15 to 45 s). This effect, was however, more evident at lower aroma concentrations (0.5 mg/L). Contrarily, β -ionone did not increase with the extraction time. In the same way, ethyl decanoate did not increase with the extraction time either, and this compound only experienced a significant increase (from 30 s to 45 s) at the lowest tested concentration (0.5 mg/L) (Table 2). However, β -ionone and ethyl decanoate should be good candidates for extraction by the apolar polymeric phase (PDMS) of the twister based on their hydrophobicity expressed as log P values (3.8 and 4.8 respectively) (Ochiai, Sasamoto, David, & Sandra, 2018). To explain this, it is important to bear in mind that once the wine has been swallowed (or as in our experimental conditions, once it has been spat-off), *in-mouth* aroma release depends on the adsorption (to oral mucosa) / desorption (to the air contained in the mouth) kinetics. Previous works have proven the high capacity of hydrophobic compounds to bind to oral mucosa (Esteban-Fernández, et al., 2016). This capacity seems to be dependent on the available binding sites for specific aroma compounds of oral mucosa. At high aroma concentrations, all the potential binding sites

could already be occupied by aroma molecules, thus, the effect of an increase of extraction time has no consequence on aroma release. On the contrary, at lower concentrations, there could still be binding sites available, proving the extraction time effect. This hypothesis might explain why the rise of ethyl decanoate with increasing extraction times only happened at a low aroma concentration. In the case of β -ionone, it has been shown that this compound strongly interacts with oral mucosa being slowly and progressively released in the mouth (Esteban-Fernández, et al., 2016). This behaviour seems to agree with the lack of variation observed for this compound with little changes in the *-in mouth* extraction time.

3.1.2 Repeatability

Table 2 also shows the repeatability of the *in-mouth* HSSE extraction procedure at the two tested concentrations using the aromatised synthetic wine. Repeatability was estimated by the relative standard deviation (RSD %) of the absolute peak areas of each compound after performing three independent *in-mouth* analyses for both aroma concentrations (0.5 and 1 mg/L) applying the three extraction times (15, 30 and 45 s). Results showed RSD values ranged from the very low variation (RSD = 0.2 %) found for ethyl butanoate at the lowest aroma concentration (0.5 mg/L) and 45 s of *in-mouth* extraction, and the highest variation (RSD = 34.1%) determined for ethyl decanoate at the highest concentration (1 mg/L) and 45 s of *in-mouth* extraction. The highest variation was found for the most hydrophobic compounds (β -ionone and ethyl decanoate). These repeatability values are quite similar to those obtained in HSSE wine aroma studies (Weldegergis, et al., 2007) and they were deemed good enough, considering that this is an *in-vivo* analysis performed under physiological conditions, in which a total voluntary control of many physiological events is not always achieved (e.g. more or less rinsing movements, slightly different amounts of expectorated

samples, changes in the *in mouth* saliva volume, etc.), even after following strict experimental procedures (Esteban-Fernández, et al., 2016).

Although many of the tested compounds experienced an increase with the extraction time, it was finally decided to select 30 s, since the sensitivity was sufficient to detect most of them, also the repeatability was adequate, and the use of short extraction times in the mouth was valued more important, since this will allow the possibility of performing several extractions of the same wine sample, and thus, in future studies to reconstruct the aroma release kinetics in the course of wine consumption, which could better relate with sensory studies.

3.1.3. *Effect of wine aroma concentration*

To check the effect of wine aroma concentration on the amount of oral aroma determined using *in-mouth* HSSE, the same synthetic wines spiked with increasing amounts of the eighteen target aroma compounds (ranging from 0.15 to 2.52 mg/L) described in section 2.2 were assayed. From the eighteen compounds, three of them were not detected in the mouth. Guaicol and γ -nonalactone were not detected at any of the tested concentrations, while *cis*-3-hexen-1-ol was only found in the assays performed at the highest wine aroma concentrations (> 1 mg/L). The low extraction of polar compounds by PDMS twisters has already been described (Ochiai, et al., 2018). For some wine HSSE applications, this could be resolved by increasing the extraction time (Weldegergis, et al., 2007), but the short *in-mouth* extraction time selected in the present work (only 30 s) did not allow the extraction of these very polar compounds. Similarly, longer extraction times should have been necessary for the extraction of a relatively low volatile compound like γ -nonalactone. Nonetheless, other

tested lactones with similar hydrophobicity and boiling points (such as cis-and trans-whisky-lactones) were extracted in the HS of the mouth, even at the lowest assayed concentration. Differences in the molecular structure of compounds from the same chemical class can affect the binding of aroma molecules to the oral mucosa and might explain these results (Esteban-Fernández, et al., 2018).

For the other 15 aroma compounds, the relationship between the amount of *in-mouth* aroma (after rinsing and spitting-off the wine) and wine aroma concentration (in the synthetic wine) is shown in Figure 2. The figure also includes the regression models that better fit this behaviour. As it can be seen, a general increase in oral aroma release with an increase in wine aroma concentration was found. However some differences depending on the type of aroma compound were also observed. For instance, all the ethyl esters and 1-hexanol showed the same trend, characterised by a slow increase in their oral amount with an increase in wine aroma concentration at concentration values lower than 0.5 mg/L. However, a rapid rise in the signal at concentrations higher than 0.5 mg/L was also observed. The three terpenes (limonene, 1-linalool and geraniol) also showed a similar behaviour. The only difference was that the inflexion point at which the extraction started to rise was at higher concentrations (above 1mg/L) for terpenes, compared to the first group of compounds (above 0.5 mg/L). This could be related to the amount of aroma compounds in the headspace of the oral cavity available for twister extraction. Aroma compounds contained in the *in-mouth* HS can be absorbed onto the twister but they can also be adsorbed/ desorbed by the oral mucosa, modifying the amount of aroma available in the HS for twister extraction. In other words, the twister has to compete with oral mucosa for the binding of these volatiles. As mentioned above, the limited binding capacity of the aroma compounds in the oral mucosa may be responsible for the fact that the higher the aroma concentration, the more free aroma

there will be in the HS of the mouth, because these compounds cannot interact with the oral mucosa, and therefore there could be more aroma available for the twister extraction. The aroma concentration at which oral mucosa cannot bind more aroma depends on the type of aroma compound (Buettner & Welle, 2004; Esteban-Fernández, et al., 2016). Previous studies showed higher oral aroma binding for hydrophobic compounds. However, hydrophobicity is not the only driving force for aroma-oral mucosa interactions. The strength of these interactions determines the kinetics of aroma release from oral mucosa and the final amount of aroma in the oral HS to interact with the twister. In this sense, it has been shown that esters can easily bind to the oral mucosa, but they are more quickly released compared to other compounds such as linalool, which is more retained (Esteban-Fernández, et al., 2016). This is in agreement with the results of this work, and might explain, the different behaviour between these two groups of compounds. Besides this, a third group of compounds formed by β -ionone, eugenol, and cis- and trans- whisky lactone was also observed. Interestingly, the profiles obtained for these four compounds (Figure 1) were almost identical, as are their chemical properties (high hydrophobic and low volatile compounds). As it can be seen in the figure, there was an increase in the extracted amount of these volatiles in the mouth at lower concentrations, followed by a drastic drop at a concentration around 0.5 mg/L and a further increase at higher wine aroma concentrations. These compounds have a relatively high hydrophobicity and they should be well extracted by the apolar polymeric phase of the twister, but they also have a good ability to interact with oral mucosa. Nonetheless, they also have a low volatility and they could be less released from the oral mucosa to the *in-mouth* HS. For instance, using intra-oral SPME, it has been shown that β -ionone was very strongly retained by oral mucosa being slowly and progressively released in the mouth (Esteban-Fernández, et al., 2016). Thus, if this

compound is scarcely released into the oral cavity, the amount extracted by the twister would be also small. Only when the concentration increases, this effect could be counteracted following linear *in-mouth* extraction behaviour (Figure 2). Finally, β -phenylethanol followed a very odd trend at low wine aroma concentrations. This compound was hardly retained by oral mucosa (Esteban-Fernández, et al., 2016), therefore a high amount of this compound in the HS of the mouth could be expected. However, its low polarity ($\log P = 1.57$) and volatility (218 °C) might make twister extraction difficult, at least at low wine aroma concentrations (<1mg/L).

3.2. Wine chemical and volatile composition

To check if the *in-mouth* HSSE technique can be used to look for differences in oral aroma release between real wine samples, this technique was applied to 10 different commercial wines (Table 1). The non-volatile and volatile chemical wine composition was previously determined. Considering their chemical composition (pH, total polyphenols, procyanidins, neutral polysaccharides, free amino acids), significant differences among wines and depending on wine type (white, red or rosé wine) were found (Table 1).

In addition, in order to determine differences in volatile composition among the wines, a targeted HSSE-GCMS analysis was also performed. For this, forty one compounds characteristic of the volatile profile of the wines were quantified in the ten wines. These compounds belonged to different chemical classes, such as esters, ketones, alcohols, lactones, furanic compounds, C13-norisoprenoids, volatile phenols and volatile fatty acids. Their concentration in the wines is shown in **table 3**. Although most of these volatiles were found in all the wines at a higher or lower concentration, some of them such as ethyl cinnamate, nerol, cis--and trans- whisky lactones, γ -nonalactone, β -

damascenone and 4-ethylguaicol were only detected in red wines. A principal component analysis (PCA) to check differences in wine volatile profile among the wines was also carried out. The first three components obtained from this analysis explained about 67% of data variation. **Figure 3a** shows the representation of the data in the plane obtained with the two first components (F1 and F2). F1 explained 36.1 % of data variation and separated W3, W9, W4 and W10 wines, located in the right side of the graph from the rest. All of them were red wines positively correlated with this component.. On the contrary, white and rosé wines are located on the left side of the graph. As it can be seen in the figure, the differences among them are lower than among red wines. F1 was strongly and negatively correlated (factor loadings >0.6) with some volatile compounds, such as esters (ethyl butanoate, ethyl hexanoate, ethyl octanoate) and decanoic acid. In addition, it was positively correlated with diethyl succinate, β -damascenone, ethyl dodecanoate, α -ionone, trans-whisky lactone, cis-whisky lactone, β -ionona, γ -ionone, ethyl cinnamate, eugenol, ethyl dodecanoate and β -citronellol. Therefore, except W10, the rest of red wines were characterized by higher concentrations of these volatile compounds. W4 and W9 wines, located close together in the graph had higher concentrations of these compounds compared to W3 and W10 red wines. This is in agreement with the oak aging step that W4 and W9 wines underwent, in which typical wood aroma compounds (lactones, furans, etc.) can be released into the wines (Pérez-Coello & Díaz-Maroto, 2009). On the contrary, white and rosé wines with negative correlation with F1, had a minor concentration of these aging related aroma compounds.

In addition, the second component (F2), explained a minor data variation (16%) (Figure 3a), but it was also interesting, since it could differentiate W3 red wine, with high and positive values of F2, from W10 red wine. This component was positively correlated

(factor loadings > 0.6) with some terpenes, such as α -pinene and limonene, and some esters such as butyl acetate and ethyl pentanoate. Thus, despite that both were young red wines from the same grape variety (Tempranillo); W3 had higher concentrations of these compounds compared to W10. In addition, F2 also allowed the separation of W5 and W2 wines, with higher concentrations of the above mentioned volatile compounds from the rest of the white and rosé wines (W1, W6, W8 and W7).

3.3. Application of *in-mouth* HSSE to wines

The *in-mouth* HSSE procedure using the optimised conditions (30 seconds of mouth rinsing with wine and 30 s of twister extraction after spitting-off the wine) was also applied to the same ten wines. The experiment was performed three times with each wine. Between each assay, a cleaning of the oral cavity (using water and a pectin solution 1g/L) was done and a blank of the oral cavity was also run.. The blank analysis showed that some of the target aroma compounds (Table 3) were already present in the oral cavity. For instance some traces of alcohols, such as 1-butanol, furfuryl alcohol, geraniol, β -phenylethanol and β -ionone were found, which could come from the toothpaste for teeth cleaning. In addition, some carboxylic acids, such as hexanoic, decanoic and dodecanoic acids already described in the saliva volatolome (Amann et al., 2014) were also found. Although the amount of these compounds in the mouth blanks was very small, these values were taken into account and corrected from the peak areas obtained after rinsing with the wines. By applying *in-mouth* HSSE after rinsing the oral cavity with the ten wines, most of the volatile compounds previously determined in the headspace of the wines (using HSSE) were also found in the mouth. Their concentration in the mouth (absolute peak areas) after rinsing with the different wines is shown in Table 2S. Some of these compounds were not detected in any of the tested wines. These compounds appear with an asterisk in table 3 and were the three terpenes (linalool, α -

terpineol and nerol), the three lactones (cis-whisky lactone, trans-whisky lactone and γ -nonalactone), one C13-norisoprenoid (β -damascenone) and the two volatile phenols (4-ethylguaicol and eugenol). As shown in the volatile composition of the wines (Table 3), linalol and terpineol were detected together in the white Albariño wines (W5 and W6) at very low concentrations (0.45-0.54 $\mu\text{g/L}$), but the former was also detected in the red W4 and W10 wines; and the later, in the rose W2 wine and in the red wine W9 at very low concentration (Table 3). Nerol was only found in W4 red wine (1.21 $\mu\text{g/L}$). Considering that the *in-mouth* aroma extraction was performed once the wine disappeared from the oral cavity, the remaining oral amount of these compounds should have been too low and the extraction time (30 s) too short to allow enough extraction of these compounds. In addition, although the three lactones (cis-whisky lactone, trans-whisky lactone and γ -nonalactone) were identified in all the red wines, none of them were detected in the mouth headspace after rinsing with these wines (Table 2S). The low aroma concentration of these compounds in the wine, and their low volatility could be the reason. Similarly, β -damascenone was only detected in the red W4 wine and at very low concentration in W3 (Table 3), but it was not found in the mouth after rinsing with this wine (Table 2S). Nonetheless, this compound had similar physicochemical characteristics (a relatively high hydrophobicity and boiling point) as the other two lactones (α - and β -ionone) identified in the mouth after rinsing with white (W1 and W6) and red (W4 and W9) wines (Table 2S). Therefore, its low concentration in the wine made its *in-mouth* extraction difficult. Interestingly, from the three volatile phenols (Table 3), only 4-ethylphenol was detected in the mouth after rinsing with W4 and W9 red wines. This compound was also quantified in some white wines (W5, W6, W8), but at lower concentrations, which confirms that the original compound concentration in the wine, is the most limiting factor for the *in-mouth* aroma extraction. Similarly, 4-

ethylguaicol that was present in W9 red wine at a very low concentration was not found either during the *in-mouth* sampling (Table 2S). In the case of eugenol, this compound was not found in the mouth despite appearing in the red W4 wine at a similar concentration as 4-ethylphenol (Table 3). Since both compounds have similar hydrophobicity values (log P 2.3 and 2.5 for eugenol and ethyl phenol respectively), the higher volatility of 4-ethylphenol (210.6 °C) compared to eugenol (253 °C) might have favoured the *in-mouth* extraction.

The rest of the aroma compounds depicted in Table 3 were found in the mouth, at higher or lower amounts depending on the wine type. These data (absolute peak area) were submitted to a PCA in order to confirm if discrimination between wine types could be also possible using the *in-mouth* HSSE volatile profile. Similarly to that described for the PCA of the wines using HSSE data, from this statistical treatment, three components explaining 66.33 % of data variation were obtained. Figure 3b shows the wine samples in the plane defined by the first two principal components. The first one (F1) explained 31.8 % of data variation and it could differentiate white wines (W1, W5, W6 and W8) on the right side of the graph and positive values for this component, from red wines (W4, W9, W3) with negative values and located on the left side of the graph. W10 wine exhibited positive values for PC1 but closer to 0. Likewise, results from the PCA with HSSE (Figure 3a), showed the same situation for W10 red wine with values for F1 closer to zero (Figure 3b). The rosé wine W7, was also closer to the white wines; while the other rosé wine (W2) was more similar to the red wines (Figure 3 b). This was also observed in the first PCA (Figure 3a). When looking in Figure 3b, white wines were more correlated (factor loadings >0.6), with some compounds such as ethyl butanoate, isoamyl acetate, ethyl hexanoate, ethyl octanoate, ethyl decanoate and β -phenylethanol, which were found in higher amounts in the oral cavity after rinsing with

these wines (Figure 3b). All of them are typical compounds found in white wines and are associated to fruity and floral aromatic notes (Francis & Newton, 2005). These aroma compounds also allow us to discriminate between both types of wines using HSSE data (Figure 3a). Remarkably, both types of wines (white and red) were even better separated in the second PCA with data obtained from the *in-mouth* HSSE. Figure 3b, also shows that the oral amounts of the above mentioned aroma compounds were higher in the white Verdejo white wine (W1) compared to the rest of white wines. Furthermore, red wines located on the left side of the graph were positively correlated (factor loadings >0.6) with compounds such as cis-3-hexen-1-ol, diethyl succinate, β -damascenone, ethyl cinnamate, 4-ethyl phenol and dodecanoic acid. All of them were found in the mouth in higher amounts after rinsing with red wines and they also discriminated between red and white types using HSSE data (Figure 3a).

Finally, the second principal component explained a minor percentage of data variation (18.81 %), however, it allowed us to discriminate specific wines from the same wine type (white, red and rosé). For instance, white Godello wine (W8) exhibited higher and positive values of this component compared to the rest of the white wines (W1, W5 and W6). In addition, this component could differentiate W4 and W9 red wines with high values of F2, from W3, with considerably lower values of F2. The former were submitted to an oak aging process, but not the latter. That means that some aroma compounds, like ethyl propanoate, butyl acetate, α -ionone and β -ionone, hexanoic and decanoic acids were found at higher concentrations in the mouth after rinsing with oak aged red wines. This discrimination among young and aged red wines was however better observed in the first PCA, in which some lactones (cis-whisky lactone, trans-whisky lactone) and other aroma compounds, such as eugenol, were highly correlated with these wines, but they could not be detected by using *in-mouth* HSSE.

4. Conclusion

In spite of the very short sampling time (30 s) used in the optimised *in-mouth* HSSE extraction procedure and the very low concentration of wine volatile compounds remaining in the headspace of the mouth after wine rinsing and spit-off, the optimised procedure provided a good representation of the *in-mouth* volatile profile during wine tasting. Above all, the simplicity, sensitivity and good precision of the *in-mouth* HSSE procedure, the possibility of detecting more than 30 wine volatile compounds present in the wine at very low aroma concentrations in a single run and the automatization of the desorption step (using a TDU-CIS device), makes this technique a reliable and feasible tool which can be implemented in many labs to monitor the aroma compounds in the oral cavity, in order to better understand the chemical and biochemical changes that these compounds might experience during wine consumption and how this can affect aroma perception and ultimately consumers choices.

Conflict of interest

The authors declare no competing financial interest.

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Figure Captions

Figure 1. Glass device employed for the *in-mouth* HSSE procedure

Figure 2. Relationship between oral aroma release monitored using *in-mouth* HSSE and wine aroma concentration of typical wine odorant compounds (continuous line). On the Y axis, absolute peak areas (ABAs) are represented, meanwhile, the X axis indicates aroma compound concentration in the synthetic wine (mg/L). Error bars represent $\pm$ SD. The figure also shows the best regression model that fit the experimental data, the estimated regression line (dashed line), and the determination coefficient (R^2). All the measurements were performed three times by the same panellist.

Figure 3. PCA biplot of the ten wines using (a) HSSE data of wine aroma composition (b) *in-mouth* HSSE data of oral aroma composition after wine intake. Only compounds with factor loadings $|\geq 0.6|$ are depicted. **EtBut:** ethyl butanoate; **EtPro:** ethyl propanoate; **EtPen:** ethylpentanoate; **EtHex:** Ethyl hexanoate; **EtOct:** ethyl octanoate; **EtDec:** Ethyl decanoate; **EtDod:** ethyl dodecanoate; **EtCin:** ethyl cinnamate; **Et.Mbut** : ethyl 2-methyl butanoate; **IsoAc:** isoamyl acetate; **DiSuc:** diethyl succinate; **PheAc:** 2-phenylethyl acetate; **HexAct:** hexyl acetate; **ButAct:** butyl acetate; **Ehexol:** (E)-3-hexanol; **Zhexol:** (Z)-3-hexanol; **α -Pin:** α -pinene; **Lim:** limonene; **β -cit:** β -citronellol; **Ner:** nerol; **β -dam:** β -damascenone; **β -ion:** β -ionone; **α -ion:** α -ionone; **γ -non:** γ -nonalactone; **ZwLac:** cis-whisky lactone; **EwLac:** trans-whisky lactone; **EtPhen:** 4-ethylphenol; **Eug:** eugenol **MethFur:** 5-methyl furfural; **Hxacid:** hexanoic acid; **Dcacid:** decanoic acid; **Ddacid** dodecanoic acid.

Appendix A. Supplementary data

Table 1S. Regression lines used for the quantification of aroma compounds in the ten wines using the HSSE method.

Table 2S.. In-mouth aroma release (mean values of absolute peak areas) determined by using in-mouth HSSE GCMS after the oral rinsing with the ten wines of this study.

References

- Amann, A., de Lacy Costello, B., Miekisch, W., Schubert, J., Buszewski, B., Pleil, J., . . . Risby, T. (2014). The human volatilome: volatile organic compounds (VOCs) in exhaled breath, skin emanations, urine, feces and saliva. *Journal of breath research*, 8(3), 034001.
- Buettner, A. (2004). Investigation of potent odorants and afterodor development in two Chardonnay wines using the buccal odor screening system (BOSS). *Journal of agricultural and food chemistry*, 52(8), 2339-2346.
- Buettner, A., & Welle, F. (2004). Intra-oral detection of potent odorants using a modified stir-bar sorptive extraction system in combination with HRGC–O, known as the buccal odour screening system (BOSS). *Flavour and fragrance journal*, 19(6), 505-514.
- Callejon, R., Clavijo, A., Ortigueira, P., Troncoso, A., Paneque, P., & Morales, M. (2010). Volatile and sensory profile of organic red wines produced by different selected autochthonous and commercial *Saccharomyces cerevisiae* strains. *Analytica Chimica Acta*, 660(1-2), 68-75.
- Doi, E., Shibata, D., & Matoba, T. (1981). Modified colorimetric ninhydrin methods for peptidase assay. *Analytical biochemistry*, 118(1), 173-184.
- Esteban-Fernández, A., Muñoz-González, C., Jiménez-Girón, A., Pérez-Jiménez, M., & Pozo-Bayón, M. Á. (2018). Aroma release in the oral cavity after wine intake is influenced by wine matrix composition. *Food chemistry*, 243, 125-133.
- Esteban-Fernández, A., Rocha-Alcubilla, N., Muñoz-González, C., Moreno-Arribas, M. V., & Pozo-Bayón, M. Á. (2016). Intra-oral adsorption and release of aroma compounds following in-mouth wine exposure. *Food chemistry*, 205, 280-288.
- Francis, I., & Newton, J. (2005). Determining wine aroma from compositional data. *Australian Journal of Grape and Wine Research*, 11(2), 114-126.
- Genovese, A., Piombino, P., Gambuti, A., & Moio, L. (2009). Simulation of retronasal aroma of white and red wine in a model mouth system. Investigating the influence of saliva on volatile compound concentrations. *Food chemistry*, 114(1), 100-107.
- Mayr, C. M., Parker, M., Baldock, G. A., Black, C. A., Pardon, K. H., Williamson, P. O., . . . Francis, I. L. (2014). Determination of the importance of in-mouth release of volatile phenol glycoconjugates to the flavor of smoke-tainted wines. *Journal of agricultural and food chemistry*, 62(11), 2327-2336.
- Monagas, M., Gómez-Cordovés, C., & Bartolomé, B. (2006). Evolution of the phenolic content of red wines from *Vitis vinifera* L. during ageing in bottle. *Food chemistry*, 95(3), 405-412.
- Muñoz-González, C., Cueva, C., Pozo-Bayón, M. Á., & Moreno-Arribas, M. V. (2015). Ability of human oral microbiota to produce wine odorant aglycones from odourless grape glycosidic aroma precursors. *Food chemistry*, 187, 112-119.
- Muñoz-González, C., Feron, G., Guichard, E., Rodríguez-Bencomo, J. J., Martín-Alvarez, P. J., Moreno-Arribas, M. V., & Pozo-Bayón, M. A. n. (2014). Understanding the role of saliva in aroma release from wine by using static and dynamic headspace conditions. *Journal of agricultural and food chemistry*, 62(33), 8274-8288.
- Muñoz-González, C., Martín-Álvarez, P. J., Moreno-Arribas, M. V., & Pozo-Bayón, M. Á. (2013). Impact of the nonvolatile wine matrix composition on the in vivo aroma release from wines. *Journal of agricultural and food chemistry*, 62(1), 66-73.
- Muñoz-González, C., Rodríguez-Bencomo, J. J., Moreno-Arribas, M. V., & Pozo-Bayón, M. Á. (2011). Beyond the characterization of wine aroma compounds: looking for analytical approaches in trying to understand aroma perception during wine consumption. *Analytical and bioanalytical chemistry*, 401(5), 1497-1512.

- Ochiai, N., Sasamoto, K., David, F., & Sandra, P. (2018). Recent Developments of Stir Bar Sorptive Extraction for Food Applications: Extension to Polar Solutes. *Journal of agricultural and food chemistry*, 66(28), 7249-7255.
- Parker, M., Black, C. A., Barker, A., Pearson, W., Hayasaka, Y., & Francis, I. L. (2017). The contribution of wine-derived monoterpene glycosides to retronasal odour during tasting. *Food chemistry*, 232, 413-424.
- Pérez-Coello, M. S., & Díaz-Maroto, M. C. (2009). Volatile compounds and wine aging *Wine chemistry and biochemistry* (pp. 295-311): Springer.
- Pérez-Jiménez, M., Rocha-Alcubilla, N., & Pozo-Bayón, M. Á. (2018). EFFECT OF SALIVA ESTERASE ACTIVITY ON ESTER SOLUTIONS AND POSSIBLE CONSEQUENCES FOR THE IN-MOUTH ESTER RELEASE DURING WINE INTAKE. *Journal of texture studies*.
- Poinot, P., Arvisenet, G., Ledauphin, J., Gaillard, J.-L., & Prost, C. (2013). How can aroma-related cross-modal interactions be analysed? A review of current methodologies. *Food Quality and Preference*, 28(1), 304-316.
- Polášková, P., Herszage, J., & Ebeler, S. E. (2008). Wine flavor: chemistry in a glass. *Chemical Society Reviews*, 37(11), 2478-2489.
- Pozo-Bayón, M. Á., Muñoz-González, C., & Esteban-Fernández, A. (2016). Wine preference and wine aroma perception *Wine Safety, Consumer Preference, and Human Health* (pp. 139-162): Springer.
- Robinson, A. L., Boss, P. K., Solomon, P. S., Trengove, R. D., Heymann, H., & Ebeler, S. E. (2014). Origins of grape and wine aroma. Part 1. Chemical components and viticultural impacts. *American Journal of Enology and Viticulture*, 65(1), 1-24.
- Segarra, I., Lao, C., López-Tamames, E., & De La Torre-Boronat, M. C. (1995). Spectrophotometric methods for the analysis of polysaccharide levels in winemaking products. *American Journal of Enology and Viticulture*, 46(4), 564-570.
- Sémon, E., Arvisenet, G., Guichard, E., & Le Quéré, J. L. (2018). Modified proton transfer reaction mass spectrometry (PTR-MS) operating conditions for in vitro and in vivo analysis of wine aroma. *Journal of Mass Spectrometry*, 53(1), 65-77.
- Shepherd, G. M. (2015). Neuroenology: how the brain creates the taste of wine. *Flavour*, 4(1), 19.
- Singleton, V. L., & Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture*, 16(3), 144-158.
- Spitaler, R., Araghipour, N., Mikoviny, T., Wisthaler, A., Dalla Via, J., & Märk, T. D. (2007). PTR-MS in enology: Advances in analytics and data analysis. *International Journal of Mass Spectrometry*, 266(1-3), 1-7.
- Ubeda, C., Callejón, R., Troncoso, A., Peña-Neira, A., & Morales, M. (2016). Volatile profile characterisation of Chilean sparkling wines produced by traditional and Charmat methods via sequential stir bar sorptive extraction. *Food chemistry*, 207, 261-271.
- Weldegergis, B. T., Tredoux, A. G., & Crouch, A. M. (2007). Application of a headspace sorptive extraction method for the analysis of volatile components in South African wines. *Journal of agricultural and food chemistry*, 55(21), 8696-8702.

726 TABLES:

727 **Table 1.** Wine types used in this study and chemical composition (average values). Results
728 from the Tukey test to check compositional differences among wines are also shown.

Wine	Type	Grape variety	Alcohol (%)	pH	TPI (mg/L)	Pr (mg/L)	NP (mg/L)	FAA (mg/L)
W1	White	Verdejo	12.5	3.3 ^{ab}	176.5 ^a	35.8 ^a	6886.5 ^{de}	201.6 ^{cd}
W2	Rosé	Tempranillo	12	3.2 ^a	361.4 ^b	116.9 ^a	3549.9 ^a	184.4 ^{bc}
W3	Red	Tempranillo	13	3.7 ^e	1622.8 ^c	953.4 ^b	6256.7 ^{cd}	225.9 ^{de}
W4	Red	Tempranillo, Graciano, Mazuelo	13.5	3.8 ^e	1712.8 ^c	926.5 ^b	5405.2 ^{bc}	247.6 ^{ef}
W5	White	Albariño	12.5	3.6 ^d	201.5 ^{ab}	36 ^a	7435.3 ^e	268.7 ^f
W6	White	Albariño	12.5	3.5 ^c	248.4 ^{ab}	42.4 ^a	6893.3 ^{de}	267.8 ^f
W7	Rosé	Garnacha	13.5	3.3 ^a	295.1 ^{ab}	102.5 ^a	3308.8 ^a	103 ^a
W8	White	Godello	13.5	3.4 ^{bc}	169.3 ^a	32.2 ^a	6099.1 ^{bcd}	189.9 ^{bc}
W9	Red	Tempranillo	14	3.8 ^e	2138.5 ^d	1198.6 ^c	5313.5 ^b	187.3 ^{bc}
W10	Red	Tempranillo	14	3.7 ^e	1690.3 ^c	992.5 ^b	6778 ^{de}	170.4 ^b

729 **TPI:** Total polyphenols; **Pr:** procyanidins; **NP:** neutral polysaccharides; **FAA:** free amino acids;
730 different superscripts in the same column denote significant differences ($p < 0.05$).

731

732 **Table 2:** Average values of oral aroma release (absolute peak area) obtained using the *in-mouth* HSSE
733 procedure obtained with an aromatised synthetic wines at two aroma concentration (0.5 and 1 mg/L) and
734 different *in-mouth* extraction times (15, 30 and 45 s).

Aroma compounds	In-mouth extraction						Repeatability (R)			
	0.5 mg/L			1 mg/L			0.5 mg/L		1 mg/L	
	15s	30s	45s	15s	30s	45s	15s	30s	45s	15s
Isoamyl acetate	94743 a	245215 a	279817 b	233038 a	369933 b	492094 c	9.1	1.2	4.3	7.9
Ethyl butanoate	61259 a	139906 a	149634 b	158273 a	167163 b	282115 b	11.3	0.5	0.2	3.6
Ethyl pentanoate	90796 a	204877 a	221988 b	235098 a	278448 b	426790 b	2.8	6.8	2.9	9.6
Ethyl hexanoate	99816 a	261417 b	331716 c	259975 a	385097 b	574383 c	2.5	0.6	7.5	4.8
Ethyl octanoate	295307 a	809384 b	1062978 c	612638 a	954754 b	1435143 b	2.4	8.2	2.1	4.2
Ethyl decanoate	175382 a	410475 b	495671 c	336339 a	579068 a	875668 a	6.4	9.8	0.3	20.3
Linalool	22954 a	41624 b	70957 c	62775 a	87189 ab	114062 b	1.3	10.2	0.7	11.5
β-Phenylethanol	39379 a	36233 b	40296 a	40913 b	132434 a	61177 b	2.0	0.8	1.3	24.5
β-Ionone	1366133 a	1016773 a	1469521 a	1067912 a	1393643 a	1215342 a	5.0	15.9	33.4	9.6

735

736 For each compound and concentration assayed, different letters (a-c) denotes significant
737 differences ($p < 0.05$) across extraction times (15, 30 and 45 s).

Table 3. Aroma composition (mean $\pm$ SD mg/L) of the wines (n = 10) of this study obtained by using HSSE-GCMS.

Aroma compounds	White wines								Rosé wines				Red wines							
	W1		W5		W6		W8		W2		W7		W3		W4		W9		W10	
	Mean	$\pm$ SD	Mean	$\pm$ SD	Mean	$\pm$ SD	Mean	$\pm$ SD	Mean	$\pm$ SD	Mean	$\pm$ SD	Mean	$\pm$ SD	Mean	$\pm$ SD	Mean	$\pm$ SD	Mean	$\pm$ SD
Esters																				
Ethyl propanoate	2.34	0.38	1.68	0.11	3.19	0.28	3.62	0.59	2.84	0.18	2.72	0.24	2.18	0.07	2.35	0.08	2.05	0.11	2.14	0.03
Ethyl butanoate	5.71	0.23	4.31	0.27	5.05	0.32	5.72	0.67	3.61	0.04	6.01	0.51	3.39	0.05	2.31	0.26	2.59	0.07	3.97	0.21
Ethyl 2-methylbutanoate	0.11	0.01	0.17	0.09	0.18	0.01	0.38	0.03	0.33	0.04	0.13	0.02	0.42	0.02	0.23	0.03	0.29	0.01	0.07	0.00
Butyl acetate	0.17	0.02	0.38	0.21	0.10	0.01	0.32	0.06	0.38	0.03	0.13	0.01	0.74	0.11	0.10	0.04	n.d.		n.d.	
Isoamyl acetate	62.44	2.52	26.50	0.63	11.96	0.53	16.53	0.42	4.43	0.24	29.50	2.46	11.81	0.03	3.45	0.23	3.84	0.13	40.78	2.20
Ethyl pentanoate	0.28	0.04	0.53	0.36	0.07	0.00	0.61	0.16	0.57	0.09	0.17	0.04	1.40	0.14	0.18	0.21	0.16	0.21	0.15	0.02
Ethyl hexanoate	8.97	0.14	6.83	0.40	9.18	0.27	9.27	0.07	4.39	0.14	7.93	0.66	4.22	0.08	2.84	0.22	3.13	0.06	4.82	0.16
Hexyl acetate	3.41	0.12	1.80	0.33	0.63	0.02	0.73	0.10	0.43	0.05	1.79	0.14	0.99	0.08	0.17	0.11	0.15	0.14	0.90	0.01
Ethyl octanoate	4.72	0.12	4.72	1.64	3.74	0.00	5.48	0.27	1.06	0.03	1.69	0.13	1.79	0.06	0.99	0.15	0.73	0.04	1.71	0.10
Ethyl decanoate	1.12	0.04	1.32	0.65	0.72	0.02	1.19	0.11	0.21	0.03	0.17	0.01	0.40	0.01	0.25	0.10	0.10	0.03	0.40	0.02
Diethyl succinate	4.68	0.18	8.40	1.32	20.74	2.75	33.59	0.23	33.92	0.97	6.20	0.74	62.02	8.13	134.21	9.07	149.33	8.55	53.98	11.09
2-Phenylethyl acetate	3.56	0.28	1.97	0.13	0.78	0.03	0.88	0.04	0.53	0.01	1.70	0.38	0.92	0.00	1.01	0.60	0.66	0.58	2.56	0.17
Ethyl dodecanoate	0.09	0.01	0.07	0.02	0.04	0.01	n.d.		0.15	0.03	n.d.		0.11	0.02	0.26	0.17	0.14	0.18	0.02	0.00
Ethyl cinnamate	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		4.79	3.96	2.64	4.39	n.d.	
Ketones																				
Diacetyl	57.71	6.35	n.d.		23.69	1.34	47.11	10.89	n.d.		n.d.		28.28	0.97	n.d.		26.20	0.61	70.52	5.88

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750 **Table 1S.** Regression lines used for the quantification of aroma compounds in the ten
751 wines using HSSE-GCMS.

Aroma compounds	Linear range (mg/L)	Calibration curve	R ²
<i>Esters</i>			
Ethyl propanoate	0.03 – 1.50	y = 5.1131x	0.99
Ethyl butanoate	0.05 – 2.49	y = 11.297x	0.99
Ethyl 2-methylbutanoate	0.02 – 1.17	y = 21.84x	0.99
Butyl acetate	0.03 – 1.67	y = 9.9516x	0.99
Isoamyl acetate	0.05 – 2.62	y = 22.995x	0.99
Ethyl pentanoate	0.06 – 3.00	y = 15.795x	0.99
Isobutyl acetate	0.02 – 0.99	y = 0.1666x	0.98
Ethyl hexanoate	0.06 – 3.00	y = 25.65x	0.99
Hexyl acetate	0.05 – 2.55	y = 29.156x	0.98
Ethyl octanoate	0.05 – 2.00	y = 21.121x	0.97
Ethyl decanoate	0.03 – 0.88	y = 46.132x	0.99
Diethyl succinate	0.04 – 2.18	y = 0.9789x	0.97
2-Phenylethyl acetate	0.04 – 1.79	y = 8.7202x	0.95
Ethyl dodecanoate	0.02 – 0.62	y = 84.683x	0.91
Ethyl cinnamate	0.03 – 1.14	y = 13.68x	0.98
<i>Terpens</i>			
α-Pinene	0.02 – 0.65	y = 71.646x	0.99
β-Pinene	0.01 – 0.58	y = 133.56x	0.99
Limonene	0.02 – 0.71	y = 48.152x	0.98
Linalool	0.01 – 0.72	y = 4.5982x	0.98
Terpinen-4-ol	0.02 – 1.18	y = 3.5412x	0.99
α-Terpineol	0.02 – 1.22	y = 1.5709x	0.99
β-Citronellol	0.02 – 0.72	y = 1.9571x	0.96
Nerol	0.02 – 0.97	y = 1.8173x	0.97
Geraniol	0.02 – 0.75	y = 1.0487x	0.98
<i>Alcohols</i>			
1-Butanol	0.06 – 2.94	y = 0.2413x	0.99
1-Hexanol	0.07 – 3.34	y = 2.1601x	0.98
Trans-3-hexenol	0.02 – 0.97	y = 2.274x	0.98
Cis-3-hexenol	0.04 – 1.76	y = 1.6257x	0.98
Benzyl alcohol	0.05 – 2.52	y = 0.2492x	0.97
β-Phenylethanol	0.05 – 2.40	y = 0.6812x	0.94
<i>Lactones/furanic acids</i>			
Furfural	0.07 – 3.74	y = 0.9375x	0.99
5-Methylfurfural	0.07 – 3.69	y = 1.2232x	0.98
Furfuryl alcohol	0.16 – 2.03	y = 0.106x	0.86
Cis-whisky lactone	0.04 – 1.30	y = 0.7163x	0.91
Trans-whisky lactone	0.04 – 1.73	y = 0.5639x	0.80
γ-Nonalactone	0.02 – 0.83	y = 1.1399x	0.91
<i>C13 norisoprenoids</i>			

β -Damascenone	0.01 – 0.35	$y = 7.546x$	0.91
α -Ionone	0.02 – 0.62	$y = 10.875x$	0.98
β -Ionone	0.01 – 0.50	$y = 11.351x$	0.98
<i>Volatile phenols</i>			
Guaiacol	0.03 – 1.43	$y = 0.8796x$	0.99
4-Ethylguaiacol	0.03 – 1.32	$y = 2.0456x$	0.98
Eugenol	0.02 – 0.96	$y = 0.6921x$	0.94
4-Ethylphenol	0.03 – 1.72	$y = 1.3559x$	0.96
2-Methoxy-4-vinylphenol	0.02 – 1.17	$y = 0.0219x$	0.76
<i>Ketones</i>			
2,3-Butanedione	0.10 – 4.88	$y = 0.1402x$	0.92
<i>Acids</i>			
Hexanoic acid	0.05 – 1.49	-	-
Decanoic acid	0.04 – 1.52	$y = 0.3916x$	0.73
<i>Benzenic compounds</i>			
Vanillin	0.03 – 1.27	-	-
Methyl vanillate	0.01 – 0.33	-	-
Ethyl vanillate	0.02 – 0.88	-	-
Acetovanillone	0.01 -0.51	-	-

Methyl nonanoate was used as internal standard for the calibration curves of all compounds.

Table 2S. In-mouth aroma release (mean values of absolute peak areas) determined by using in-mouth HSSE GCMS after the oral rinsing with the ten wines of this study.

	White wines				Rosé wines		Red wines			
	W 1	W 5	W 6	W 8	W 2	W 7	W 3	W 4	W 9	W 10
Esters										
Ethyl propanoate	83890.3 ^{abc}	149013.3 ^{de}	121529.7 ^{bcd}	170855.0 ^e	133213.3 ^{cde}	129961.5 ^{cde}	61289.0 ^a	154395.5 ^{de}	111863.8 ^{abcd}	69390.3 ^{ab}
Ethyl butanoate	407182.3 ^c	250041.0 ^{ab}	259234.3 ^{ab}	302263.7 ^b	252749.3 ^{ab}	451648.0 ^c	162741.7 ^a	215028.8 ^{ab}	156938.0 ^a	263810.0 ^{ab}
Ethyl 2-methylbutanoate	0.0 ^a	0.0 ^a	35160.0 ^b	40405.3 ^b	45516.3 ^b	0.0 ^a	0.0 ^a	52493.3 ^b	41423.7 ^b	0.0 ^a
Butyl acetate	0.0 ^a	0.0 ^a	51419.0 ^a	498700.3 ^b	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a
Isoamyl acetate	8096087.8 ^f	2880731.3 ^{de}	1105192.0 ^{abc}	1744890.3 ^{bcd}	726323.7 ^{ab}	3842926.3 ^e	1332292.5 ^{abc}	646812.3 ^{ab}	490795.0 ^a	2480980.0 ^{cde}
Ethyl pentanoate	27467.5 ^b	7320.0 ^{ab}	0.0 ^a	20688.0 ^b	21469.0 ^b	0.0 ^a	26136.0 ^b	22076.5 ^b	8613.3 ^{ab}	0.0 ^a
Ethyl hexanoate	2339017.5 ^d	1482825.3 ^{abc}	1594298.3 ^{bc}	1811394.5 ^{cd}	1224409.0 ^{abc}	1841075.7 ^c	785501.0 ^a	879769.3 ^a	744535.0 ^a	905044.7 ^{ab}
Hexyl acetate	951005.0 ^d	391579.3 ^{bc}	141398.7 ^{ab}	103367.3 ^a	38298.3 ^a	516553.5 ^c	56157.0 ^a	38048.7 ^a	16229.7 ^a	234103.5 ^{ab}
Ethyl octanoate	2488113.8 ^{cde}	2875402.7 ^{de}	2146377.7 ^{bcd}	3194139.3 ^e	836681.3 ^{ab}	1580812.7 ^{abcd}	1107274.3 ^{ab}	833762.3 ^a	598421.3 ^a	1184428.5 ^{abc}
Ethyl decanoate	563512.5 ^{cd}	733853.0 ^d	498839.7 ^{abcd}	572571.7 ^{bcd}	250110.0 ^{abc}	471508.7 ^{abcd}	185849.0 ^a	125856.7 ^a	123302.5 ^{ab}	236298.5 ^{abc}
Diethyl succinate	35596.8 ^a	79225.7 ^a	78764.5 ^a	271554.7 ^a	277912.7 ^a	50068.0 ^a	412254.3 ^a	1409928.5 ^b	1155921.7 ^b	475104.0 ^a
2-Phenylethyl acetate	227359.0 ^c	134159.3 ^{bc}	35177.3 ^{ab}	59219.5 ^{ab}	39598.5 ^{ab}	107528.3 ^{ab}	42816.7 ^{ab}	23331.5 ^{ab}	25419.7 ^a	90402.7 ^{ab}
Ethyl dodecanoate	37397.5 ^{ab}	26100.5 ^{ab}	22674.3 ^{ab}	0.0 ^a	69365.5 ^b	0.0 ^a	66908.0 ^b	27271.5 ^{ab}	29231.7 ^{ab}	25302.5 ^{ab}
Ethyl cinnamate	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a	191800.5 ^b	0.0 ^a	0.0 ^a
Ketones										
Diacetyl	138685.3 ^b	0.0 ^a	140043.7 ^d	183350.3 ^{bc}	0.0 ^a	237163.5 ^d	267890.0 ^{cd}	0.0 ^a	134906.8 ^b	226684.5 ^{cd}
Terpenes										
α -Pinene	274121.3 ^b	0.0 ^a	0.0 ^a	0.0 ^a	33620.5 ^a	0.0 ^a	48127.3 ^a	0.0 ^a	0.0 ^a	0.0 ^a
Limonene	151932.0 ^b	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a
β -Citronellol	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a	61369.0 ^b	0.0 ^a	0.0 ^a	0.0 ^a
Geraniol	258550.0 ^d	0.0 ^a	213922.7 ^{cd}	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a	36448.0 ^{ab}	174990.0 ^{bcd}	118381.0 ^{abc}
Alcohols										
1-Butanol	44004.0 ^a	52751.0 ^a	28862.5 ^a	38966.3 ^a	45079.3 ^a	45103.0 ^a	53329.7 ^a	53811.3 ^a	43124.7 ^a	40465.7 ^a
1-Hexanol	345213.0 ^{bcd}	365714.3 ^{cd}	255030.0 ^{abc}	232310.5 ^a	225561.7 ^{abc}	264518.7 ^{abc}	402308.3 ^d	535279.5 ^e	305424.7 ^{abcd}	163923.0 ^{ab}
Trans-3-hexenol	33296.5 ^c	18180.7 ^b	17754.0 ^b	32441.5 ^c	0.0 ^a	28948.0 ^{bc}	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a
Cis-3-hexenol	22231.7 ^b	23766.0 ^b	18070.0 ^b	0.0 ^a	53579.3 ^c	19012.7 ^b	28976.0 ^b	43559.3 ^c	30555.7 ^b	21373.7 ^b
Benzyl alcohol	11258.5 ^{ab}	23229.3 ^b	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a	26467.0 ^{ab}	28922.7 ^b	12848.3 ^{ab}	0.0 ^a
β -Phenylethyl ethanol	317905.0 ^a	495098.0 ^{ab}	358753.3 ^{ab}	750754.7 ^{abc}	456082.7 ^{ab}	489586.3 ^{ab}	676601.7 ^{abc}	1320701.7 ^{bcd}	1975062.3 ^d	1625361.0 ^{cd}
Lactones / furanic compounds										
Furfural	68175.0 ^b	0.0 ^a	55748.5 ^b	61389.3 ^b	73170.7 ^b	64709.7 ^b	54530.0 ^b	69893.3 ^b	51471.0 ^b	47767.0 ^b
5-Methylfurfural	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a	5332.5 ^{ab}	0.0 ^a	15029.5 ^b	7736.0 ^{ab}	0.0 ^a	11904.0 ^{ab}
Furfuryl alcohol	110916.3 ^{abc}	139022.7 ^{bc}	96243.3 ^{ab}	129649.3 ^{abc}	110503.0 ^{abc}	103219.3 ^{abc}	129774.3 ^{abc}	161423.7 ^c	93282.3 ^{ab}	75629.0 ^a
C13 norisoprenoids										
β -Damascenone	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a	21674.0 ^b	0.0 ^a	0.0 ^a
α -Ionone	36476.3 ^{bc}	36419.7 ^{bc}	32170.0 ^{bc}	54016.3 ^c	0.0 ^a	0.0 ^a	0.0 ^a	39838.7 ^{bc}	23772.0 ^b	0.0 ^a
β -Ionone	11115.3 ^{ab}	0.0 ^a	0.0 ^a	25825.0 ^b	0.0 ^a	0.0 ^a	0.0 ^a	20541.5 ^b	23269.5 ^b	0.0 ^a
Volatile phenols										
4-Ethylphenol	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a	43142.3 ^b	82428.3 ^b	0.0 ^a
Fatty acids										
Hexanoic acid	103153.8 ^a	281737.0 ^{abcd}	291590.7 ^{abcd}	676850.5 ^d	110235.0 ^{ab}	484000.0 ^{cd}	205887.0 ^{abc}	343455.0 ^{bcd}	246762.3 ^{abcd}	360723.0 ^{bcd}
Decanoic acid	257557.3 ^{ab}	295711.3 ^{ab}	216172.7 ^a	420595.5 ^{bc}	309258.7 ^{ab}	222975.3 ^a	271913.3 ^{ab}	456472.7 ^c	283713.0 ^{ab}	257390.0 ^{ab}
Dodecanoic acid	77035.0 ^a	272923.3 ^{abc}	142577.0 ^{ab}	190853.5 ^{ab}	374914.5 ^{bc}	107644.7 ^a	116497.3 ^{ab}	389296.3 ^c	138952.5 ^{ab}	147610.0 ^{ab}

Different superscripts letters for the same compound denote significant statistical differences (p<0.05) among wines from the application of Tukey test.

785 Figure 1

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Figure 2

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Figure 3