Chapter 10

In-Mouth Wine Aroma Analysis

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Abstract

In-mouth aroma analysis under wine-tasting conditions is a challenging task. By combining the use of analytical tools such as solid phase micro-extraction (SPME) or stir bar sorptive extraction (SBSE) with the design of different devices and/or methodological approaches, it is possible to monitor the aroma release in the mouth at different times while simulating the consumption of wine. Two recently employed techniques to monitor in-mouth aroma release from wine are intra-oral SPME and in-mouth headspace sorptive extraction (HSSE). These procedures consist in the intra-oral extraction of wine volatiles into adsorbent/absorbent polymers after the oral exposure to the wine by using an SPME fiber or a stir bar also called a twister. Then, aroma compounds contained in both types of polymers are desorbed and analyzed by gas chromatography-mass spectrometry (GC-MS). Using these tools, the total amount of aroma released by each odorant can be determined. Through this, it is possible to compare the intra-oral amounts and release patterns of different types of wine volatiles, which can be useful for determining their oral aroma persistence. This chapter provides a step-by-step guideline for the extraction, desorption, and analysis by GC-MS of the wine aroma compounds released in the mouth after wine tasting by using intra-oral SPME or in-mouth HSSE procedures.

Key words Intra-oral aroma analysis, Aroma release, Retronasal aroma, Aroma persistence, GC-MS, Wine oral processing

1 Introduction

The intra-oral solid phase microextraction (SPME) and the in-mouth headspace sorptive extraction (HSSE) techniques coupled to gas chromatography-mass spectrometry (GC-MS) allowed us to determine the presence of aroma molecules in the mouth after wine tasting [1, 2]. Both techniques are based on the monitoring of the in-mouth headspace immediately after oral exposure to wine by using adsorbent/absorbent polymers, such as SPME fibers or magnetic stir bars also called twisters, normally used for SBSE. Both techniques rely upon considering that during the in-mouth extractions, the oral cavity works as a closed system as the velum tongue is

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closed, and thus, there is no airflow circulation between the nasal and the oral cavities [3]. Additionally, these techniques allow us to monitor the headspace of the mouth at different and specific times after wine tasting [4, 5]. Like this, it can be possible to obtain the release kinetics of different aroma molecules in the mouth and determine both the immediate and the prolonged retronasal wine aroma, also called aroma persistence. Both techniques have recently been used in different works to investigate the impact of wine composition [6, 7] and/or individual factors [8, 9] on the release and persistence of different types of wine volatiles.

The use of SPME fibers or twisters allows us in a single step the extraction and concentration of the aroma compounds contained in the breath after wine tasting in the polymers before the GC-MS analysis of odorants. Unlike other in vivo and online methods, such as proton-transfer-reaction mass spectrometry (PTR-MS) or atmospheric-pressure chemical ionization (APCI-MS), in which volatiles released in the breath are detected in the MS at real time, both, intra-oral SPME and in-mouth HSSE methods are *offline* (cumulative) methods based on a previous chromatographic separation of the breath aroma compounds prior to detection. The main advantage of the intra-oral SPME procedure over the HSSE procedure is that SPME is more affordable and easier to apply. Nonetheless, its main weakness is the limited sensitivity of the method, due to the reduced amount of polymer in the fiber. This is why this technique could be mainly used for major wine volatiles of for working in spiked model wines, with a reinforced aroma profile. Another drawback is that the technique is not fully automated, since it requires the manual desorption of the fibers (one-by-one) in the GC inlet, which limit the number of analyses that can be done per day. On the other hand, the main advantage of the in-mouth HSSE procedure is that is more sensitive, due the large amount of polymer in the twisters, which allow us to work with real wines at natural aroma concentration without the necessity to reinforce the wine aroma profile [2]. Furthermore, in case of having a TDU (thermal desorption unit) combined with an autosampler, another advantage of the in-mouth HSSE procedure is that once all the in-mouth aroma extractions have been completed, the twisters with the breath extracts can be automatically desorbed in the TDU, which enable to analyze several samples per day and to work with larger groups of volunteers [5].

As previously described in the literature, the main steps of both the intra-oral SPME and the in-mouth HSSE procedures consist in: (1) monitor the in-mouth headspace before the oral exposure to wine; (2) in-mouth aroma extraction after the oral exposure to wine, (3) desorption from the polymer, and (4) analysis by GC-MS [1, 2].

2 Materials

The laboratory reagents should be of food grade and material exclusively for using in studies with human subjects. Once the in-mouth extractions have been performed, in the subsequent stages of sample processing, is not necessary to use food grade reagents and materials.

2.1 General Laboratory Material and Reagents

Laboratory material: graduated flasks with cups, precision balance, spatula, glass pipettes, test tubes, wine glasses, glass or plastic containers to store stocks.

Reagents: ethanol, aroma standards.

2.2 Volunteers

For the selection of volunteers (*see* **Note 1**), some aspects related to their physiology should be considered, such as not having known illnesses, allergies to wine components or being pregnant, and to be non-smokers.

Before to starting the assays, the experimental procedure must be explained to the volunteers in detail. Like this, volunteers should be informed about the aims and procedures of the study, which will be conducted according to the guidelines of the Declaration of Helsinki. They should provide their written consent before their participation. Additionally, is important to train the volunteers in the correspondent procedure (intra-oral SPME or in-mouth HSSE) before the beginning of the test to obtain the most accurate possible results.

2.3 Wine and Palate Cleansers

For the wine, serve 15 mL of wine or synthetic wine (depending on the study) (*see* **Note 2**) in a wine glass and cover it to prevent the alteration of the wine (*see* **Note 3**).

For the application of the intra-oral SPME procedure, it is recommended to use spiked (aromatized) wines in order to enrich their volatile profile. While, the in-mouth HSSE method can be applied directly to wine for testing its aroma release, thus an aromatization steps is not needed.

In case of working with aromatized wines or synthetic wines, it is recommended that the aromatization step can be done immediately before the experiment. The final concentration of each target aroma compound may vary depending on the aim of the study and on the characteristics of the odorants (*see* **Note 4**). Previous studies have used concentrations between 1 and 4 mg/L in 15 mL of wine (Table 1). For the aromatization, it is recommended that each aroma compound can be individually added to the wine. For that, it is advisable to prepare two stock solutions of each aroma compound separately. The first solution (Solution 1) should have a high concentration (e.g., 1000–1500 mg/L) (*see* **Note 5**). A highly concentrated solution is easy to prepare, as it is quite difficult to

Table 1
Aroma compounds, concentrations, and monitoring times employed in the literature in different studies using the intra-oral SPME method to monitor oral aroma release during wine tasting

Aroma compounds	Concentration	N° of in-mouth samplings	References
Ethyl hexanoate, β-ionone, linalool, guaiacol, β-phenylethanol and isoamyl acetate	0.5, 1, 1.5, or 2 mg/L	1	Esteban-Fernández et al. [1]
Ethyl hexanoate, β-ionone, linalool, guaiacol, β-phenylethanol and isoamyl acetate	1 mg/L	1	Esteban-Fernández et al. [13]
Ethyl hexanoate, β-ionone, linalool, guaiacol, β-phenylethanol and isoamyl acetate	2 mg/L	2 (t1 = 0 min; t2 = 4 min)	Perez-Jiménez et al. [4]
Ethyl butyrate, isoamyl acetate, ethyl pentanoate, ethyl hexanoate, ethyl octanoate, ethyl decanoate	2 mg/L	2 (t1 = 0 min; t2 = 4 min)	Pérez-Jiménez et al. [14]
Ethyl butyrate, isoamyl acetate, ethyl pentanoate, ethyl hexanoate, ethyl octanoate, ethyl decanoate	4 mg/L	2 (t1 = 0 min; t2 = 4 min)	Muñoz-González et al. [15]
Ethyl butyrate, isoamyl acetate, ethyl pentanoate, ethyl hexanoate, ethyl octanoate, ethyl decanoate	4 mg/L	2 (t1 = 0 min; t2 = 4 min)	Muñoz-González et al. [16]

t1 and t2 are the in-mouth sampling times (in min) after wine spat out

weigh very small quantities of aroma compounds. From Solution 1, a second stock solution (Solution 2) with a lower concentration (e.g., between 200 and 300 mg/L) can be prepared. This Solution 2 will be the working solution to spike the wines.

Palate cleansers: prepare in three different bottles a solution of a teaspoon of bicarbonate in mineral water, a pectin solution of 1 g/L in mineral water, and finally mineral water to clean the mouth of the volunteers before each assay and between wine samples (*see* **Note 6**).

2.4 Intra-Oral Wine Aroma Extraction

In case of the intra-oral SPME procedure, DVB/CAR/PDMS (divinylbenzene/Carboxen/polydimethyl siloxane 50/30 μm film thickness, 2 cm length) coated SPME fibers are usually employed for the extraction of aroma compounds in the mouth. If the analysis requires monitoring aroma release at different times after expectoration of the wine, different fibers will be used for each sampling time (one fiber to monitor immediately after rinsing, another fiber to monitor 5 min later, etc.). The number of fibers needed will depend on the number of in-mouth aroma extractions, considering that fibers are manually injected in the GC (*see* **Note 7**).

To monitor the headspace of the mouth, the fibers should be placed in manual holders for SPME. In order to assure that the fiber does not touch the oral surfaces during the intra-oral

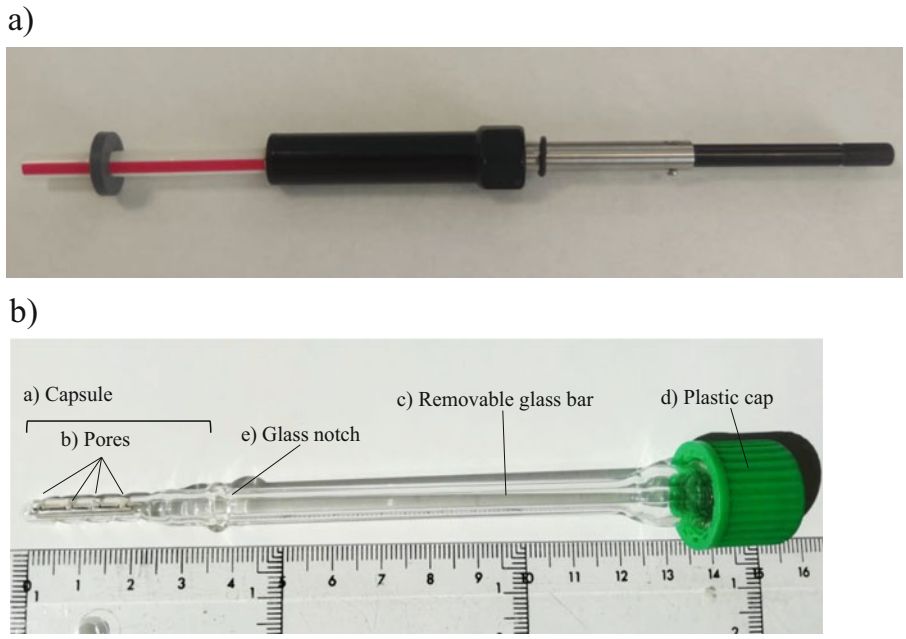

Fig. 1 Devices employed for the intra-oral wine aroma extraction. (**a**) The device employed for the intra-oral SPME. (**b**) The glass device employed for the in-mouth HSSE procedure. (From Perez-Jimenez and Pozo-Bayon [2])

monitorization, a home-made plastic adaptor can be placed above the fiber [1]. This can be a plastic/Teflon tube (like a piece of a drinking straw) with a mark in one of its sides to place the lips always in the same position (Fig. 1a). Plastic tubes should be discarded after the test. Each volunteer might use his/her own adaptor throughout the study.

In the case of the in-mouth HSSE procedure, PDMS twisters of 20 mm length × 0.5 mm thickness are usually employed for the extraction of aroma compounds in the mouth. Different twisters should be employed for each in-mouth extraction. The number of twisters needed for the experiment will depend on the number of in-mouth aroma extractions and on the number of analyses to be performed per day, considering that twisters can be automatically desorbed in the GC (*see* **Note 7**).

For the in-mouth headspace twister extraction, it is necessary to place the twister in a holder device [2]. For instance, in Perez-Jimenez and Pozo-Bayón (2019) they used a tailored made glass holder device developed by Segainvex-UAM (Madrid, Spain) (Fig. 1b). This was a hollow glass tube (14.5 cm long) with a capsule (a) at one end into which the twister was placed. The capsule was homogeneously perforated with eight pores (2–3 mm diameter) (b). The dimensions of the capsule (27 mm long × 5 mm diameter) prevent the movement of the twister during the in-mouth aroma extraction. A removable glass bar (c) was placed

inside the hollow tube to prevent air flow during the extraction. At the end of the tube, a plastic cap (d) closed the device. The device also had a glass notch (e) outside which indicates the position where the lips should be placed during the extraction.

For handling the twisters without touching them, a magnet bar can be used.

3 Equipment for the Desorption and Analysis of Aroma Compounds

For the analysis of aroma compounds, a GC-MS equipment can be used. Alternatively, other chromatographic techniques such as GC-olfactometry (GC-O), two-dimensional GC, or other detectors like Flame Ionization Detector (FID) could be used for the identification of volatiles [10–12].

For the intra-oral SPME procedure, the SPME fiber should be manually injected in the split/splitless GC injector. For the separation of volatiles, it is recommended to use a capillary column with high polarity and polyethylene glycol as a stationary phase. For instance, a DB-WAX column (Agilent, j&WScientific, Folsom, CA, USA) with dimensions of 60 m × 0.25 mm and film thickness of 0.50 μm [1]. Nonetheless depending on the type of volatiles of interest other columns might be also used. The carrier gas is usually Helium.

For the in-mouth HSSE procedure, the automated injection of twisters should be done in the autosampler of the GC that must contain the injector adaptor for twisters. Additionally, a thermal desorption unit (TDU) in combination with a CIS-4 (cooled injection system) injector is required. This system allows the thermal desorption of volatiles in the TDU first and then, cryo-focusing the analytes in the CIS-4 system using low temperature (e.g., liquid Nitrogen) prior to their transfer onto the analytical column. For the separation of volatiles, the same column and chromatographic conditions recommended for intra-oral SPME can be used.

4 Methods

It is recommended to perform each analysis at least three times with each volunteer (*see* **Note 8**). The volunteers must be instructed not to drink, eat or smoke 2 h before the assay.

Fifteen minutes before each experiment, the volunteers must perform vigorous rinses with the palate cleanser solutions in the order: bicarbonate solution, pectin solution, and mineral water, to have the most similar oral conditions among them when starting the assay.

The volunteers must introduce the 15 mL of the wine or aromatized wine in the mouth in a single zip doing soft rinses during 30 s (*see* **Note 9**). During rinsing, the lips must be closed and swallowing is not allowed, in order to avoid opening the velum–tongue border prior to expectoration. After rinsing, volunteers should spit out the wine, perform a single swallowing of the remaining saliva in the mouth and wait for 5 s until the first intra-oral aroma extraction.

5 Intra-Oral Wine Aroma Extraction

5.1 Intra-Oral SPME

A schematic representation of the intra-oral SPME procedure is shown in (Fig. 2a).

Once the wine sample has been expectorated and the remaining saliva swallowed, place the SMPE fiber contained in the manual holder and with the plastic protector tube into the oral cavity of the volunteer for 2 min (*see* **Note 10**), as it is shown in Fig. 3a. During the in-mouth extraction, the lips should be kept closed around the plastic tube containing the SPME fiber, and swallowing should be avoided (*see* **Note 11**).

After 2 min of extraction, remove the fiber from the mouth and swallow once.

Remove the fiber from the manual holder and keep it in the freeze (4 °C) until its analysis by GC-MS. For that, introduce the SPME fiber in a sealed glass test tube to assure the proper preservation of the extracted aroma compounds from the breath (*see* **Note 12**).

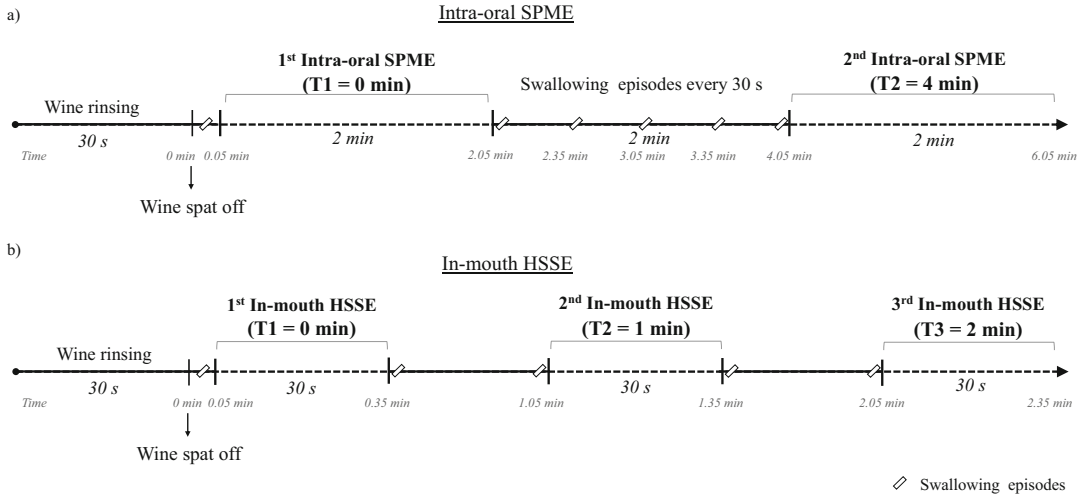

Fig. 2 Scheme of the procedures followed for the aroma extraction in the oral cavity. (**a**) The intra-oral SPME; (**b**) the in-mouth HSSE. (Modified from Perez-Jiménez et al. [4, 5])

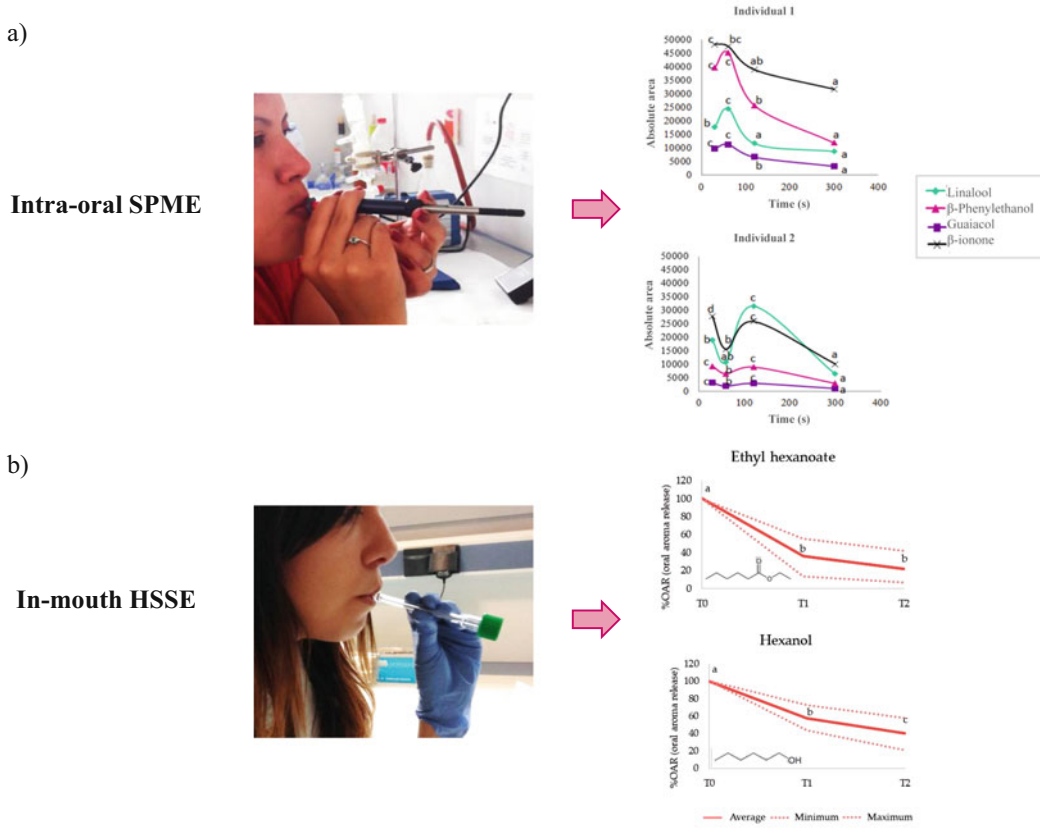

Fig. 3 Picture of the aroma release kinetics of different types of wine aroma compounds obtained by using intra-oral SPME (3a) and in-mouth HSSE procedures (2b). (From Pérez-Jiménez [17])

After the first monitoring, wait 2 min until the second expectoration (*see* **Note 13**). During waiting, the volunteer must breathe normally through the nose and with the mouth closed (*see* **Note 14**) and perform one swallowing every 30 s (five times in total) (Fig. 1a).

Then, 4 min after wine expectoration, perform the second intra-oral monitoring by using a second SPME fiber and following the same instructions than for the first monitorization. After 2 min of intra-oral extraction, remove the fiber from the mouth and desorbed it immediately in the GC system for the analysis of odorants. When the run has finished, analyze the fiber with the breath aroma extract corresponding to the first monitorization.

5.2 In-Mouth HSSE

A schematic representation of the in-mouth HSSE procedure is shown in Fig. 2b.

Expose the glass device containing the twister to the headspace of the mouth and keep the lips closed and placed on the surface of the glass notch (*see* **Note 10**). During the in-mouth extraction swallowing is not allowed (*see* **Note 11**). Keep this position during

30 s as it is shown in Fig. 3b, then, remove the device from the mouth and swallowing once. After 1 min of waiting (*see* **Notes 13** and **14**), swallow once and place into the mouth the second glass device with the second twister inside for the second in-mouth aroma extraction. Keep this position during 30 s, then remove the device from the mouth, swallow once and wait during 1 min until the third breath monitoring. For the third (and followings) in-mouth aroma extractions, repeat the procedure explained for the second monitoring.

Once the in-mouth extractions are finished, remove the twisters from the glass devices using a magnetic bar (*see* **Note 15**), dry them softly with a tissue and place them in their correspondent glass tubes in a refrigerated tray for their thermal desorption.

6 Desorption and Analysis of Aroma Compounds

6.1 Desorption and Chromatographic Analysis

The desorption of the fibers must be in splitless mode for 1.5 min at 270 °C in the injector port of the GC (*see* **Note 16**). The Helium flow is usually set at 1 mL/min. Different chromatographic conditions can be used. For instance, the oven temperature ramp can be start at 40 °C for 2 min, then increased at 8 °C/min to 240 °C and hold it for 15 min.

The desorption of twisters in the TDU is in splitless mode, and the ramp temperature could be start at 40 °C, then increases to 240 °C at 60 °C/min and hold for 5 min (*see* **Note 17**). For the cryofocusing in CIS by using liquid nitrogen the ramp temperature can be start at −100 °C, then heated to 240 °C at 12 °C/min, and hold for 5 min. The injection is usually configured in solvent vent mode. The chromatographic conditions can be similar to those described for the intra-oral SPME.

6.2 MS Identification of Aroma Compounds

Different conditions of MS can be used. An example can be: transfer line at 270 °C, quadrupole at 150 °C and ion source at 230 °C. Electron impact mass spectra is usually recorded at 70 eV and the ionization current is 10 μA. For the acquisitions, both selected ion mass monitoring (SIM) and full scan mode (mass range of 35–350 m/z) can be used depending on the objective of the study.

For the identification of compounds, the mass spectra and retention times are compared with those present in MS libraries (e.g., NIST 2.0 database).

In the intra-oral SPME and in-mouth HSSE methods, the absolute peak areas of aroma compounds are obtained which are used to express the amount of aroma release. These procedures allow to compare the extent of intra-oral aroma release among wine samples by comparing the absolute peaks areas of the same aroma compounds among samples, individuals, etc. Figure 3 shows an example of the release kinetic that can be obtained with the intra-oral SPME and the in-mouth HSSE procedures.

7 Notes

1. For studies aiming to investigate the effect of wine matrix, it would be recommended to recruit at least 8 volunteers. While, for studies aiming to investigate inter-individual differences, a larger number of volunteers would be recommended for their recruitment, although this number should not be too high either (e.g., more than 40). For instance, for the intra-oral SPME it would be recommended to recruit up to 20 volunteers (more than 20 participants will extend too much the duration of the study), whereas for the in-mouth HSSE a large number of volunteers (e.g., 30) could be recruited without extending too much the duration of the study.
2. When working with wines that require to reinforce the aroma profile, it is recommended to use a wine with a low aromatic profile for a better detection of the target aroma compounds added to the wine.
3. To cover the glass wine, a piece of aluminum foil or a Petri dish lid could be used.
4. Before the experiments, it is recommended to check if the selected aroma concentrations (in case of spiked or synthetic wines) provide enough sensitivity. It is important to keep the in mind that differences in the physicochemical characteristics of the aroma molecules (e.g., volatility, polarity) can affect the affinity for the PDMS polymer and the chromatographic response.
5. Having a small amount of ethanol at the bottom of the flask helps the aroma compound to dissolve better.
6. Rinses with bicarbonate can be optional, while rinses with pectin solution and water are highly recommended to clean the palate when tasting wines with a high concentration of polyphenols. It is recommended to perform rinses during around 30 s with each of the palate cleansers (bicarbonate, pectin solution, and water). After rinses with all the palate clean solutions, it is recommended to wait 15 min until starting the next assay (in-mouth extraction) with wine.
7. It is recommended to check and select all the SPME fibers and twisters that will be used through the study, considering their similarity in volatile recovery rates, bearing in mind that differences between them should not exceed 5%.
8. A maximum of three wine samples per day, including sampling replicates (e.g. a total of 9 wine samples), and per volunteer is recommended to avoid participant fatigue. Although this number may vary depending on the ethanol/polyphenol content of the wine samples used.

9. To make this task easier for the volunteers, it is recommended that the person in charge of the experiment give the precise instructions of each step of the extraction procedure.
10. It is important to place the fiber or the glass device containing the twister in the headspace of the mouth avoiding any contact with oral surfaces. During the in-mouth aroma extraction, it is also important to adopt a comfortable posture, for example sitting with both elbows on the table and holding the fiber holder or the glass device with both hands. Volunteers must be also informed to breathe through the nose, not to blow through the mouth, not to swallow and not to make abrupt movements during the intra-oral monitoring. It is recommendable trying to follow always a similar protocol of movements and breathing in all the repetitions of the test in order to obtain a good repeatability (less than 15% of variation among replicates). For training the volunteers in the in-mouth extraction techniques, 2 or 3 additional sessions may be necessary.
11. It may be possible the generation and accumulation of saliva in the mouth during the in-mouth aroma extraction. In this case, gently blot the remaining saliva with a paper tissue, trying to not alter the position and without open the mouth.
12. Preliminary experiments have been performed in order to ensure that there were no significant losses of aroma during the storage of the fiber, which was not more than 1 h.
13. The waiting time between the in-mouth monitorization can be modified depending on the aims of the study.
14. During this waiting time between the first and second intra-oral aroma extraction do not talk or open the mouth. Swallowing is only possible when indicated and it is recommended to be quite and keep in the same place and position moving as little as possible (e.g., do not stand up).
15. For the cleaning of the glass devices after in-mouth extractions, a solution of ethanol in water at 60% is used. For that, all the glass devices and the removable glass bar from inside can be immersed in an ethanolic solution for a few minutes and then dry with clean paper.
16. For cleaning the fibers after each injection avoiding any memory effect, the fibers can be placed in the injector of the GC at 270 °C during 10 min.
17. For cleaning the twisters after the in-mouth aroma extractions avoiding possible residual aroma compounds a GC method is used. For this, the TDU can be configured in split mode at 240 °C for 10 min and the CIS temperature ramp set from 180 to 240 °C. The oven temperature can be start in 50 °C during 2 min, increases up to 240 °C and hold for 15 min. This cleaning procedure also allow the cleaning of the glass liners in which the twisters are placed for their desorption.

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