Optimization of stir bar sorptive extraction (SBSE) and multi-stir bar sorptive extraction (mSBSE) to improve must volatile compounds extraction

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ABSTRACT

The aromatic compounds present in grapes are found in very low concentrations, since, for their determination, a previous step of selective extraction is necessary. In this work we optimize and compare, for the first time, the techniques of extraction by stir bar sorption (SBSE) and multi-stir bar sportive extraction (mSBSE), with the aim of analyzing the must volatile composition. For this purpose, two randomized factorial designs were carried out in which the following factors and levels were combined and optimized: for SBSE, extraction mode (headspace (HS), direct immersion (DI), and both at the same time), extraction speed (500/1000 rpm), extraction time (1/3/6 h), extraction temperature (20/40/60 °C) and NaCl addition (with and without NaCl, and sequential); and for mSBSE: extraction speed (500/1000 rpm), extraction time (1/3/6 h), extraction temperature (20/40/60 °C), and NaCl addition (with and without). The results showed that SBSE technique provided a higher extraction of volatile compounds than mSBSE. After performing principal component analysis (PCA) and analysis of variance (ANOVA) multifactorial, it was concluded that the best conditions for SBSE were: HS, 500 rpm, 6 h, 60 °C and adding NaCl (sequential); and for mSBSE were: 500 rpm, 6 h, 60 °C and without NaCl.

1. Introduction

Grape volatile composition is one of the most important parameters to determine the quality of must and wine (Aleixandre-Tudo, Weightman, Panzeri, Nieuwoudt, & Du Toit, 2015; Robinson et al., 2014). Grape aromas, also called primary aromas, are composed of varietal and pre-fermentative compounds, and their content depend mainly on the grape variety, climatic conditions and viticultural practices (Marín-San Román, Garde-Cerdán, Baroja, Rubio-Bretón, & Pérez-Álvarez, 2020). The main compounds that form the varietal aroma are: monoterpenoids, C_{13} norisoprenoids, benzenoids, esters, thiols and methoxypyrazines (Rubio-Bretón et al., 2019). Within these compounds, monoterpenoids and C_{13} norisoprenoids are the compounds that contribute most to wine aroma. On the other hand, within the pre-fermentative aroma, C6 compounds, known as "green leaf volatiles", can be found (Black, Parker, Siebert, Capone, & Francis, 2015; Rubio-Bretón et al., 2019; Styger, Prior, & Bauer, 2011).

In order to understand the chemical nature of wine aroma, it is necessary to determine and know the aromatic compounds present in the grape. These compounds are found in very low concentrations, so, in order to carry out their identification and quantification, an efficient pre-concentration method is required prior to their analysis (Marín-San Román, Rubio-Bretón, Pérez-Álvarez, & Garde-Cerdán, 2020; Perestrelo, Barros, Rocha, & Câmara, 2011; Sánchez-Palomo, Alañón, Díaz-Maroto, González-Viñas, & Pérez-Coello, 2009). Sample preparation is a critical step, since a large amount of analyte can be lost (Andrade-Eiroa, Canle, Leroy-Cancellieri, & Cerdà, 2016). In recent years, sample preparation techniques have been evolving, searching for greater accuracy, precision, and sensitivity (Serrano de la Hoz, 2014). For years, different techniques have been used for the extraction of volatile compounds in grapes and wine. The most conventional techniques, such as liquid-liquid extraction (LLE) or solid phase extraction (SPE), have several drawbacks: low selectivity and reproducibility, loss of analytes, difficult automation, and large volumes of solvent and sample (Marín-San Román, Rubio-Bretón, et al., 2020). These reasons encouraged the emergence of solid phase microextraction (SPME) and stir bar sportive extraction (SBSE) which used less solvent volume, could be automated, improved sensitivity, etc … (Kataoka, Lord, & Pawliszyn,

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2000; Marín-San Román, Rubio-Bretón, et al., 2020). Both techniques improve the sensitivity since the devices are introduced directly into the thermal desorption system (TD) of the GC. However, SBSE increases the sensitivity by a factor of 50–250 and is much more robust compared to SPME (Marín-San Román, Rubio-Bretón, et al., 2020).

SBSE was first introduced in 1999 (Baltussen, Sandra, David, & Cramers, 1999), is based on the use of a magnetic bar (Twister), encapsulated in a glass cover coated with an absorbent material, which can be used by head space (HS) or by immersion (DI) (Baltussen et al., 1999, 2002). This technique is simple, straightforward to use, environmentally friendly, only require small sample volume, and have low operation costs (Huang et al., 2020). The most used polymeric material for this technique is polydimethylsiloxane (PDMS), due to PDMS has inert character, and is ease of synthesize, has high extraction performance, and its degradation products are easily identifiable (Marín-San Román, Rubio-Bretón, et al., 2020). It has been proven that PDMS Twister does not lose its absorbent capacity until approximately 100 uses. The amount of absorbent can vary from 24 μL to 126 μL depending on the length of the Twister (10 mm or 20 mm). This large phase volume is what allows it to increase the sensitivity with respect to SPME (Bicchi, Iori, Rubiolo, & Sandra, 2002; Lanças, Queiroz, Grossi, & Olivares, 2009; Nogueira, 2012, 2015). A variant of this extraction technique is multi-SBSE (mSBSE). mSBSE is the only technique so far that combines two magnetic bars with different absorbents, ethylene glycol (EG) and PDMS (Ochiai, Sasamoto, Ieda, David, & Sandra, 2013). EG has a higher affinity for polar compounds than PDMS coating. Combining both of them, it is possible to cover a greater range of polarities (Marín-San Román, Rubio-Bretón, et al., 2020; Ochiai, Sasamoto, David, & Sandra, 2018).

Since the beginning of SBSE, the technique has been widely used for the analysis of volatile compounds in different matrices such as roasted Arabica coffee (Bicchi et al., 2002), water (Ochiai, Sasamoto, Kanda, & Pfannkoch, 2008; Sampedro, Goicolea, Unceta, Sánchez-Ortega, & Barrio, 2009), vinegar (Marrufo-Curtido et al., 2012), drug products (Scherer, Marcseková, Posset, & Winter, 2019), among others. SBSE has also been used, and even optimized, in the analysis of volatile compounds in wine samples, in particular in oak-aged wines (Marín, Zalacain, De Miguel, Alonso, & Salinas, 2005), in Pinot Noir wines (Fang & Qian, 2006), in white wines of six varieties (Zalacain, Marín, Alonso, & Salinas, 2007), in Bordeaux wines (Franc, David, & de Revel, 2009), in Riesling wines (Doneva-Sapceska, Sponholz, & Tasev, 2011), in Graciano wines (Gómez-Caballero et al., 2013), in 15 white, rosé, and red wines (Cacho, Campillo, Viñas, & Hernández-Córdoba, 2013), in Monastrell wines (Pardo-Garcia, De La Hoz, Zalacain, Alonso, & Salinas, 2014), in Chilean sparkling wines (Ubeda, Callejón, Troncoso, Peña-Neira, & Morales, 2016), etc … Some studies have compared the use of the polymeric phase of EG versus PDMS for the aromatic characterization of wines (Serrano de la Hoz, Salinas, & Ferrandino, 2016; Tang, Hu, Fan, Xu, & Li, 2020; Zhou, Qian, & Qian, 2015). In all of them, better yields are obtained for more polar solutes using the EG phase. However, very few works have been found that study the volatile composition of grape or must using SBSE (Carlomagno, Schubert, & Ferrandino, 2016; Caven-Quantrill & Buglass, 2006, 2011; Martínez-Gil, Garde-Cerdán, Martínez, Alonso, & Salinas, 2011; Pedroza, Zalacain, Lara, & Salinas, 2010; Salinas, Zalacain, Pardo, & Alonso, 2004; Vasile-Simone et al., 2017).

On the other hand, the mSBSE technique has been used for the analysis of volatile compounds in aqueous samples (Ochiai et al., 2013), in beverages (Ochiai, 2018), and in bacterial cultures (Berrou, Dunyach-Remy, Lavigne, Roig, & Cadiere, 2019). In the case of the articles of beverage and aqueous samples, a much higher recovery of polar solutes was obtained with mSBSE than with the conventional SBSE technique. However, no papers have been found that use mSBSE to analyze aromatic compounds in grapes or wines, and seeing the good results obtained in samples of similar characteristics to wine and must, it was decided to also optimize the mSBSE for must samples.

Therefore, the objective of this work was to optimize the SBSE and mSBSE methods for the extraction of volatile compounds in Tempranillo must. The factors and conditions to be tested were chosen based on the existing literature and the Twister manufacturer's recommendations. A design of experiments (DoE) was performed to collect the factors and conditions for each factor. Once the compounds of interest were extracted, they were identified and quantified by gas chromatography-mass spectrometry (GC-MS). Finally, a comparison was made between the best conditions obtained for SBSE and the best conditions obtained for mSBSE, in order to choose which method best extracts the volatile compounds in Tempranillo must.

2. Materials and methods

2.1. Materials and reagents

Chromatographic standards linalool, α-terpineol, geraniol, β-ionone, β-damascenone, 2-phenylethanol, ethyl octanoate, ethyl hexanoate ethyl decanoate, isoamyl acetate, 2-phenethyl acetate, hexanoic acid, octanoic acid, isobutanol (2-methyl-1-propanol), 2-octanol (I.S.), hexanal, amyl alcohol (2-methyl-1-butanol), isoamyl alcohol, and 1-hexanol were purchased from Merck (Darmstadt, Germany).

The reagents ethanol (EtOH), and NaCl were purchased from Merck, water was purified through a Milli-Q system Millipore (Bedfords, MA, USA).

Twister for SBSE with PDMS (film thickness 0.5 mm, length 10 mm), Twister for SBSE with EG-Silicone (length 10 mm, 32 μL phase volume), liners packed with Tenax TA™, and borosilicate magnetic stirrers were obtained from GERSTEL GmbH & Co (Mülheim an der Ruhr, Deutschland). The capillary column BP21 (50 m length, 0.22 mm i.d., and 0.25 μm film thickness) was obtained from SGE (Ringwood, Australia).

Ultra-Turrax was purchased from IKA-Werke GmbH & Co. KG (Staufen, Germany). Gas chromatograph was purchased from Agilent Technologies (Palo Alto, CA, USA). Multi-purpose autosampler (MPS) and Automated thermal desorption unit (TDU) were provided from GERSTEL. The autosampler system consisted of a multi-purpose sampler (MPS), equipped with tube tray, thermal desorption unit (TDU), and cooled injection system (CIS-4) connected to a N_2 ranger.

2.2. Grape paste

The grapes were harvested from a Tempranillo vineyard located in Logroño (La Rioja). For this purpose, 50 vines from this vineyard were marked. The grapes were harvested during the 2019 vintage. A total of 44.4 kg of grapes were obtained. The bunches of grapes were shelled in a box and all the grapes were mixed. Once shelled, all the berries were crushed with the Ultra-Turrax until a homogeneous paste was obtained. Then, 450 Falcon tubes of 50 mL were obtained from this paste, which were frozen for later use.

2.3. Standards solution

A solution in which all standards was added. This solution was prepared in 12% EtOH. The concentration of the standards was: 0.001 mg/mL of linalool, α-terpineol, geraniol, β-ionone, isoamyl acetate, hexanoic acid, octanoic acid, isobutanol, hexanal, amyl alcohol, isoamyl alcohol, and 1-hexanol; 0.0002 mg/mL of ethyl octanoate, ethyl hexanoate and ethyl decanoate; and $9.99 \cdot 10^{-5}$ mg/mL of β-damascenone.

2.4. Design of experiments (DoE)

A randomised factorial design was used for the two extraction techniques. The software used to perform the DoE was Minitab 18 (Minitab Inc, Pennsylvania, USA).

SBSE. A 5-factor randomised factorial design with 2, 3, 3, 3, and 3 levels each was performed. The factors were: stirring speed (500, and

Table 1

Design of experiments with their factors and levels of the two extraction techniques (SBSE and ^{m}SBSE).

	FACTORS	LEVELS		
SBSE	**Stirring speed (rpm)**	500		1000
	Extraction time (h)	1	3	6
	Extraction temperature (°C)	20	40	60
	Addition of NaCl	YES	NO	Sequential
	Extraction mode	HS-SBSE	DI-SBSE	HS+DI
^{m}SBSE	**Stirring speed (rpm)**	500		1000
	Extraction time (h)	1	3	6
	Extraction temperature (°C)	20	40	60
	Addition of NaCl	YES		NO

1000 rpm), extraction time (1, 3, and 6 h), extraction temperature (20, 40, and 60 °C), NaCl addition (Yes, No, and Sequential), and extraction mode (Head Space (HS), Direct Immersion (DI), and HS+DI) (Table 1). Sequential indicates that NaCl was added at half the extraction time. In order to favor the extraction of less polar compounds at the beginning, and more polar at the end. Samples were run in duplicate, giving a total of 486 assays.

^{m}SBSE. A randomised factorial design of 4 factors of 2, 3, 3, and 2 levels each was performed. The factors were: stirring speed (500, and 1000 rpm), extraction time (1, 3, and 6 h), extraction temperature (20, 40, and 60 °C), and NaCl addition (Yes, and No) (Table 1). Samples were run in duplicate, giving a total of 108 assays.

Initially 1500 rpm was tried, but the stirring magnets and Twisters were breaking, so we stopped working at that stirring speed.

2.5. Conditioning and cleaning of twisters

SBSE. The PDMS Twisters were conditioned before the first use and after each use. Stir bars were placed in a desorption tube and thermally desorbed using an autosampler coupled to the gas chromatography (GC) system. These were controlled with GERSTEL MAESTRO software. Stir bars were thermally desorbed using helium as carrier gas at a flow rate of 75 mL/min; TDU was programmed from 40 °C to 300 °C (45 min) at a rate of 100 °C/min for following the supplier's recommendations. The temperature of the CIS-4 was 300 °C (5 min), which was reached at a rate of 12 °C/s.

^{m}SBSE. EG Twisters were conditioned before the first use and after each use. Stir bars were thermally desorbed using helium as carrier gas at a flow rate of 75 mL/min; the TDU temperature programme was as follows: 40 °C to 240 °C (30 min) at a rate of 100 °C/min for following the supplier's recommendations. The CIS-4 temperature was programmed to reach 240 °C (5 min) at 12 °C/s. The GC oven temperature was programmed at 40 °C (5 min), raised to 170 °C (2 °C/min, held for 0 min) then to 220 °C (10 °C/min, held 15 min).

2.6. Optimization of volatile compounds extraction

2.6.1. Stir bar sorptive extraction (SBSE)

Different amounts of sample were added depending on the extraction mode (HS or DI). This is due to the fact that, when working with grape must, and the PDMS Twister works by DI, the sample has to be diluted so that no remains of sugars adhere to it.

Head Space SBSE (HS-SBSE). An aliquot of 8 mL of centrifuged must sample (15 min, 4500 rpm), 1 mL of the standards solution, and 25 µL of the 2-octanol solution (5 µL 2-octanol/100 mL EtOH) were added to a

Table 2

Maximum and minimum values of the relative area with respect to 2-octanol (I.S.) of each compound within each method (SBSE and ^{m}SBSE).

	SBSE		^{m}SBSE	
	maximum value	minimum value	maximum value	minimum value
Terpenoids				
Linalool	7.12 (HS_500_6_20_SEQ)	0.17 (HS_500_3_40_YES)	0.14 (1000_6_20_YES)	0.08 (500_1_60_YES)
α-Terpineol	1.70 (DI_500_6_60_SEQ)	0.01 (HS_500_1_20_YES)	0.11 (500_6_60_NO)	0.03 (1000_1_40_NO)
Geraniol	2.50 (HS+DI_500_6_20_NO)	0.05 (HS_1000_3_40_NO)	0.16 (1000_3_20_NO)	0.04 (1000_1_40_NO)
C$_{13}$ norisoprenoids				
β-Damascenone	2.13 (DI_1000_6_60_YES)	0.02 (HS_500_1_20_YES)	0.12 (500_6_60_NO)	0.01 (500_3_20_YES)
β-Ionone	16.35 (DI_1000_1_20_NO)	0.02 (HS_500_6_20_YES)	0.15 (500_6_20_NO)	0.01 (500_3_20_YES)
Benzenoid compounds				
Benzaldehyde	0.22 (HS+DI_500_6_60_YES)	0.004 (HS_500_3_40_YES)	0.03 (500_3_20_NO)	0.002 (1000_1_40_YES)
2-Phenylethanol	0.50 (HS+DI_1000_3_40_NO)	0.01 (HS_500_1_20_YES)	0.04 (1000_6_60_YES)	0.01 (500_1_60_YES)
Esters				
Isoamyl acetate	27.57 (HS_500_6_20_SEQ)	0.12 (DI_1000_6_40_YES)	0.10 (500_3_20_NO)	0.01 (1000_6_60_YES)
Ethyl hexanoate	6.74 (HS_500_6_20_SEQ)	0.06 (DI_1000_6_40_YES)	0.06 (500_3_20_NO)	0.01 (500_6_60_YES)
Ethyl octanoate	2.29 (HS_500_6_20_SEQ)	0.01 (DI_500_1_20_YES)	0.04 (1000_3_20_NO)	0.002 (500_3_20_YES)
Ethyl decanoate	1.85 (HS+DI_1000_3_20_NO)	0.01 (DI_500_1_40_YES)	0.03 (1000_6_20_NO)	0.001 (1000_3_20_YES)
Fatty acids				
Hexanoic acid	4.98 (HS+DI_500_3_60_SEQ)	0.02 (HS_500_6_20_YES)	0.35 (500_3_60_NO)	0.01 (500_3_40_YES)
Octanoic acid	0.89 (DI_500_6_60_SEQ)	0.01 (HS_500_1_20_YES)	0.07 (500_3_20_NO)	0.01 (1000_3_20_NO)
Higher alcohols				
Isoamyl alcohol	1.16 (HS_1000_3_40_NO)	0.01 (HS_500_3_40_YES)	0.01 (500_3_40_NO)	0.0003 (1000_6_60_NO)
Amyl alcohol	0.83 (HS_500_6_20_SEQ)	0.01 (HS_500_3_40_YES)	0.06 (1000_6_20_YES)	0.001 (1000_1_40_NO)
2-Ethyl-1-hexanol	0.81 (DI_1000_3_40_SEQ)	0.01 (HS_1000_3_60_NO)	0.19 (500_1_20_NO)	0.004 (500_1_20_NO)
C6 compounds				
(Z)-3-Hexen-1-ol	1.60 (HS_500_6_20_SEQ)	0.02 (HS_500_3_40_YES)	0.02 (1000_6_60_YES)	0.005 (500_1_20_NO)
Hexanal	33.58 (HS_500_6_20_SEQ)	0.03 (HS_1000_1_40_SEQ)	0.17 (1000_3_20_NO)	0.02 (500_3_20_NO)
1-Hexanol	26.03 (HS_500_6_20_SEQ)	0.32 (HS_500_3_40_YES)	0.45 (500_3_40_NO)	0.06 (1000_1_20_NO)
2-Hexenal	7.85 (HS_500_6_20_SEQ)	0.01 (HS_1000_1_40_SEQ)	0.05 (500_6_20_NO)	0.01 (500_3_40_NO)
2-Hexen-1-ol	6.23 (HS_500_6_20_SEQ)	0.01 (DI_1000_3_40_YES)	0.08 (1000_3_40_YES)	0.01 (500_1_20_NO)
Other compounds				
Decanal	0.86 (HS+DI_500_3_60_NO)	0.004 (HS_500_3_40_YES)	0.10 (500_1_20_NO)	0.002 (1000_1_40_YES)
Furanmethanol			0.09 (500_6_20_NO)	0.0003 (500_1_40_YES)
Acetol			0.09 (500_6_20_NO)	0.003 (1000_6_40_YES)
Methyl jasmonate			14.06 (1000_6_20_YES)	0.08 (1000_6_60_NO)

The conditions that gave these values are shown in parentheses. HS: head-space. DI: direct-immersion. HS+DI: one Twister for HS and one for DI. Stirring speed: 500 or 1000 rpm. Time: 1, 3, 6 h. T^a: 20, 40, 60 °C. YES: With NaCl. NO: without NaCl. SEQ: NaCl was added halfway through the reaction time.

Table 3

Variables (compounds) of each principal component (PC) for each method (SBSE and ^{m}SBSE).

PC	SBSE	^{m}SBSE
1	(Z)-3-Hexen-1-ol (0.97)	Ethyl hexanoate (0.96)
	Isoamyl acetate (0.95)	Ethyl octanoate (0.95)
	Amyl alcohol (0.94)	Isoamyl acetate (0.93)
	Hexanal (0.93)	β-Ionone (0.81)
	2-Hexenal (0.92)	Ethyl decanoate (0.77)
	2-Hexen-1-ol (0.91)	α-Terpineol (−0.76)
	1-Hexanol (0.88)	Benzaldehyde (0.72)
	Ethyl hexanoate (0.86)	2-Hexenal (0.70)
	Linalool (0.70)	
2	α-Terpineol (0.93)	1-Hexanol (0.87)
	Octanoic acid (0.90)	2-Hexen 1-ol (0.86)
	2-Phenylethanol (0.79)	Isoamyl alcohol (0.67)
	Geraniol (0.74)	(Z)-3-Hexen-1-ol (0.64)
	2-Ethyl-1-hexanol (0.73)	Hexanal (−0.55)
3	Ethyl decanoate (0.94)	Octanoic acid (0.90)
	β-Ionone (0.93)	2-Phenylethanol (0.67)
	Ethyl octanoate (0.75)	(E)-β-Damascenone (0.61)
4	Decanal (0.84)	Decanal (0.73)
	Benzaldehyde (0.78)	Hexanoic acid (0.56)
	(E)-β-Damascenone (0.63)	
5	Hexanoic acid (0.83)	Furanmethanol (0.80)
	Isoamyl alcohol (0.62)	Linalool (0.68)
		Acetol (0.66)
6	-	Amyl alcohol (0.77)
7	-	Methyl jasmonate (0.82)
		2-Ethyl-1-hexanol (0.61)

The percentage weight of each variable within each component is shown in parentheses.

20 mL extraction vial. If NaCl had to be added, 2.5 g was added. Compounds were extracted by introducing the PDMS coated stir bar into a insert for headspace, and this insert was put into the 20 mL vial. A borosilicate magnetic stirrer was added.

Direct immersion SBSE (DI-SBSE). An aliquot of 0.8 mL of centrifuged must sample (15 min, 4500 rpm), 0.1 mL of the standards solution, 25 μL of the 2-octanol solution (5 μL 2-octanol/100 mL EtOH), and 8.1 mL of Milli-Q water were added in a 20 mL vial. If NaCl had to be added, 2.5 g was added. Compounds were extracted by introducing the PDMS coated stir bar into 20 mL vial. Twister works as a stirrer.

All samples were stirred at the working speed (500, or 1000 rpm), at the specified time (1, 3, or 6 h) and at the definite temperature (20, 40, or 60 °C). At the end of the extraction time, the stir bar was removed from the sample, rinsed with distilled water and dried with a tissue paper, and later transferred into a thermal desorption tube for GC-MS analysis. The TDU tube was sealed with a transport adapter and placed in a 40 position Twister rack on the MPS robotic for automated analysis.

2.6.2. Multi-stir bar sorptive extraction (^{m}SBSE)

In this case, DI was always used, since the PDMS Twister is always submerged. A 0.8 mL aliquot of centrifuged sample (15 min, 4500 rpm), 0.1 mL of the standards solution, 25 μL of the 2-octanol solution (5 μL 2-octanol/100 mL EtOH), and 8.1 mL of Milli-Q water were added in a 20 mL vial. If NaCl had to be added, 2.5 g was added. Compounds were extracted by introducing the PDMS coated stir bar (0.5 mm film thickness, 10 mm length) into the 20 mL vial, and the EG coated stir bar (10 mm length, 32 μL phase volume) into de insert for headspace 20 mL vial. The Twister works as a stirrer.

All samples were stirred at the working speed (500, or 1000 rpm), at the specified time (1, 3, or 6 h) and at the specified temperature (20, 40, or 60 °C). At the end of the extraction time, the stirs bars were then removed from the sample, rinsed with distilled water and dried with a cellulose tissue, and later transferred into a thermal desorption tube for GC-MS analysis. The TDU tube was sealed with a transport adapter and placed in a 40 position Twister rack on the MPS robotic for automated

analysis.

2.7. Desorption conditions

The volatile analysis was performed using an automated TDU. The method used for the determination of must volatile composition is based on that described by Sánchez-Gómez, Zalacain, Alonso, and Salinas (2014) with some modifications.

SBSE. Stir bars, coating with PDMS, were thermally desorbed using helium as carrier gas at a flow rate of 75 mL/min; TDU was programmed from 40 °C to 295 °C (5 min) at a rate of 60 °C/min for following the supplier's recommendations. The analytes were focused on the CIS-4, containing a packed liner (20 mg of Tenax), at −40 °C with liquid N$_2$ cooling prior to injection. After desorption and focusing, the CIS-4 temperature was programmed to reach 260 °C (5 min) at 12 °C/s to transfer volatiles onto the analytical column. The TDU operated in the splitless desorption mode, the CIS-4 operated in PTV solvent vent mode (purge flow to split vent of 80 mL/min, vent 75 mL/min and pressure 20.85 psi).

^{m}*SBSE.* Stir bars were thermally desorbed using helium as carrier gas at a flow rate of 75 mL/min; the desorption was carried out with the following TDU temperature program: beginning at 40 °C, increased to 200 °C at 60 °C/min and held at 200 °C for 5 min. Desorbed compounds were then transferred to the CIS-4 system, and cryo-focused at −40 °C on a Tenax TA-packed liner (20 mg of Tenax). After desorption and focusing, the CIS-4 inlet was programmed to increase to 230 °C at 12 °C/s from the initial temperature of −40 °C and held at 230 °C for 5 min to transfer volatiles onto the analytical column. The TDU operated in the splitless desorption mode, the CIS-4 operated in PTV solvent vent mode (purge flow to split vent of 80 mL/min, vent 75 mL/min and pressure 20.85 psi).

2.8. Chromatographic conditions

The desorbed volatile compounds were separated in an Agilent 7890A gas chromatograph system (GC) coupled to a triple quadrupole (QqQ) Agilent 7000C electron ionization mass spectrometric detector (Agilent Technologies), operating in simple quadrupole (Q).

The GC oven temperature was programmed at 40 °C (2 min), raised to 80 °C (5 °C/min, held for 2 min), then to 130 °C (10 °C/min, held 5 min), then to 150 °C (5 °C/min, held for 5 min) and finally to 230 °C (10 °C/min, held 5 min). The MS operated in scan mode (35–300 amu) with ionization energy set at 70 eV. The temperature of the MS transfer line was 230 °C. Each molecule was identified by comparing mass spectrum with those of the NIST library and chromatographic retention index of each standard. Compounds for which no standard was added were identified by comparing their mass spectra with NIST. To avoid matrix interferences, the GC peak area of each compound was obtained from the ion extraction chromatogram (EIC) by selecting target ions for each one. The target ions were m/z 41 for 2-hexenal, m/z 43 for isoamyl acetate, decanal, and acetol, m/z 45 for 2-octanol (I.S.), m/z 55 for isoamyl alcohol, m/z 56 for 1-hexanol, and hexanal, m/z 57 for amyl alcohol, 2-ethyl-1-hexanol, and (E)-2-hexen-1-ol, m/z 59 for α-terpineol, m/z 60 for hexanoic acid, and octanoic acid, m/z 67 for (Z)-3-hexen-1-ol, m/z 69 for β-damascenone, and geraniol, m/z 71 for linalool, m/z 77 for benzaldehyde, m/z 83 for methyl jasmonate, m/z 88 for ethyl hexanoate, ethyl octanoate, and ethyl decanoate, m/z 91 for 2-phenylethanol, m/z 98 for furanmethanol, and m/z 177 for β-ionone. Finally, a semi-quantification was carried out by comparing the area of each compound with that of 2-octanol (I.S.), thus obtaining the relative area.

2.9. Statistical analysis

In accordance with the objective of maximizing the overall extraction of volatiles, a statistical analysis has been proposed in which the values of relative area of all the volatile compounds obtained in each of
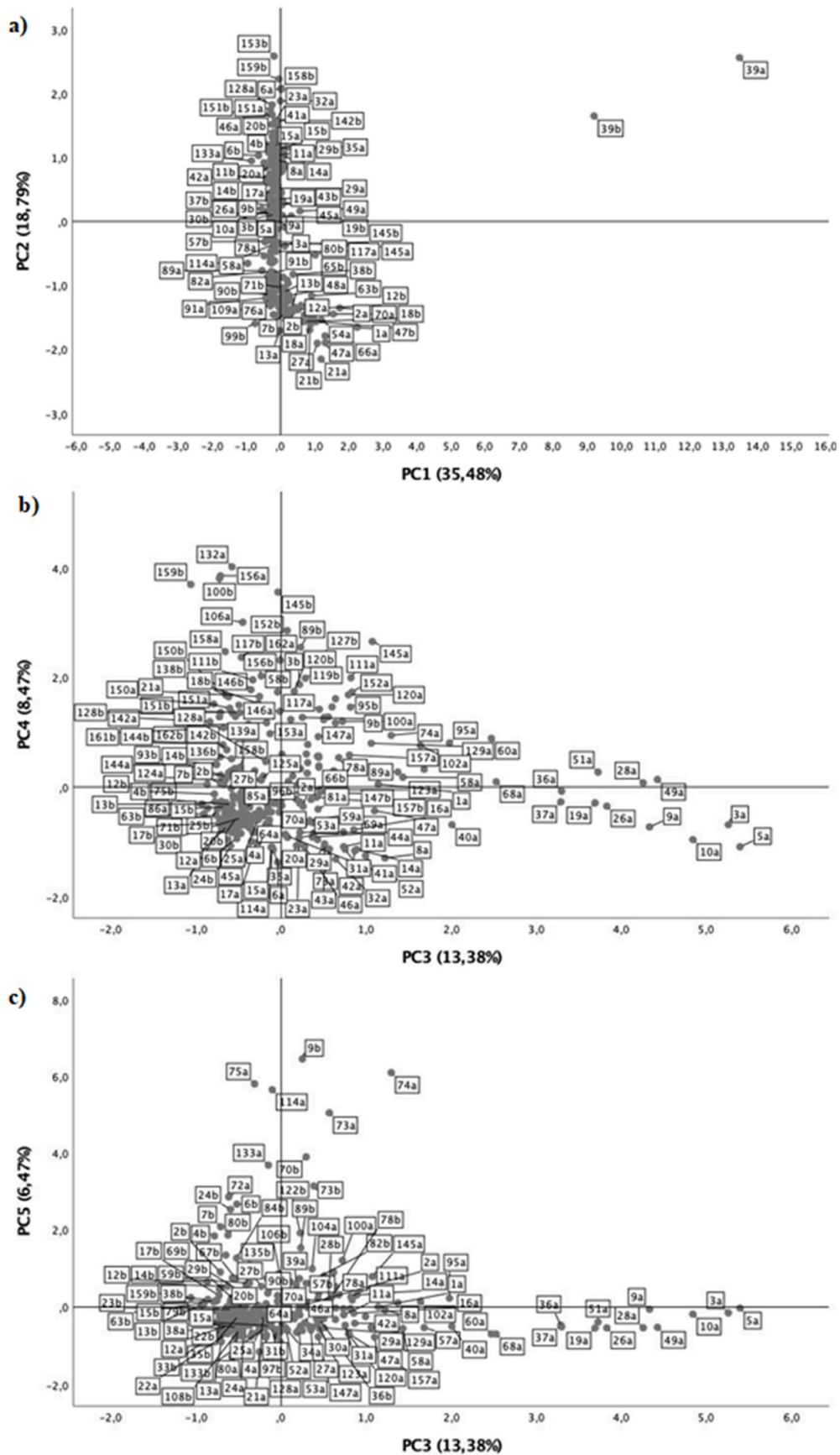
Fig. 1. Principal component analysis plots for SBSE method. a) PC1 *vs* PC2, b) PC3 *vs* PC4, and c) PC5 *vs* PC6. Each number shown in the graphic corresponds to a combination of conditions. The letters "a" and "b" after each number correspond to the two replicates of each combination of conditions.
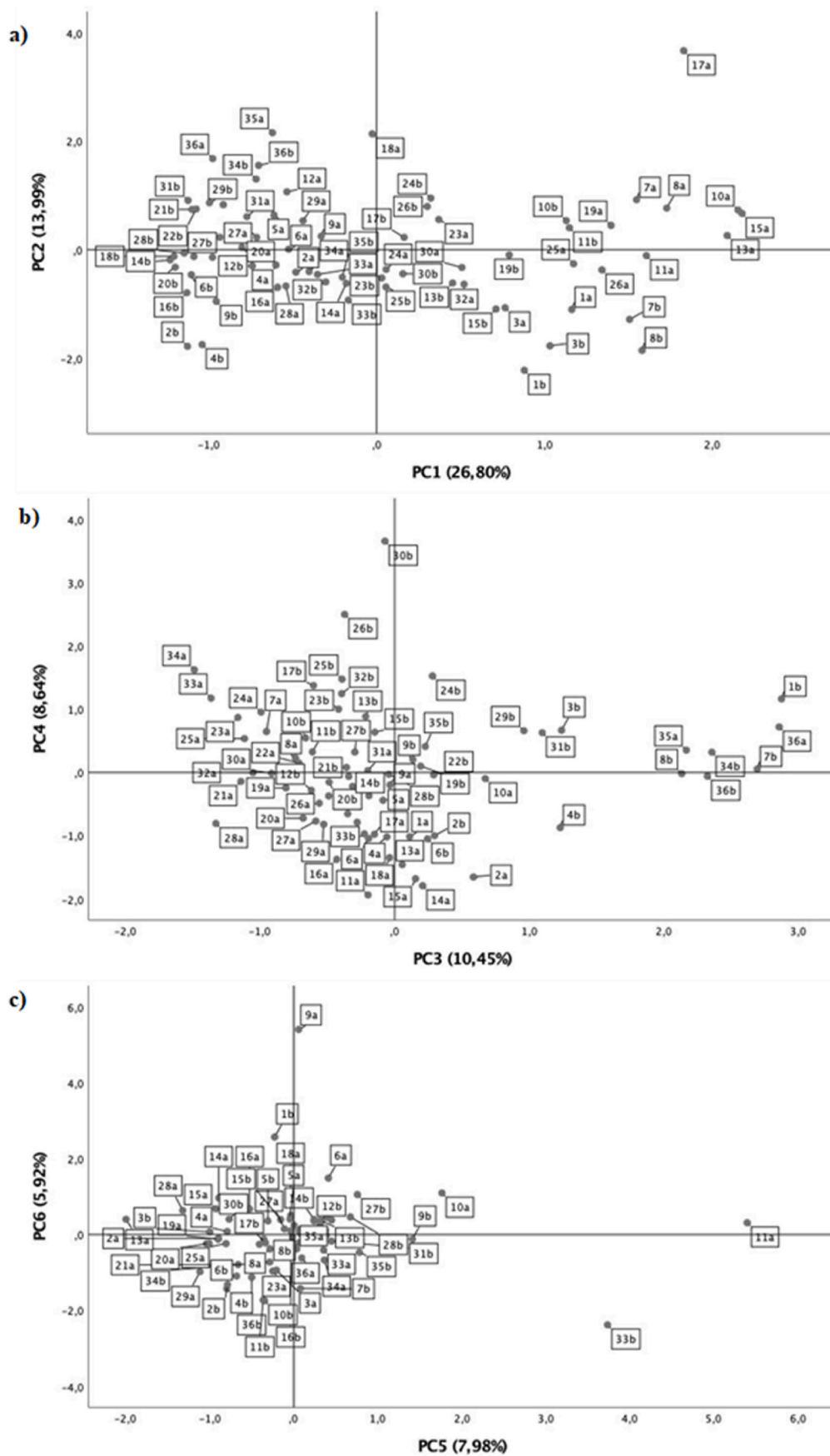
Fig. 2. Principal component analysis plots for ^{m}SBSE method. a) PC1 *vs* PC2, b) PC3 *vs* PC4, and c) PC5 *vs* PC6. Each number shown in the graphic corresponds to a combination of conditions. The letters "a" and "b" after each number correspond to the two replicates of each combination of conditions.

Table 4

Tests of inter-subject effects obtained from multifactorial analysis of variance (ANOVA) for ^{m}SBSE.

	PC	1		2		3		4		5	
	Factors	F	p	F	p	F	p	F	p	F	p
Simples	RPM	0.120	0.731	0.002	0.962	0.003	0.958	0.145	0.705	0.004	0.952
	Time	1.872	0.168	8.351	**0.001***	0.056	0.946	2.024	0.147	5.785	**0.007***
	T^{a}	8.418	**0.001***	4.01	**0.027***	2.586	0.089	4.566	**0.017***	1.031	0.367
	NaCl	181.815	**0.000***	1.231	0.275	0.148	0.702	8.913	**0.005***	0.921	0.344
Second-order interactions	RPM*time	0.075	0.928	0.537	0.589	0.014	0.986	0.276	0.761	0.094	0.911
	RPM*T^{a}	0.029	0.971	0.095	0.910	0.015	0.985	0.714	0.497	0.497	0.613
	RPM*NaCl	0.001	0.976	1.858	0.181	2.172	0.149	0.03	0.863	0.001	0.980
	Time*T^{a}	2.448	0.064	1.043	0.398	3.405	**0.018***	0.871	0.491	1.025	0.408
	Time*NaCl	2.485	0.098	0.244	0.784	0.735	0.487	1.372	0.267	1.474	0.242
	T^{a}*NaCl	7.331	**0.002***	1.507	0.235	2.442	0.101	0.026	0.975	0.608	0.550

*Indicates significant effect of simple factor or second-order interaction ($p \leq 0.05$).

the described experimental conditions are studied simultaneously. Before approaching this analysis, an exhaustive descriptive analysis was carried out which allowed the detection and elimination of outliers, when necessary. In this analysis, the significant correlation between volatile compounds has also been verified, which justifies the need to analyze all the values of the volatiles using a multivariate treatment.

For the collective study of volatile compounds, a principal component analysis (PCA) was performed to reduce the dimensionality of the problem by synthesizing the initial data vectors into a reduced number of principal components (PCs) (James, Witten, Hastie, & Tibshirani, 2013). In order to facilitate the interpretation of the PC obtained, a varimax rotation was applied. For this purpose, and in coherence with the experimental design described above, a multifactorial analysis of variance (ANOVA) was used, taking the scores of each PC as the response variable.

Finally, another ANOVA was performed to compare the best conditions of the two methods (SBSE, and ^{m}SBSE). In this case, the volatile compounds were not treated as a set, but the relative area values of each were compared individually.

3. Results and discussion

3.1. Must volatile composition

The maximum and minimum values of relative area obtained for each volatile compound with each of the methods can be seen in Table 2. Due to the large amount of data obtained, only the conditions that provided the maximum and minimum value for each compound are shown. The compounds listed in Table 2 are all those that could be identified in the chromatogram, regardless of whether standard was added or not.

A total of 22 compounds were identified for SBSE, and 25 for ^{m}SBSE. The terpenoids identified were: linalool, α-terpineol, and geraniol. The C_{13} norisoprenoids identified were β-damascenone, and β-ionone. The benzenoid compounds identified were: benzaldehyde, and 2-phenylethanol. Terpenoids, C_{13} norisoprenoids, and some benzenoid compounds are the most important grape aroma compounds present in the pulp and skin of the berries in both free and glycoside forms (Gutiérrez-Gamboa, Pérez-Álvarez, Rubio-Bretón, & Garde-Cerdán, 2019). Terpenes are the constituents responsible for the characteristic floral and fragrant Muscat aroma in grapes and wines (Marín-San Román, Garde-Cerdán, et al., 2020).

In addition, 4 esters were identified: isoamyl acetate, ethyl hexanoate, ethyl octanoate, and ethyl decanoate, 2 fatty acids: hexanoic acid, and octanoic acid, and 3 higher alcohols: isoamyl alcohol, amyl alcohol, and 2-ethyl-1-hexanol. The ethyl esters contribute to the pleasant aroma (Rubio-Bretón et al., 2019).

Five C6 compounds were also identified: (Z)-3-hexen-1-ol, hexanal, 1-hexanol, 2-hexenal, and 2-hexen-1-ol. C6 compounds, which are referred to as "green leaf volatiles," are characterized by a "herbaceous"

and "green" aroma (Rubio-Bretón et al., 2019).

Finally, other compounds were identified, in the case of SBSE, decanal, and in the case of ^{m}SBSE, decanal, furanmethanol, acetol, and methyl jasmonate. These compounds are not of special relevance to grape aroma.

SBSE. It can be seen in Table 2 that many of the compounds maximized their extraction with the same conditions (HS_500 rpm_6h_20 °C_Secuencial). Subsequently, the necessary statistics were performed to confirm these results.

^{m}*SBSE.* In view of the results presented in Table 2, it was not possible to choose conditions that would maximize the extraction of most of the compounds, in contrast to what occurred in SBSE. Therefore, in order to determine these conditions, it was necessary to use a multivariate statistical analysis.

Table 2 shows that the maximum values for the SBSE method were much higher than those for the ^{m}SBSE method. This result was contrary to expectations, since the objective of using the ^{m}SBSE was to improve the extraction of the more polar compounds, without affecting the extraction of the less polar ones (Serrano de la Hoz et al., 2016; Tang et al., 2020).

3.2. Principal component Analysis (PCA)

A PCA was performed to reduce the total number of variables (compounds) to a reduced number of principal components (PCs). The number of variables used was 22 (SBSE), and 25 (^{m}SBSE) (Table 2). In all cases it was found that the Kaiser-Meyer-Olkin (KMO) test of sampling adequacy obtained sufficiently large values (between 0 and 1): in all cases values above 0.8 were obtained (Hair, Anderson, Black, & Babin, 2010). Barlett's test of sphericity was also significant in all cases. Both tests indicate that the underlying PC extraction method is adequate. The values of the communalities of each of the variables were high in all cases. This analysis has allowed us to identify groups of volatile compounds with correlated performance, which has simplified the study of the relationship between the values obtained in the aromatic profile with the experimental factors.

Table 3 shows the number of resulting PCs for each method, as well as the variables that form each PC, and the weights of each one of them.

SBSE. Five PCs were retained, with an overall retained variance of 82.96%. The compounds with highest weight in each PC were; PC1: (Z)-3-Hexen-1-ol, isoamyl acetate, amyl alcohol, hexanal, 2-hexenal, 2-hexen-1-ol, 1-hexanol, ethyl hexanoate, and linalool, which are divided into: 5 C6 compounds, 2 esters, 1 higher alcohol, and 1 terpenoid. PC2: α-terpineol, octanoic acid, 2-phenylethanol, geraniol, and 2-ethyl-1-hexanol, which are divided into: 2 terpenoids, 1 fatty acid, 1 benzenoid, and 1 higher alcohol. PC3: ethyl decanoate, β-ionone, y ethyl octanoate, which are divided into: 2 esters, and 1 C_{13} norisoprenoid. PC4: decanal, benzaldehyde, and β-damascenone, which are divided into: 1 aldehyde, 1 benzenoid, and 1 C_{13} norisoprenoid. Finally, PC5: hexanoic acid, and isoamyl alcohol, i.e., 1 ester and 1 higher alcohol, respectively.
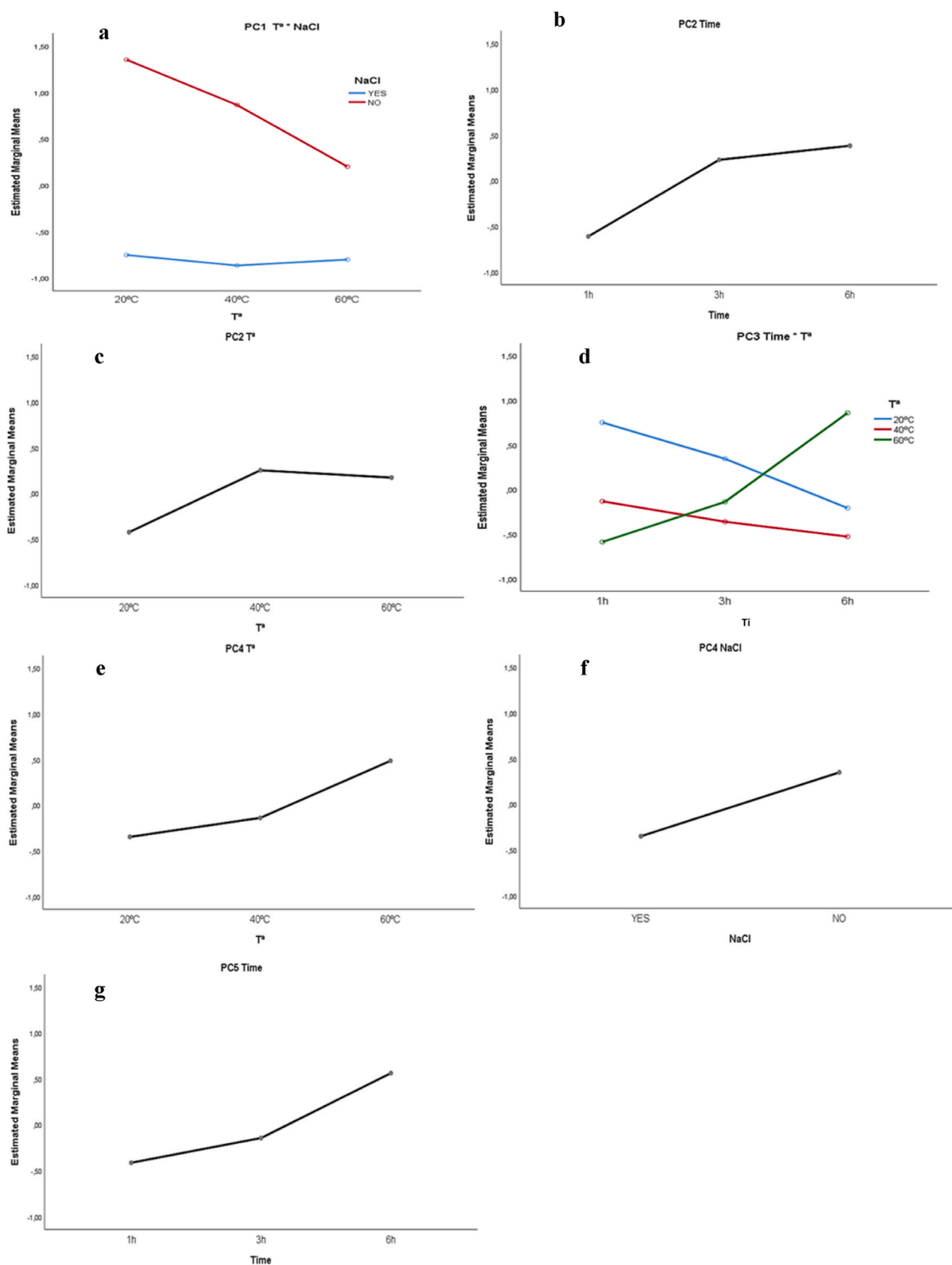
Fig. 3. Plots of estimated marginal means for [m]SBSE method. a) T[a]*NaCl interaction for PC1, b) Time factor for PC2, c) Temperature factor for PC2, d) Time*T[a] interaction for PC3, e) Temperature factor for PC4, f) NaCl factor for PC4, and g) Time factor for PC5.

Fig. 1 shows the plots of the sample scores on the PCA for the SBSE method. Fig. 1a shows the PCA for PC1 and PC2. PC1 explained a variance of 35.48%, and PC2 18.79%. In this case it was clearly observed that samples 39a and 39b (replicates), maximized the response of the volatile compounds belonging to PC1 and PC2. The conditions corresponding to these samples are: head space, 500 rpm, 6 h, 20 °C, and NaCl sequential addition (HS_500_6_20_SEQ). This confirmed what was observed in Table 2.

Since a randomized factorial design was followed, these samples, despite being two replicates of the same conditions, were performed on different days, which confirmed that the HS_500_6_20_SEQ conditions were the best for extracting the volatile compounds belonging to PC1 and PC2. Fig. 1b shows the PCA plots for PC3, PC4 and PC4. PC3 explained a variance of 13.38%, and PC4 explained a variance of 8.47%. Fig. 1c shows the plots of the PCA for PC3 and PC5. PC5 explained a variance of 6.47%. For the compounds belonging to these PCs, no pair of

Table 5
Best conditions within each principal component chosen from inter-subject tests and plots of estimated marginal means, and global conditions for ᵐSBSE.

PC	Compounds	Best conditions	Optimal conditions
1	Ethyl hexanoate Ethyl octanoate Isoamyl acetate β-Ionone Ethyl decanoate α-Terpineol Benzaldehyde 2-Hexenal	20°C-No NaCl > >40°C-No NaCl > >**60°C-No NaCl**	
2	1-Hexanol 2-Hexen 1-ol Isoamyl alcohol (Z)-3-Hexen-1-ol Hexanal	**6h** 40°C ≈ **60°C**	
3	Octanoic acid 2-Phenylethanol (E)-β- Damascenone	**6h-60°C**	500_6_60_NO
4	Decanal Hexanoic acid	**60°C** **No NaCl**	
5	Furanmethanol Linalool Acetol	**6h**	
6	Amyl alcohol	[a]	
7	Methyl jasmonate 2-Ethyl-1-hexanol	[a]	

Stirring speed: 500 or 1000 rpm. Time: 1, 3, 6 h. Tᵃ: 20, 40, 60 °C. YES: With NaCl. NO: without NaCl. [a]Indicates that no significant factors or interactions exist for these principal components.

Table 6
Values of relative area with respect 2-octanol (I.S) obtained with the global conditions of the two methods (SBSE and ᵐSBSE) for each of the compounds.

	SBSE	ᵐSBSE	p
Terpenoids			
Linalool	0.04 ± 0.01	0.03 ± 0.01	0.72
α-Terpineol	**0.23 ± 0.03**	0.11 ± 0.04	**0.01***
Geraniol	**0.69 ± 0.01**	0.13 ± 0.00	**0.00***
C₁₃ norisoprenoids			
β-Damascenone	**0.17 ± 0.02**	0.11 ± 0.01	**0.01***
β-Ionone	**0.03 ± 0.00**	0.00 ± 0.00	**0.00***
Benzenoid compounds			
Benzaldehyde	**0.72 ± 0.05**	0.04 ± 0.01	**0.00***
2-Phenylethanol	0.05 ± 0.03	0.01 ± 0.00	0.08
Esters			
Isoamyl acetate	**2.32 ± 0.24**	0.07 ± 0.01	**0.00***
Ethyl hexanoate	**0.32 ± 0.01**	0.09 ± 0.01	**0.00***
Ethyl octanoate	0.07 ± 0.01	**0.12 ± 0.00**	**0.00***
Ethyl decanoate	**0.05 ± 0.01**	0.02 ± 0.00	**0.01***
Fatty acids			
Octanoic acid	**0.09 ± 0.01**	0.01 ± 0.00	**0.00***
Hexanoic acid	**0.15 ± 0.04**	0.07 ± 0.01	**0.03***
Higher alcohols			
Isoamyl alcohol	**1.79 ± 0.16**	0.23 ± 0.17	**0.00***
Amyl alcohol	**0.28 ± 0.05**	0.02 ± 0.00	**0.00***
2-Ethyl-1-hexanol	**0.15 ± 0.01**	0.07 ± 0.01	**0.00***
C6 compounds			
(Z)-3-Hexen-1-ol	0.06 ± 0.00	0.06 ± 0.01	0.37
Hexanal	**1.71 ± 0.56**	0.08 ± 0.00	**0.01***
1-Hexanol	**0.07 ± 0.01**	0.00 ± 0.00	**0.00***
2-Hexenal	**0.10 ± 0.04**	0.03 ± 0.01	**0.04***
2-Hexen-1-ol	**0.04 ± 0.01**	0.02 ± 0.00	**0.02***
Other compounds			
Decanal	0.04 ± 0.02	0.04 ± 0.00	0.69
Furanmethanol	0.22 ± 0.00	**0.69 ± 0.08**	**0.00***
Acetol	0.27 ± 0.14	0.05 ± 0.02	0.07

Data are shown as mean ± standard deviation (n = 3). *Indicate significant differences between methods (p ≤ 0.05). Bold indicates the highest value of the compound showing significant differences.

conditions was observed that clearly differed from the rest. For this reason, and since in Fig. 1a the differences were so clear, the conditions chosen for the SBSE method were HS_500_6_20_SEQ, so it was not necessary to perform ANOVA.

ᵐSBSE. A total of 7 PC were retained, with an overall retained variance of 73.78%. The compounds with the highest weight in PC1 were: ethyl hexanoate, ethyl octanoate, isoamyl acetate, β-ionone, ethyl decanoate, α-terpineol, and benzaldehyde, which were divided into: 4 esters, 1 C₁₃ norisoprenoid, 1 terpenoid, 1 benzenoid, and 1 C6 compound. PC2: 1-hexanol, 2-hexen 1-ol, isoamyl alcohol, (Z)-3-hexen-1-ol, hexanal, and 2-hexenal, which were divided into: 4 C6 compounds, and 1 higher alcohol. PC3: the fatty acid, octanoic acid, the benzenoid, 2-phenylethanol, and the C₁₃ norisoprenoid, β-damascenone. PC4: the other compound, decanal, and the fatty acid, hexanoic acid. PC5: furanmethanol, linalool, and acetol, which were divided into 2 other compounds, and 1 terpenoid. PC6: the higher alcohol, amyl alcohol. PC7: the other compounds, methyl jasmonate, and the higher alcohol, 2-ethyl-1-hexanol.

Fig. 2 shows the PCA corresponding to the ᵐSBSE method. Fig. 2a shows the PCA of PC1 and PC2. PC1 explained a variance of 26.80%, and PC2 explained a variance of 13.99%. Fig. 2b shows the PCA of PC3 and PC4. PC3 explained a variance of 10.45%, and PC4 explained a variance of 8.64%. Fig. 2c shows the PCA of PC5 and PC6. PC5 explained a variance of 7.98%, and PC6 explained a variance of 5.92%. In this case, no graph showed the existence of a pair of conditions that clearly differed from the rest, for which ANOVA was used.

3.3. Selection of optimal conditions for ᵐSBSE

The selection of the conditions that optimized the extraction of volatile compounds in ᵐSBSE was performed with an ANOVA. It was verified that all the parametric hypotheses necessary for the use of this technique were satisfied. Table 4 shows the results of the test of inter-subject effects, showing the F value as well as the p for each PC.

Table 4 shows that, for PC1, the factors Tᵃ and NaCl were significant, as well as the interaction Tᵃ*NaCl. For PC2, the factors time and Tᵃ had a significant effect. In the case of PC3, the interaction time*Tᵃ was significant. In PC4, the factors Tᵃ and NaCl were significant. Finally, for PC5, the time factor had a significant effect. In the case of PC6 and PC7, neither factor or interaction was significant.

Once the factors or interactions that have a significant effect for each PC are known, we look at the plots of marginal means of these factors (Fig. 3).

Fig. 3 shows the plots of marginal means of the factors and interactions that have a significant effect, obtained in the ANOVA of the ᵐSBSE method. Fig. 3a shows the graph of the Tᵃ*NaCl interaction for PC1. It was observed that the best results were obtained at 20 °C and without adding NaCl, followed by 40 °C without NaCl, and followed by 60 °C without NaCl. Fig. 3b and c shows the graphs of the individual factors time and Tᵃ for PC2. In this case, the best results were 6h, and 40 °C or 60 °C. Fig. 3d shows the graph of the time*Tᵃ interaction for PC3. The combination of conditions that provided a higher extraction of the volatile compounds belonging to this group were 6h and 60 °C. Fig. 3e and f represent the Tᵃ and NaCl factors respectively for PC4. The Tᵃ value that provided the highest extraction for PC4 compounds was 60 °C. Besides, the highest values for the compounds belonging to the PC4 group were obtained when NaCl was not added. Finally, Fig. 3g shows the time factor graph for PC5. In this case the value of this factor that optimized the extraction of the compounds forming PC5 was 6h.

Table 5 shows the best conditions within each PC, as well as the optimal conditions finally chosen.

Once the best conditions were chosen for each PC, a compromise situation was reached, in order to find a global maximization. In this

case it was simple since in most of the PCs the best conditions were 6h, 60 °C, and without NaCl. Therefore, in the case of PC1, the third option was chosen instead of the first one. However, in Fig. 3a it can be seen how despite being the third option, this combination of conditions still gives a great response compared to the samples that carried NaCl, regardless of the T^a. Thus, this choice did not greatly compromise the extraction of compounds belonging to this PC, which remained high. The optimum conditions chosen for this method were: 500 rpm, 6h, 60 °C, without NaCl (500_6_60_NO) (Table 5). Since rpm was not a significant factor in any PC, 500 rpm was chosen as it is less aggressive to the magnets.

3.4. Comparison of SBSE and ^{m}SBSE optimal conditions

Finally, a comparison was made between the optimal conditions chosen for SBSE and the optimal conditions chosen for ^{m}SBSE. For this, 3 replicates were prepared with the SBSE method, by HS, at 500 rpm, for 6h, at 20 °C, adding NaCl at half time, and 3 replicates with the ^{m}SBSE method, adding the PDMS Twister by DI, the EG Twister by HS, at 500 rpm, for 6h, at 60 °C, without NaCl.

The results of this comparison are shown in Table 6, which shows the means of the relative area results obtained for each compound, as well as their standard deviation (n = 3). The optimum conditions chosen for SBSE provide better extractions of a-terpineol, geraniol, β-damascenone, β-ionone, benzaldehyde, isoamyl acetate, ethyl hexanoate, ethyl decanoate, octanoic acid, hexanoic acid, isoamyl alcohol, Amyl alcohol, 2-ethyl-1-hexanol, hexanal, 1-hexanol, 2-hexenal, and 2-hexen-1-ol. On the other hand, mSBSE better extracts ethyl octanoate, and furanmethanol. Therefore, it can be concluded that the SBSE provides a higher extraction of volatile compounds than ^{m}SBSE, under the conditions: head space (HS), 500 rpm, 6 h, 20 °C, and NaCl sequential addition (HS_500_6_20_SEQ).

4. Conclusions

For the first time for these techniques, so many factors with so many different conditions are optimized. It can be concluded that both methods (SBSE and ^{m}SBSE) generally provide good extraction of volatile compounds in must. The combination of principal component analysis (PCA) with multifactorial analysis of variance (ANOVA) was used to choose the best conditions for each method, being, for SBSE: Head-space (HS), stirring at 500 rpm for 6 h at 20 °C, and adding NaCl at 3 h of extraction (HS_500_6_20_SEQ). For ^{m}SBSE: stirring at 500 rpm for 6 h at 60 °C, without adding NaCl (500_6_60_NO). Finally, the comparison between the optimal conditions of each method confirmed that the best method for the extraction of volatile compounds in must is the classical SBSE, using only the PDMS Twister.

CRediT authorship contribution statement

Sandra Marín-San Román: Investigation, Methodology, Formal analysis, Writing – original draft. **José Miguel Carot-Sierra:** Formal analysis, Writing – review & editing. **Itziar Sáenz de Urturi:** Investigation, Writing – review & editing. **Pilar Rubio-Bretón:** Investigation, Writing – review & editing. **Eva P. Pérez-Álvarez:** Investigation, Writing – review & editing. **Teresa Garde-Cerdán:** Supervision, Funding acquisition, Investigation, Methodology, Writing – review & editing.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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