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SPME Method Optimized by Box-Behnken Design for Impact Odorants in Reduced Alcohol Wines

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Abstract: The important sampling parameters of a headspace solid phase microextraction-gas chromatography-mass spectrometry (HS-SPME-GC-MS) procedure, extraction temperature, extraction time and sample volume were optimized to quantify 23 important impact odorants in reduced alcohol red and white wines. A three-factor design of Box-Behnken experiments was used to determine optimized sampling conditions for each analyte, and a global optimized condition at every ethanol concentration of interest determined using a desirability function that accounts for a low signal response for compounds. Shiraz and Chardonnay wines were dealcoholized from 13.7 and 12.2% v/v ethanol respectively, to 8 and 5% v/v, using a commercially available membrane-based technology. A sample set of the reduced alcohol wines were also reconstituted to their natural ethanol level to evaluate the effect of ethanol content reduction on volatile composition. The three-factor Box-Behnken experiment ensured an accurate determination of the headspace concentration of each compound at each ethanol concentration, allowing comparisons between wines at varying ethanol levels to be made. Overall, the results showed that the main effect of extraction temperature was considered the most critical factor when studying the equilibrium of reduced alcohol wine impact odorants. The impact of ethanol reduction upon the concentration of volatile compounds clearly resulted in losses of impact odorants from the wines. The concentration of most analytes decreased with dealcoholization compared to that of the natural samples. Significant differences were also found between the reconstituted volatile composition and 5% v/v reduced alcohol wines, revealing that the dealcoholization effect is the result of a combination between the type of dealcoholization treatment and reduction in wine ethanol content.

Keywords: reduced-alcohol wine; solid-phase microextraction; gas chromatography; chemometrics

1. Introduction

Increasing medical and social behavior issues associated with immoderate alcohol consumption have led the World Health Organization (WHO) to introduce in 2010 a global strategy to reduce the alcohol intake in the population [1]. This has directed many governments to enforce pricing and taxation policies on alcohol production, with the wine sector being financially impacted [2]. Alcohol is also the main source of caloric content in wine and nutritional mandatory labelling is occurring in many countries such as South Africa, France and Germany [3]. Although warning health labelling in some countries such as the United States is currently voluntary, for large chain restaurant nutritional

labelling is likely to be mandatory for all foods and beverages served. This development is likely to impact the export market with analytical information as well as a reason to reduce the alcohol content in wine to be competitive with other products. All these considerations have stimulated a greater interest among researchers in order to implement new technologies for reducing the alcohol content in wine [4-6]. Several techniques have been experimented to reduce wine alcohol content, but dealcoholization (i.e. removal of alcohol from wine) using membrane-based devices remains the most employed technique at a commercial scale. From the literature, dealcoholization is also one of the most studied techniques in the field, in part because ethanol removal causes significant losses of important impact odorants, which researchers are committed to minimizing [7].

Impact odorants such as ethyl esters, acetates of higher alcohols, and terpenes, are of primary importance for understanding consumer acceptability of wines, due to their contribution to desirable aroma attributes at very small concentrations (10^{-4}-10^{-12} g/L) [8,9]. Several extraction techniques such as distillation, solvent extraction and solid-phase have been used prior to the analysis of these volatile compounds; however, headspace solid-phase extraction (HS-SPME) is currently the most used for dealcoholized wine trials [7]. Sampling headspace vapors by SPME essentially includes two operating steps: (i) partitioning of analytes between the fiber coating and the headspace gas phase, and (ii) desorption of the concentrated analytes into an analytical instrument such as a gas chromatograph-mass spectrometer (GC-MS). Compared to conventional extraction techniques, SPME is relatively simple, fast and cost-efficient. Moreover, SPME requires no-solvent and a limited manipulation of the sample [10,11].

Despite the advantages of SPME, comparisons of HS-SPME-GC-MS results between two or more treatments (e.g., natural and dealcoholized wines) may be confounded by varying experimental conditions, such as fiber exposure time, sample temperature and volume, type and uniformity of the sample matrix including ethanol content [12-14]. In particular, ethanol, the main component of wine alcohol content, has a great impact on the partitioning coefficient of other volatile compounds, thus on their concentration in the wine headspace [15]. In addition, the competition between ethanol and other analytes for binding sites on the SPME fiber during headspace sampling will be altered with varying sample ethanol concentrations, which may confound characterization studies and may lead to misinterpretation of results [16]. Optimization of experimental conditions using multivariate statistical approaches such as full three-level factorial Box-Behnken designs, a response surface methodology, helps to overcome these problems [17]. Nevertheless, Box-Behnken designs have not been widely used for the optimization of SPME methods [18].

The aim of this study was to optimize an SPME-GC-MS method to identify and quantify important volatile compounds in red and white reduced alcohol wines. In addition to evaluating the overall effect on reduced alcohol wine's volatile composition, a sample set of the reduced alcohol wines were reconstituted to their natural ethanol level to evaluate the effect of ethanol content reduction on volatile composition. A full three-factor Box-Behnken design was used for the optimization of SPME conditions. The quantitative methodology was validated for 23 impact odorants of relevance in wines. Based on a literature survey, this appears to be the first time that a quantitative method for the analysis of the volatile fraction of red and white wines is developed and validated for dealcoholization trials.

2. Materials and Methods

2.1. Chemicals

Ethyl butyrate, ethyl-2-methyl butyrate, ethyl-3-methyl butyrate, isoamyl acetate, 3-methyl-1-butanol, ethyl hexanoate, ethyl-s-lactate, (z)-3-hexenol, methyl octanoate, ethyl octanoate, propanoic acid, linalool, methyl decanoate, ethyl decanoate, isoamyl octanoate, 3-(methylthio)-1-propanol, β-phenyl ethyl acetate, ethyl dodecanoate, geraniol, β-phenyl ethanol, octanoic acid, decanoic acid and vanillin were purchased from Fulka (Buchs, Switzerland). Analyte's identification number for Box-Behnken design, odors and olfactory thresholds, and boiling points of each compound are presented in Table 1. Ethanol (VWR Prolabo, Fontenay Sous Bois, France), L-(+) tartaric acid (Sigma, Steinheim,

Germany) and potassium hydrogen tartrate (BHD Chemicals Ltd., Poole, UK) were used for the preparation of model wine solutions. Deionized water was obtained from a Milli-Q mixed bed resin system (18 MΩ/cm, 25°C).

Table 1. Compound identification for Box-Behnken design, odors and olfactory thresholds, and boiling point.

Code	Compounds	Odors [1]	OT [1] (μg/L)	BP (°C)
1	ethyl butyrate	apple	20	121
2	ethyl-2-methyl butyrate	apple	18, 1	138
3	ethyl-3-methyl butyrate	sweat, acid, rancid	33.4	134
4	isoamyl acetate	banana	30	130
5	3-methyl-1-butanol	whiskey, malt, burnt	30000	132
6	ethyl hexanoate	apple peel, fruit	14, 2	167
7	ethyl-s-lactate	fruit, milk [2]	154000	154
8	(z)-3-hexenol	green (cut grass)	400	156
9	methyl octanoate	waxy, apple peel [2]	-	192
10	ethyl octanoate	fruit, fat	5, 2	207
11	propanoic acid	pungent, rancid, soy	8100	141
12	linalool	flower, lavander,	25.2	198
13	methyl decanoate	wax, soap, fruit [2]	-	108
14	ethyl decanoate	grape	200	245
15	isoamyl octanoate	wax, soap, pear [2]	-	267
16	3-(methylthio)-1-propanol	sweet, potato	1000	90
17	β-phenyl ethyl acetate	rose, honey	250	229
18	ethyl dodecanoate	wax, soap [2]	-	269
19	geraniol	rose, geranium	30	230
20	β-phenyl ethanol	honey, rose	14000, 10000	219
21	octanoic acid	sweat, cheese	500	240
22	decanoic acid	rancid, fat	1000	268
23	vanillin	vanilla	200	285

OT, odor threshold; BP, boiling point.

[1] From Francis and Newton [19] except where specified.

[2] From Antalick, *et al.* [20].

2.2. Instrumentation

An Agilent 7890A (Palo Alto, CA) gas chromatograph, equipped with a Gerstel multipurpose sampler with automated SPME capability and Peltier cooled sample tray, interfaced to an Agilent 5975C triple axis mass detector, was used for confirmation of compound identity, method development and final sample analysis. MSD ChemStation® E.02.00.493 (Agilent Technologies, Ltd.) and NIST MS Search 2.0, version 2008, (Agilent Technologies, Ltd.) were used to control the instrument performance and for mass spectra assessment. Samples (10 mL) were placed into the Peltier cooler tray set at 8°C until analysis, whereupon vials were transferred to a heater block with a 2-min pre-incubation time before insertion of the SPME fiber. A fused silica capillary column (DB-Waxetr, 60 m × 0.25 mm inner diameter, 0.25 μm film thickness, J&W Scientific, Folsom, CA) was used for compound separation by GC. The injector block was fitted with a 2-mm internal diameter borosilicate liner (SGE), and the injector temperature set to 260°C in splitless mode. The fiber was inserted into the injector for 1 min, withdrawn and injected into a second injector set at 270°C with a 50:1 split for 10 min with a 15 mL/min purge flow to clean the fiber, prior to the next sample analysis. The oven temperature program commenced at 40°C for 5 min and increased to 230°C at a rate of 6°C/min. The total run time was 45 min. The flow rate of ultra-high-purity helium gas was constant

at 3 mL/min. The MS source, quadrupole and transfer line temperatures were set to 230, 150, and 275°C, respectively.

2.3. Compound Identification and Elution Profiles

The solutions of 23 compounds were prepared at an approximate midpoint of the calibration range, in a model wine solution containing tartaric acid (0.008 M), potassium hydrogen tartrate (0.011 M) and ethanol (5% v/v) to confirm compound elution times and ion profiles. Samples (10 mL) were transferred to the heater block set at 50°C with an agitation rate of 250 revolutions per minute (rpm) and allowed to equilibrate for 1 min. A divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber was preconditioned at 270°C for 60 min, before the insertion into the GC injector. Mass spectral data was collected in selective ion monitoring (SIM) at an ionization voltage of 70 eV. Final elution profiles were confirmed by matching mass spectral data with the NIST mass spectral search program (version 2.0), MS library (version 8.0) and Kovat's retention indices (RIs). RIs were checked for each compound using a commercial mixture of *n*-alkanes (Sigma, Steinheim, Germany), an identical oven ramp profile and gas flow rates as used for the final analyses. This formula was used to calculate RI [21]:

$$RI = 100z + 100\ (t_{R(i)} - t_{R(z)}/(t_{R(z+1)} - t_{R(z)}) \quad (1)$$

RI = relative index of compound i;
z = carbon number of the alkane z;
$t_{R(i)}$, $t_{R(z)}$, and $t_{R(z+1)}$ = retention times of the compound i, the compound z, and the alkane $z + 1$, respectively

2.4. Optimization of Sample Extraction Conditions

Three model wines were prepared with tartaric acid (0.008 M), potassium hydrogen tartrate (0.011 M) and ethanol (5, 8 or 13% v/v), with the compounds of interest added at concentrations that approximately match the mid-point of calibration curves. Sample extraction temperature (°C), time (min) and volume (mL), in addition to the ethanol content (% v/v), were optimized using a three-factor Box-Behnken design (Table 2). Optimum sample incubation temperature, SPME fiber exposure time and sample volume combinations at each ethanol concentration were based on a previously described method [13]. A quadratic equation with second-order interactions for the three factors was used to determine the maximum predicted signal for each analyte using the following equation:

$$\hat{y} = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 \quad (2)$$

$\hat{y}$ = predicted response; b_0 is the intercept or average response;
$b_1x_1 + b_2x_2 + b_3x_3$ = linear terms associated with each factor (temp, time, sample vol.);
$b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3$ = second-order interaction terms between each factor;
$b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2$ = quadratic terms for each factor;
x_1 = factor extraction temperature;
x_2 = factor extraction time;
x_3 = factor sample volume in 20 mL vial.

The significance of each experimental factor coefficient (b) in the quadratic equations was determined using a Students t-test following calculation of a coded design matrix, predicted responses and residuals [22] as described in the supplementary materials (Tables ST1 and ST2), with insignificant factors dropped from the final modelled response. The predicted maximum response and optimized conditions for each analyte were then used to determine the final global optimized sample incubation time, fiber exposure time and sample volumes at each ethanol concentration. This was completed according to previously published methods [23] in which a desirability function is used to maximize the sampling sensitivity for analytes with low detector responses within the calibration range (presented separately for 5, 8 and 13% v/v ethanol concentration in Supplementary

Tables TS3, TS4 and TS5, respectively). All statistical testing and modeling were conducted in Matlab R2007a (The Mathworks, Natick, MA).

Table 2. Box-Behnken (three-factor) design.

Parameter/Conditions	Levels
Extraction temperature (°C)	30, 50, 70
Extraction time (min)	15, 30, 45
Sample volume (mL) in 20 mL vial	7, 10, 13

2.5. Calibration Curves

Calibration curves were obtained by diluting the highest concentration mixture of pure compounds to eight concentrations over the analytical range. Calibration samples of different concentration were prepared for the 5, 8 and 13% v/v ethanol content model solutions, and the samples were injected into the GC-MS instrument according to the optimized exposure time, temperature and volume of the three different ethanol content solutions. The calibration curves for the compounds of interest at three different ethanol concentrations were prepared using the ChemStation® (Agilent Technologies, Ltd.) software. The linear regression equation for each compound was determined from the calibration curve of peak area ratio (volatile compound/internal standard) versus the compound concentrations in solution. The limit of quantification (LOQ) was evaluated from the determination of signal to noise ratio for all compounds with a minimum ratio of 10 used as the criteria.

2.7. Natural and Reduced Alcohol Wines

Both Shiraz and Chardonnay wines were supplied by the Charles Sturt University's commercial winery and contained 13.7 and 12.2% v/v ethanol respectively. Both wines were produced according to a conventional winemaking produced, which also included an oaked component as part of the final blend. Two reduced alcohol wines containing 8 and 5% v/v ethanol were produced from both Shiraz and Chardonnay wines. Ethanol content was measured using an Anton Paar Alcoholyser (Graz, Austria) NIR-density meter. Partial dealcoholization was performed using a Memstar AA MEM-066 (Oakleigh, Australia) bench-top laboratory membrane filtration system, which consisted of a reverse osmosis (RO) unit followed by evaporative perstraction (EP) as previously described [24]. Briefly, this two-stage filtration process ensures that the wine is first separated by reverse osmosis into retentate (concentrate; alcohol reduced) and permeate (filtrate; alcohol enriched) streams. The permeate and stripping solution (water) counter-flowed on either side of the membrane contactor while alcohol is discharged by means of perstraction (evaporation and diffusion) through the membrane. The dealcoholized permeate is then cooled and recombined with the feed wine. Prior to bottling into dark green 750 mL glass bottles, the concentration of molecular sulfur dioxide of all samples was adjusted to 0.5-0.8 mg/L. Bottles were screw-capped and stored at 15°C until further analysis.

2.8. Reconstituted Wines

Reconstituted wines, consisting of 5% v/v reduced alcohol wines adjusted back to their natural ethanol content, were prepared by adding 8.7 mL ethanol (VWR, Prolabo) per 100 mL of Shiraz wine, and 7.2 mL ethanol per 100 mL of Chardonnay. Prior to bottling into dark green 750 mL glass bottles, the concentration of molecular sulfur dioxide was adjusted to give 0.5-0.8 mg/L. Bottles were screw-capped and stored at 1.8°C for 24 h and 14 days prior to GC-MS analysis. Reconstituted wines allow us to differentiate the effect of partial dealcoholization by RO-EP treatment from the effect of ethanol content reduction on wine volatile composition. The times chosen allowed assessment of any

immediate impact on the volatile profile (24 h), and any effect after time to permit ethanol integration (14 d).

2.9. GC-MS Analysis of Wine Samples

Samples (10 mL) of reduced alcohol treatments (i.e. 5 and 8% v/v), as well as those (8 mL) of alcoholized ones (i.e. natural and reconstituted), were accurately pipetted into 20 mL SPME vials. Internal standard mix (100 µL) was added to each vial. All vials were screw-capped, cooled at 8°C and moved to the heater block at 45°C prior to allowing equilibration for 20 min while shaken (250 rpm). The incubation temperatures of 5, 8 and 13% v/v ethanol content samples were 48, 46 and 44°C respectively. A DVB/CAR/PDMS SPME fiber was exposed into the sample vial headspace. The extraction times of volatile compounds for the 5, 8 and 13% v/v samples were 29, 43 and 42 min respectively. Extraction was performed with vial shaking and the extracted sample was inserted into the GC-MS injector at 260°C in splitless mode for 1 min.

3. Results and Discussion

3.1. Optimization of SPME Factors

Optimization of SPME sample experimental conditions is a crucial analytical step to ensure the accuracy and sensitivity of the method for headspace analysis [18]. In the present study, a quantitation method for a range of wine impact odorants was optimized by a three-level Box-Behnken design for three different ethanol content wine-model solutions (i.e. 5, 8 and 13% v/v). This multivariate statistical model minimized the number of experiments required to determine significant coefficients for linear, interactions and quadratic terms for SPME fibre exposure temperature, time and wine sample volume for each analyte responses. A global optimized condition for SPME sampling for the suite of analytes, at each ethanol concentration was then developed, by using a desirability function that accounts for a low signal response for compounds. This approach has been previously employed for determining the optimized conditions for the quantification of fungal off-flavors [23]. Ethanol is the most abundant volatile compound in wine and will compete with other compounds of interest for binding sites on the SPME fiber [25]. As wine ethanol concentrations impact the partitioning of volatile compounds from liquid to the headspace, optimized sample analysis conditions were determined at wine ethanol levels to avoid confounding analytical results associated with sample dilution. This approach ensures that headspace concentrations for each compound of interest at each ethanol concentration are accurately determined and allow comparisons between wines at differing ethanol levels to be made. The results regarding the combination of optimized fiber exposure time, temperature and sample volume at different ethanol content, are summarized in Supplementary Material (Tables TS3, TS4 and TS5 for 5, 8 and 13% v/v ethanol content model wine solutions, respectively).

Inspection of the *b*-coefficients for each compound at each ethanol concentration (Figures 1, 2 and 3 for 5, 8 and 13% v/v ethanol content model wine solutions) reveals the impact of each experimental factor upon analyte behaviour for SPME. The modulus of each *b*-coefficient is determined by the relative detector response for each analyte and the sign infers either a positive or negative impact upon analytical sensitivity. SPME temperature of fibre exposure is the most critical factor for analyte quantification in agreement with previous findings for different matrices than wine [26,27]. This is indicated by the number of analytes with a significant *b*-coefficient for the temperature linear and second order interaction terms, and this trend is evident at all ethanol concentrations. Of interest is the association of negative *b*-coefficients for temperature with early eluting compounds (1-10) and positive *b*-coefficients for late eluting compounds (15-23) and this can be correlated with the boiling point and the elution order of the analytes of interest (Supplementary Table ST6). Overall SPME fiber binding of analytes from the vial headspace is determined by mass transfer of the analytes from liquid to the volatile phase followed by mass transfer onto the fiber. Temperature has a significant impact upon the vapour pressures of analytes and therefore the headspace concentrations

in which the SPME fiber is immersed during sampling [28]. Increasing sample temperature will inevitably increase headspace concentrations of higher boiling point analytes leading to improved analytical sensitivities for these compounds and increasing competitive binding for SPME fibre space with lower boiling point analytes [13]. Increased competition between high and low boiling point compounds for fibre space, explains the negative *b*-coefficients for early eluting compounds.

SPME fiber exposure time is important for quantification of compounds and negative *b*-coefficient for linear terms are also associated with early eluting compounds, and positive coefficients for late eluting, high boiling point compounds which is evident at all ethanol concentrations. Partitioning of volatile compounds into the headspace during fiber exposure is a dynamic phenomenon as compounds absorb onto the SPME fiber from the headspace [29]. As semivolatile compound concentrations in the headspace are relatively low compared to their liquid concentration, overall mass transfer rates are low and longer extraction times lead to the increased mass transfer of these compounds from the vapour onto the fiber.

Interestingly sample volume in the headspace vial was insignificant for all compounds at 5% v/v ethanol (Figure 1), but became a significant factor for some compounds as the ethanol concentration increased (Figures 2 and 3). This observation should be considered in light of the relative concentration and partition coefficients of ethanol in the base wines compared to the analytes of interest. As the ratio of sample to headspace volume increases, compounds with high partition coefficients i.e. compounds with relatively lower headspace concentrations, partitionless into the headspace relative to compounds with low partition values. Ethanol has a relatively high partition coefficient but is present in high concentrations relative to other volatile compounds. Competition between ethanol and other volatile compounds for SPME fibre binding sites will arise which would lower sampling sensitivity. Larger sample volumes will increase the quantity of some analytes present in the headspace vial relative to ethanol thereby increasing the sensitivity of the SPME sampling for these compounds at higher ethanol concentrations.

Calibration parameters, including analyte quantification and qualification ions, elution times and method performance characteristics are presented in Supplementary Table ST6. Linearity, limits of quantification and signal to noise ratios are within acceptable thresholds for compound quantification [20].

Figure 1. *b*-coefficients for SPME optimization parameters for 23 compounds with an ethanol concentration of 5% v/v. Significant coefficients for specific compounds are indicated with an asterisk. Compound identification is cross-referenced to the numbered compounds in Table 1.

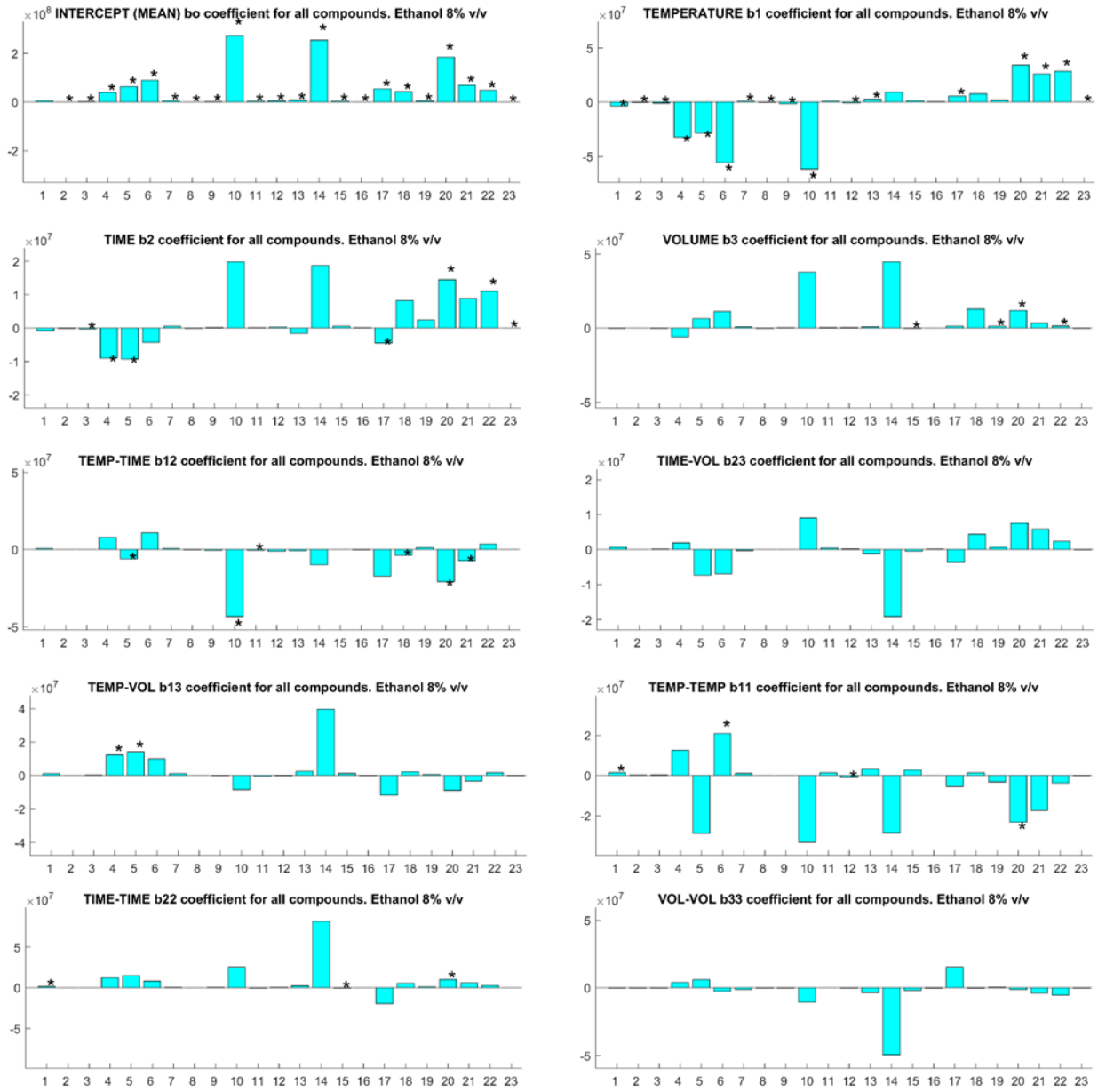

Figure 2. *b*-coefficients for SPME optimization parameters for 23 compounds with an ethanol concentration of 8% v/v. Significant coefficients for compounds are indicated with an asterisk. Compound identification is cross-referenced to the numbered compounds in Table 1.

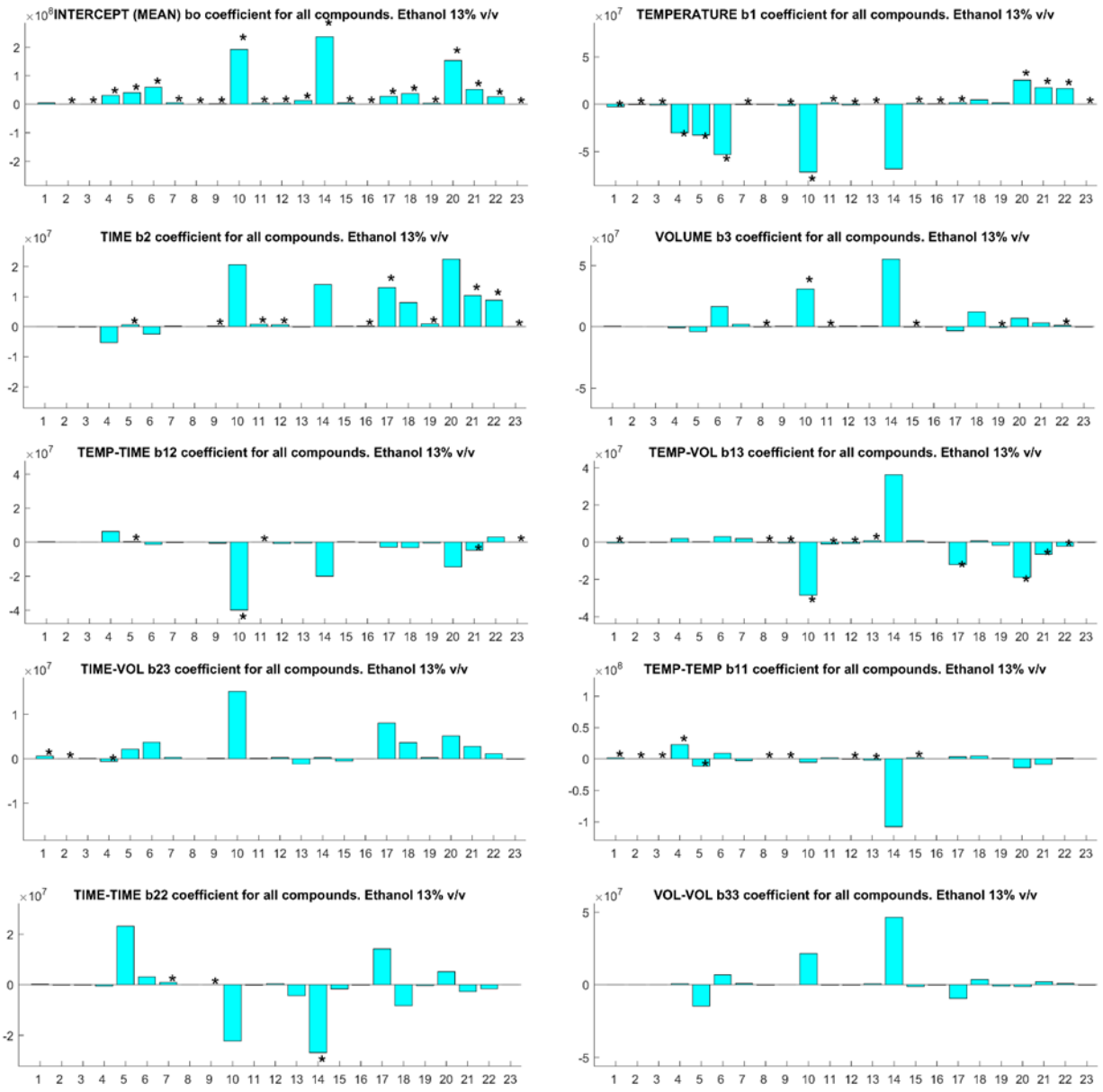

Figure 3. *b*-coefficients for SPME optimization parameters for 23 compounds with an ethanol concentration of 13% v/v. Significant coefficients for specific compounds are indicated with an asterisk. Compound identification is cross-referenced to the numbered compounds in Table 1.

3.2. Volatile Changes after RO-EP Treatment

Tables 3 and 4 report the results of the GC-MS analysis of all analytes determined in the natural and reduced alcohol wines for Shiraz and Chardonnay, respectively. A total of 23 compounds were identified and quantified in the different headspace extracts, namely: esters (13), alcohols (3), acids (3), terpenes (2), sulfur compounds (1) and phenols (1). ANOVA showed that the concentration of most analytes clearly decreased with dealcoholization by up to 82% from their natural concentration.

As reported in Tables 3 and 4, most esters decreased in both wine varieties by 20-81% from their natural concentration, except for methyl octanoate and methyl decanoate in both wine varieties at all dealcoholized levels, and isoamyl octanoate and ethyl-s-lactate in Shiraz and Chardonnay wines, respectively. The percentage losses of esters increased with increased dealcoholization extent (from 8 to 5% v/v). For example, ethyl-3-methyl butyrate and isoamyl acetate were not appreciably diminished in the 8% v/v Shiraz and Chardonnay treatments in comparison to the natural wines, but they decreased in the 5% v/v samples.

Three alcohols (other than ethanol) have been identified and quantified in the wines, originating from grape-derived precursors, such as (z)-3-hexenol, or yeast's metabolism, such as 3-methyl-1-butanol and β-phenyl ethanol. It is noteworthy that alcohols followed a similar trend for both wine varieties. Whereas 3-methyl-1-butanol and (z)-3-hexenol decreased with dealcoholization by 32-41% and 13-21% for Shiraz and Chardonnay respectively, no differences were found between 5 and 8% v/v ethanol wines.

Another additional information is that β-phenyl ethanol, which has a characteristic odor of rose-honey-like at a high concentration of ≥ 14000 μg/L [30], significantly increased with dealcoholization by up to 25% and 44% in Shiraz and Chardonnay wines, respectively. The greatest increase for β-phenyl ethanol in Shiraz and Chardonnay wines was observed in the 8% v/v treatment compared to the standard wines. For this compound, a retention effect exerted by the wine non-volatile matrix, possibly due to π-π stacking interactions with wine polyphenols, was previously suggested [14,24].

For both wine varieties, propanoic acid did not decrease at any dealcoholization level. On the other hand, octanoic acid decreased by up to 21% and 14% in Shiraz and Chardonnay wines, respectively. These results are in general agreement with the previous studies for similar membrane contactors, in which losses of up to 17% and 68% for octanoic acid and decanoic acid were respectively reported [31]. Of all quantified acids, decanoic acid was the most decreased. It decreased by up to 58% and 77% in Shiraz and Chardonnay wines, respectively. Nevertheless, no differences were observed between the 8 and 5% v/v treatments for both wine varieties.

Two monoterpenes were identified and quantified in Shiraz and Chardonnay wines, namely linalool and geraniol. Intriguingly, the concentration of linalool, which has been reported to have a characteristic lavender, flower aroma [30], increased by up to 58% in Shiraz dealcoholized wines. This was in contrast to the Chardonnay samples where the concentration of linalool decreased with dealcoholization by up to 78% from its natural concentration, possibly due to the different non-volatile matrix composition between these wines [32]. Whereas a reduced concentration in geraniol of 70-82% is perhaps not surprising for Chardonnay wines, a loss of geraniol of 64% in Shiraz dealcoholized samples is apparently in contrast with the previously suggested retention effect from wine non-volatile compounds towards aroma compounds. This may be due to differences in the type of monoterpenes, suggesting the important effect of the molecular chemical structure in the interaction with some non-volatile compounds [32].

The concentration of other compounds such as 3-(methylthio)-1-propanol did not change at any dealcoholization extent for both wine varieties. This sulfur-containing aroma compound, principally arising from the alcoholic fermentation, can impart an undesirable raw potato odor at a high concentration of ≥ 1000 μg/L [30]. Likewise, vanillin was not significantly decreased by the dealcoholization treatment in both Shiraz and Chardonnay wine varieties.

Table 3. Changes in headspace concentration of volatile compounds in Shiraz wines.

Compound (μg L^{-1})	Dealcoholized and Natural Wines			Reconstituted Wines	
				Storage Time	
	5% v/v	8% v/v	13.7% v/v	24 h	14 Days
ethyl butyrate	0.90 ± 0.01a	1.72 ± 0.08b	2.69 ± 0.06c	0.75 ± 0.01a	0.76 ± 0.02a
ethyl-2-methyl butyrate	0.33 ± 0.00b	0.46 ± 0.03c	0.47 ± 0.01c	0.23 ± 0.01a	0.17 ± 0.00a
ethyl-3-methyl butyrate	0.05 ± 0.00b	0.08 ± 0.01c	0.08 ± 0.00c	0.04 ± 0.00ab	0.03 ± 0.00a
isoamyl acetate	0.21 ± 0.01b	0.43 ± 0.03c	0.51 ± 0.03c	0.08 ± 0.01a	0.08 ± 0.01a
3-methyl-1-butanol	6.18 ± 0.06ab	6.64 ± 0.41b	9.05 ± 0.24c	5.62 ± 0.17ab	5.38 ± 0.13a
ethyl hexanoate	1.72 ± 0.02a	2.34 ± 0.23a	5.41 ± 0.88b	1.38 ± 0.11a	1.51 ± 0.05a
ethyl-s-lactate	33500 ± 720a	32300 ± 2600a	47300 ± 3060b	41900 ± 1870ab	33900 ± 1560a
(z)-3-hexenol	5.32 ± 0.10c	5.42 ± 0.04c	6.83 ± 0.05d	3.61 ± 0.14a	4.49 ± 0.08b
methyl octanoate	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	BLQ	BLQ
ethyl octanoate	3.17 ± 0.01b	4.90 ± 0.15c	7.32 ± 0.15d	2.31 ± 0.02a	2.17 ± 0.05a
propanoic acid	600 ± 20c	250 ± 10ab	220 ± 0.00a	240 ± 0.00a	290 ± 10b
linalool	0.53 ± 0b	0.59 ± 0.01b	0.25 ± 0.06a	0.27 ± 0.01a	0.27 ± 0.00a
methyl decanoate	BLQ	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
ethyl decanoate	1.66 ± 0.02bc	1.39 ± 0.10b	2.64 ± 0.46c	0.19 ± 0.13a	0.74 ± 0.09ab
isoamyl octanoate	0.02 ± 0.00b	0.02 ± 0.00b	0.02 ± 0.00b	0.01 ± 0.00a	0.01 ± 0.00a
3-(methylthio)-1-propanol	103 ± 0.42a	101 ± 0.87a	103 ± 12.0a	92.1 ± 3.44a	90.7 ± 9.40a
β-phenyl ethyl acetate	0.03 ± 0.00a	0.04 ± 0.00a	0.05 ± 0.00b	0.02 ± 0.00a	0.03 ± 0.00a
ethyl dodecanoate	0.04 ± 0.00a	0.03 ± 0.00a	0.12 ± 0.01b	0.02 ± 0.00a	0.02 ± 0.00a
geraniol	0.09 ± 0.00a	0.09 ± 0.00a	0.25 ± 0.03b	0.06 ± 0.00a	0.06 ± 0.00a
β-phenyl ethanol	280 ± 0.00d	320 ± 0.00e	240 ± 0.00c	230 ± 0.00b	200 ± 0.00a
octanoic acid	8.00 ± 0.12c	7.10 ± 0.19b	9.01 ± 0.3d	6.58 ± 0.08b	5.36 ± 0.10a
decanoic acid	0.94 ± 0.01a	0.90 ± 0.02a	2.16 ± 0.22b	1.16 ± 0.05a	0.96 ± 0.05a
vanillin	0.80 ± 0.06a	1.16 ± 0.1ab	1.13 ± 0.09ab	2.01 ± 0.40b	0.92 ± 0.08a

Data are expressed as the mean of triplicate determinations ± standard deviation. Different letters in a row discriminate the treatments significantly different from one another ($p \leq 0.05$). BLQ, below limit of quantitation.

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Table 4. Changes in headspace concentration of volatile compounds in Chardonnay wines.

Compound (μg L^{-1})	Dealcoholized and Natural Wines			Reconstituted Wines	
				Storage Time	
	5% v/v	8% v/v	12.2% v/v	24 h	14 Days
ethyl butyrate	0.96 ± 0.01a	2.42 ± 0.23b	4.12 ± 0.05c	0.87 ± 0.02a	0.88 ± 0.01a
ethyl-2-methyl butyrate	0.11 ± 0.00b	0.20 ± 0.01c	0.25 ± 0.00d	0.09 ± 0.00ab	0.07 ± 0.00a
ethyl-3-methyl butyrate	0.02 ± 0.00a	0.04 ± 0.01b	0.05 ± 0.00b	0.02 ± 0.00a	0.01 ± 0.00a
isoamyl acetate	0.04 ± 0.01a	0.23 ± 0.01b	0.21 ± 0.00b	BLQ	BLQ
3-methyl-1-butanol	3.92 ± 0.04ab	5.56 ± 0.26bc	6.68 ± 0.74c	2.78 ± 0.11a	2.92 ± 0.07a
ethyl hexanoate	3.79 ± 0.04a	5.77 ± 0.70b	11.6 ± 0.33c	2.62 ± 0.12a	2.84 ± 0.03a
ethyl-s-lactate	4790 ± 80ab	5230 ± 250ab	6560 ± 930b	4360 ± 260a	4280 ± 130a
(z)-3-hexenol	3.75 ± 0.06c	4.08 ± 0.03c	4.68 ± 0.14d	2.35 ± 0.07a	3.07 ± 0.03b
methyl octanoate	0.01 ± 0.00	0.01 ± 0.00	0.03 ± 0.00	BLQ	BLQ
ethyl octanoate	8.82 ± 0.09b	15.5 ± 0.71c	32.3 ± 0.65d	5.23 ± 0.02a	5.82 ± 0.07a
propanoic acid	200 ± 20	230 ± 10	180 ± 0.00	180 ± 0.00	210 ± 0.00
linalool	0.29 ± 0.00c	0.37 ± 0.00d	0.08 ± 0.00b	0.05 ± 0.00a	0.06 ± 0.00a
methyl decanoate	BLQ	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
ethyl decanoate	4.18 ± 0.06b	3.59 ± 0.31b	12.4 ± 0.33c	0.87 ± 0.42a	1.82 ± 0.03a
isoamyl octanoate	0.04 ± 0.00b	0.03 ± 0.00b	0.06 ± 0.00c	0.01 ± 0.00a	0.01 ± 0.00a
3-(methylthio)-1-propanol	40.9 ± 0.22b	43.3 ± 1.76b	38.2 ± 1.46b	29.1 ± 1.61a	40.4 ± 1.27b
β-phenyl ethyl acetate	0.03 ± 0.00ab	0.04 ± 0.00bc	0.04 ± 0.00c	0.03 ± 0.00a	0.03 ± 0.00a
ethyl dodecanoate	0.06 ± 0.00b	0.07 ± 0.00b	0.3 ± 0.01c	0.02 ± 0.00a	0.02 ± 0.00a
geraniol	0.20 ± 0.00c	0.12 ± 0.00b	0.67 ± 0.01d	0.14 ± 0.02b	0.06 ± 0.00a
β-phenyl ethanol	90 ± 0.00b	90 ± 0.00b	50 ± 0.00a	50 ± 0.00a	50 ± 0.00a
octanoic acid	30.3 ± 0.14b	35.7 ± 0.67c	35.2 ± 1.12c	24.6 ± 0.12a	22.1 ± 0.12a
decanoic acid	3.37 ± 0.04a	3.72 ± 0.34a	15.7 ± 0.60b	3.69 ± 0.16a	2.61 ± 0.08a
vanillin	0.08 ± 0.01a	0.12 ± 0.01a	0.19 ± 0.01ab	0.29 ± 0.05b	0.15 ± 0.02a

Data are expressed as the mean of triplicate determinations ± standard deviation. Different letters in a row discriminate the treatments significantly different from one another ($p \leq 0.05$). BLQ, below limit of quantitation.

3.4. Effect of Ethanol Content Reduction

The overall effect of dealcoholization by RO-EP treatment on wine volatile composition includes two distinct effects: (i) the RO-EP effect and (ii) the ethanol content reduction effect [7]. To quantify the effect arising from the ethanol content reduction, a comparison between reconstituted and 5% dealcoholized wines, was carried out. GC-MS analyses on reconstituted samples were performed after 24 h and 14 days of storage at 1.8°C. If ethanol content had either a suppressing or enhancing the effect on analyte responses, the addition of exogenous ethanol to 5% v/v dealcoholized samples should result in a significant difference between the GC-MS results of reconstituted and 5% reduced alcohol wines.

As indicated by Tables 3 and 4 for Shiraz and Chardonnay wines respectively, the concentration of many analytes was lower in reconstituted wines compared to that of 5% v/v ethanol samples. For example, the concentration of ethyl decanoate in reconstituted Shiraz and Chardonnay wines significantly decreased after 24 h storage by respectively 88% and 79%, compared to the corresponding 5% v/v ethanol samples. ANOVA, however, shows that the number of volatile compounds that decreased in the headspace of reconstituted wines was different between Chardonnay and Shiraz wine varieties, and slightly higher after 24 h (10/23 and 12/23 for Shiraz and Chardonnay wines, respectively) than after 14 days (8/23 and 11/23 for Shiraz and Chardonnay wines, respectively) storage. A negative relationship between ethanol and other volatile compounds is consistent with the previous studies for other wines or model solutions [15,16,33]. Differences in the number of affected analytes between Shiraz and Chardonnay wine varieties, likely arise from a different composition in wine non-volatile matrix, including an expected higher presence of phenolics in red than in white wine.

While several volatile compounds exhibited a negative relationship with ethanol, many others did not show any appreciable interaction. For example, the concentration of ethyl butyrate, ethyl-2-methyl butyrate and ethyl-3-methyl butyrate did not change at any storage extent (i.e. 24 h, 14 days) for both Shiraz and Chardonnay reconstituted wines compared to the 5% v/v ethanol samples. In the case of vanillin only, its concentration in the wine headspace of reconstituted Shiraz and Chardonnay wines increased after 24 h storage by 60% and 72%, respectively. However, no differences were observed after 14 days of storage.

Overall, these results indicate that the reduction of ethanol content in Shiraz and Chardonnay wines significantly affected the headspace composition of wine, however, the nature of changes on the different classes of analytes is not specific. These findings may be of sensory significance since the portioning of volatile compounds between the wine's headspace and liquid phases at equilibrium can change the concentration of free volatile compounds available for the perception of aromas [34].

5. Conclusions

A total of 23 compounds extracted using a DVB/CAR/PDMS SPME fiber were successfully quantified using GC-MS. The optimum HS-SPME conditions for the extraction of 23 target impact odorants in reduced alcohol red and white wine that contributed to the regression models using a Box-Behnken experimental design were determined in order to study the effect of SPME conditions, namely, extraction time, extraction temperature and sample volume. The three-factor Box-Behnken analysis showed significant ($p<0.05$) relationships between the SPME variables and the component headspace concentrations, with extraction temperature resulting in the most critical factor when studying the equilibrium of reduced alcohol wine's impact odorants.

The concentration of several analytes decreased with dealcoholization, with ethyl hexanoate, ethyl octanoate been the most affected. It was also shown that the concentration of many volatile compounds remained significantly decreased in the headspace of reconstituted wines (i.e. 5% v/v reduced alcohol wines adjusted back to their natural ethanol concentration) compared to that of 5% v/v reduced alcohol samples, confirming losses of these compounds from the wine during the ethanol removal process and revealing that the final composition of reduced alcohol wine's headspace is

mainly due to the combination of two factors: (i) type of dealcoholization treatment and (ii) ethanol content reduction.

A negative relationship between ethanol concentration and most analyte responses during the SPME sampling process is apparent and the confounding impact of competition for SPME binding sites between ethanol and other volatile compounds requires a rigorous analytical approach to enable comparisons between wines of varying ethanol concentrations. Sample dilution to a constant ethanol concentration will alleviate inconsistent competition between ethanol and analyte binding on the SPME fibre but will also alter the partitioning of compounds from liquid to headspace thereby confounding final comparisons of different wines. Researchers should consider that variation in sample conditions such as ethanol content may confound comparisons of different wines, particularly those between natural and dealcoholized treatments and for the most accurate assessment of the concentration of volatile compounds, analytical conditions that account for varying ethanol concentration must be used. Invariably this will require analytical calibrations at each important ethanol concentration for headspace analysis.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table ST1: Design matrix for the optimization experiment using experimental conditions. Table ST2: Coded design matrix where experimental factors are coded *-1, 0 1* at each level; Table ST3: Optimized SPME conditions for target aroma compounds for the 5% v/v treatment; Table ST4: Optimized SPME conditions for target aroma compounds for the 8% v/v treatment; Table ST5: Optimized SPME conditions for target aroma compounds for the 13% v/v treatment; Table ST6: Target compounds identification and calibration parameters for three different levels of ethanol content of wine.

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