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Quantification by Solid Phase Micro Extraction and Stable Isotope Dilution Assay of norisoprenoid compounds in red wines obtained from Piedmont rare varieties

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Abstract

A method for the identification and quantification of megastigmane norisoprenoid compounds in wines was developed using headspace Solid Phase Micro Extraction (SPME) coupled with gas-chromatography/mass-spectrometry (GC-MS). Three different compounds were quantified by Stable Isotope Dilution Assay (SIDA): β-damascenone, β-ionone and α-ionone.

Particular attention was paid to maximising the method's sensitivity while reducing the extraction time. To optimise the extraction conditions, a statistically designed experiment was performed using extraction time, extraction temperature and ethanol content as operating variables. Five different SPME fibres suitable for volatile compounds analysis were compared. This study confirmed that the PDMS/DVB coating performs best for the quantification of β-damascenone and β-ionone, and the crucial role of the ethanol content of the sample for the extraction effectiveness. Finally, the optimised method was applied to the study of various wines derived from rare and autochthonous grape varieties of north-western Italy.

Keywords: HS-SPME, SIDA; β-damascenone, β-ionone, α-ionone, wines, autochthonous.

1. Introduction

The aromatic compounds derived from grapes are particularly important for the olfactory characteristics of wines. These molecules have a pleasant aroma, low perception thresholds and can provide expression and originality to the wines. The aromatic compounds are present in large quantities in wines obtained from aromatic grapes, but even their presence in small quantities can positively influence the aroma of wines from non-floral grapes. During the last decade, many studies have been devoted to norisoprenoid compounds because of their very low perception threshold (Mendes-Pinto, 2009) and their powerful aroma in many fruits and foods (Winterhalter Peter & Rouseff Russell, 2011).

The norisoprenoids in wines are generally molecules with a structure of 13 carbon atoms, resulting from the enzymatic (Mathieu, Bigey, Procureur, Terrier, & Günata, 2007; Mathieu, Terrier, Procureur, Bigey, & Günata, 2005) and chemical or photochemical degradation of grape carotenoids (Baumes, Wirth, Bureau, Gunata, & Razungles, 2002). In particular, two molecules are notably important for determining the aromatic profile of wines: β-damascenone and β-ionone.

β-damascenone was identified in wine for the first time in 1974 (Schreier & Drawert, 1974). This molecule has a complex aroma suggestive of flowers and exotic fruits with honey and apple undertones, and its perception threshold in water has been estimated to be 2 ng L^{-1} (Kotseridis, Baumes, Bertrand, & Skouroumounis, 1999). In grapes, it is generated from the degradation of neoxantin and from specific glycosilated precursors during the wine aging process (Skouroumounis & Sefton, 2011). β-damascenone can be found in many wines, and its concentration can vary from 0.25 to 4.5 µg L^{-1} (Sefton, Skouroumounis, Elsey, & Taylor, 2011), which is well above its perception threshold in water.

β-ionone is an important molecule with a pleasant, typical aroma of violet. This molecule is generated by the degradation of β-carotene and is present in many flowers and fruits. Due to this compound's notably low perception threshold of 30 ng L^{-1} in water (Buttery, Ling, & Stern, 1997), it is considered to be a key aroma compound of many red wines. This compound's concentration in wine depends on the grape variety, as well as on the winemaking process (Kotseridis, Baumes, Bertrand, & Skouroumounis, 1999). Furthermore, also α-ionone has been found in wines: it has a low perception threshold and a typical aroma of violet. Its contribution to wine aroma is still uncertain, and the information on its real concentration in wines is still scarce (Mendes-Pinto, 2009).

Because these molecules are measured at ppb concentration levels in wines, their quantification requires the use of sensitive analytical techniques. Among the different sample preparation techniques is SPME (Solid Phase Micro Extraction), a technique based on the extraction of specific compounds from a complex matrix by absorption or adsorption on a thin silica fibre. Compared to other sample preparation techniques, such as liquid-liquid extraction or SPE (Solid Phase Extraction), SPME has the advantage of being solvent-free. First, this characteristic avoids exposing the operator to solvents and the need for expensive disposal procedures for toxic wastes that is typical of SPE and liquid/liquid extraction techniques. Second, SPME makes the sample preparation easier and faster, due to its simplicity and the possibility of automation. This technique, however, is extremely sensitive to experimental conditions, which must therefore be strictly controlled. Any change in the experimental conditions can influence the amount of analyte retained by the fibre and therefore can affect the reproducibility and sensitivity of the analytical method (Pawliszyn, 1999). Furthermore, the introduction of the Stable Isotope Dilution Analysis technique (SIDA) coupled with GC-MS, which is based on the use of labeled internal standards, has significantly contributed to the improvement of the accuracy of the analysis by limiting the variability related to the sample preparation procedure and to the matrix (Blank, Milo, Lin, & Fay, 1999; Sen, Laskawy, Schieberle, & Grosch, 1991).

The accurate determination of norisoprenoid compounds is important for the detailed aromatic characterisation of all wines but especially for those obtained from minor varieties with peculiarities that arise not only from their rarity but also from their chemical and physical composition. The information regarding the aromatic composition of these wines is currently inadequate, mainly due to the small number of studies specifically devoted to this subject and the large number of existing autochthonous varieties. This work was specifically aimed at optimising the extraction conditions in a SPME-GC-MS method to obtain the best reproducibility and sensitivity for the analysis. Finally, this method was applied to the quantification of β-ionone, α-ionone and β-damascenone in wines obtained from rare Piedmont grape varieties.

2 Experimental

2.1 Chemicals and reagents

The SPME fibres were purchased from Supelco (Bellefonte, PA, USA), and the headspace vials from Phenomenex (Torrence, CA, USA). The chemical standards were obtained from Sigma (Sigma-Aldrich, St. Louis, MO, USA) at the maximum purity grade available, except β-damascenone, which was generously supplied by Firmenich (Genève, Switzerland). The deuterated internal standards were generously offered by Rémi Guérin-Schneider from INRA (Montpellier, France). Ultrapure water was obtained from a Milli-Q gradient A10 instrument (Millipore Corporation, Billerica, USA).

2.2 Wines

Forty different single varietal wines were produced with 17 rare and autochthonous north-western Italian grape cvs., harvested from the ampelographic collection vineyard located in Grinzane Cavour (Cuneo, Italy) and vinified at the experimental cellar of Bonafous Centre located in Chieri, near Turin. A standard winemaking protocol with maceration was used for the vinification of Bonarda, Brunetta di Rivoli, Bubbierasco, Cellerina, Chatus, Doux D'Henry, Freisa, Fumin, Gamba di Pernice, Montanera, Nebbiolo Scarlatin, Neretto duro, Petit Rouge, Pignolo Spano, Rastajola, Rossese and Vermentino nero. In addition to these wines, a Doux d'Henry "passito" was vinified as a sweet wine. Finally, all wines were analysed for pH, ethanol content (*Commission Regulation No 2676/90)*, total anthocyanins and total flavonoids (Di Stefano, Cravero, & Gentilini, 1989). All these analysis were performed once. The wines' geographical origin and distribution is reported in **table 1** (Raimondi, Valota, & Schneider, 2009; Schneider, Mannini & Cravero, 2005; Schneider & Mannini, 2006).

2.3 Optimisation of the extraction parameters and experimental design

The extraction process of the megastigmanes was optimised during the first part of this work by choosing the fibre type and the best operative conditions. Once the analytical method was defined, the calibration curves were calculated using deuterated standards.

The type of fibre, the presence of salting-out agents, and the desorption and bakeout times were evaluated using an OFAT (One-Factor-at-A-Time) method to reduce the number of factors (Ryan, 2007). Initially, five different fibres suitable for volatile compounds analysis were compared (**table 2**). The selected fibre coatings were polydimethylsiloxane (PDMS), divinylbenzene/polydimethylsiloxane (DVB/PDMS), Carboxen/polydimethylsiloxane (CAR/PDMS), and divinylbenzene/Carboxen/polydimethylsiloxane (DVB/CAR/PDMS). Moreover, two fibres with the same coating but different internal structure were compared: fused silica core (blue, PDMS/DVB) and flexible core (pink, PDMS/DVB).

The five fibres were used to analyse two different solutions to evaluate the extraction efficiency: a Chardonnay (ethanol 15 % v/v, pH 3.5, total acidity 5.4 g L^{-1}) and a hydro-alcoholic solution (10 % v/v) with known added amounts of analytes (approximately 2 µg L^{-1}). Each analysis was performed in triplicate, and the results were processed by ANOVA and Tuckey's post hoc test (for mean comparison). The salting-out effect was tested in the same way, by evaluating the instrumental response of two different samples with the addition of sodium sulphate or sodium chloride (3.1 g), respectively, in comparison with a control without added salt. Similarly, the best conditions for bakeout and desorption times were tested. The desorption time was evaluated comparing three different times (1, 2 and 3 min), with measurements in triplicate for each selected time. In order to evaluate the presence and intensity of carryover phenomena, the measures done in the headspace of vials containing the analytes were followed by measures done in empty vials. This

procedure was repeated three times. Once these conditions were fixed, the influence of extraction time, temperature and ethanol content on the instrumental response was studied. The levels of each parameter were chosen after preliminary tests (study of the extraction kinetics) were performed to define the experimental space. A Central Composite Face Design Experiment was used (Box & Draper, 1987). The choice of this experimental plan and the statistical data processing was performed by the software MODDE 6.0 from Umetrics AB (Umeå, Sweden). For this experiment, 17 analyses were performed (**table 3**), and the instrumental response, resulting in a peak area, was processed by a complete 3-factors ANOVA, by Tukey's test (for mean comparison), and by the study of the response surfaces.

2.4 Analytical method for the determination of norisoprenoids

For this experiment, 25 mL of wine was added to 50 mL of ultrapure water in a 75 mL flask. Next, 10 mL of this solution was placed in a vial (20 mL) for headspace analysis, and 4 µL of an internal standard mixture containing 281 µg L^{-1} of [$^2H_4$]-β-damascenone, 929 µg L^{-1} of [$^2H_3$]-β-ionone, and 570 µg L^{-1} [$^2H_3$]-α-ionone was added to the sample. After brief agitation, sodium sulphate (3.1 g) was added, and the vial was capped with a crimp seal with a PTFE/silicone septum (Phenomenexm Torrence, CA, USA). The vial was placed into the heated autosampler tray (GERSTEL GmbH & Co.KG, Mülheim an der Ruhr, Germany) and was agitated at 40 °C for 15 min.

The samples were analysed by GC-MS using a 6980 HP GC coupled with a 5973N single quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). Analyses were carried out using 65 µm PDMS/DVB SPME fibres (Supelco, Bellefonte, PA, USA). Before use, each fibre was thermo-conditioned at 250 °C for 30 min according to the product instructions. The fibre was exposed in the sample headspace, and the extraction was performed using continuous stirring at 40 °C for 1 hour. The compounds were thermo-desorbed for 2 min into the GC injector (250 °C). The analyses were conducted in splitless mode, and the purge valve was opened after 3 minutes. The column was an Innowax 30 m, 0.25 mm, 0.25 µm (J&W Scientific, Folsom, CA, USA). Helium was used as carrier gas, and the column flow was set at 1 mL min^{-1}. The cleaning time after desorption was set to 10 min. The oven temperature was held at 45 °C for 2 min and raised to 80 °C at a rate of 30 °C min^{-1}, from 80 to 230 °C at a rate of 5 °C min^{-1}, and held at 230 °C for 17 min. The transfer line was set at 230 °C. The ionisation energy was set at 70 eV, and the quadrupole temperature and the ion source were set at 230 °C and at 250 °C, respectively. The quantitative determination of β-damascenone, α-ionone and β-ionone was performed in Single Ion Monitoring mode (SIM) using 100 µs of dwell time for all ions. The selected ions used for quantification were 190 and 194 m/z for β-damascenone and [$^2H_4$]-β-damascenone, respectively, 136 and 139 m/z for α-ionone and [$^2H_3$]-α-ionone, and 177 and 180 m/z for β-ionone and [$^2H_3$]-β-ionone. All these analysis were performed in duplicate for each wine.

Ultrapure water containing 4 % v/v ethanol with known amounts of added analytes was extracted as described. Six points with different concentrations were used to build the standard charts (analyses in triplicate for each point). The concentrations ranged from 20 ng L^{-1} to 10 µg L^{-1} for β-damascenone and from 15 ng L^{-1} to approximately 7 µg L^{-1} for β-ionone and α-ionone. Finally, the LOD and the LOQ of the method were calculated in accordance with the resolution of the Office International de la Vigne te du Vin regarding the estimation of the detection and quantification limits of a method of analysis (Resolution Oeno 7/2000).

Statistical analysis was performed with SPSS for Windows version 15.0 (SPSS Inc., Chicago, IL, USA) and XLSTAT version 2011.2.05 (Addinsoft New York, NY, USA).

3. Results and discussion

3.1 Selection of the SPME fibre

The results show that the SPME fibres behave differently depending on whether the extraction was performed in a hydro-alcoholic medium or in a Chardonnay wine. In a synthetic medium, the PDMS-DVB (blue hub) was the highest performing among the five SPME fibres tested. In wine, the extractive efficiency of the DVB/CAR/PDMS fibre was comparable to the PDMS-DVB with a fused silica core (**table 4**). As cited in the literature (Pawliszyn, 1999), the nature and thickness of the coating are the first factors to be assessed during the fibre choice step; moreover, the extraction efficiency of the fibre can also be strongly influenced by the physical structure of the fibre itself (Gonçalves & Alpendurada, 2002), as the different behaviour of the PDMS-DVB Stable Flex™ and the PDMS-DVB with fused silica core demonstrated. On the whole, considering the results obtained both with wine and with synthetic medium, the PDMS-DVB with fused silica core (blue hub) was the highest performing among the tested fibres.

3.2 Ionic strength

The effect of salting-out agents on the analytes extraction was evaluated. A hydro-alcoholic solution (10% v/v) containing the analytes was used as a control sample and compared to two solutions with added sodium chloride and sodium sulphate (310 g L^{-1}), respectively. The addition of salts greatly increased the volatility of norisoprenoids, and, at the chosen concentration, sodium sulphate proved to be more effective than sodium chloride, as regards in particular β-damascenone (**figure 1**).

3.3 Optimisation of the desorption step and of the cleaning conditions.

The time of permanence of the fibre in the injector was evaluated to optimise the desorption step of the analytes. Three different desorption times (1, 2 and 3 min) were compared, and the best response was obtained at two minutes: a small increase of the instrumental response was observed at 2 min of desorption time, but the difference, though statistically significant, was not high. Furthermore, a decrease of the response was observed at desorption times higher than 2 min, probably because of degradation phenomena inside the GC injector. To avoid carryover phenomena, the clean-up time in the needle-heater device of the autosampler was set to 10 minutes.

3.4 Extraction conditions

Among the tested factors, the ethanol content had a statistically significant influence on the adsorption of the molecules on the fibre and therefore on the amplitude of their chromatographic peak area (**table 5**). The decrease of the ethanol content from 12 % to 4 % v/v caused an average increase of the analytes peak area of 10 to 13 times the original area (**table 6**).

The increase of the peak areas due to the decrease of the ethanol content was greater than the decrease of the same areas due to the sample dilution; therefore, a net gain in terms of area (more than 3 times the original area) was obtained using a wine diluted from 12 % to 4 % v/v ethanol content (Table 5).

Statistically significant interactions between the factors "time" and "temperature" were observed for all of the studied molecules (**table 5**). **Figure 2** shows the response curves (peak areas) as a function of time and temperature for 4 % v/v ethanol content. As previously stated (**table 5**), the effect of the factors "time" and "temperature" was moderate compared to the factor “ethanol”. In the studied concentration range, when temperature varied between 40 °C and 60 °C and extraction time between 30 min and 90 min, the maximum variation of the peak areas was 44 %, 49 % and 42 % for α-ionone, β-ionone and β-damascenone, respectively. For α-ionone, an identical instrumental response can be obtained for values up to $3 \cdot 10^6$ (relative area) by shortening the extraction time and increasing the temperature and *vice versa*. For higher area values (over 53 °C), a decrease of the instrumental response was observed (**figure 2**). A similar behaviour was observed for β-ionone, but in this case, the threshold beyond which phenomena of desorption are observed was higher. Finally, regarding β-damascenone, a greater decrease of the peak area due to temperature was observed with the increase of contact time. Temperature most likely increases the desorption rate of the molecules from the fibre proportionally to the duration of contact.

Contact times longer than 60 min and temperatures between 40 °C and 44 °C led to better results in terms of instrumental response; consequently, wines were diluted three times to set the extraction time at 60 min (to increase the number of analysed samples) and to operate at the lowest possible temperature (40 °C).

3.5 Method validation

The main validation parameters studied are listed in **table 7**. The instrumental response was linear in the calibration range, with a coefficient of determination notably close to 1. The limits of detection and quantification for β-ionone and β-damascenone fall below the perception thresholds measured in wine. The quantification of α-ionone at very low concentrations was not possible in wine, due to the presence of a co-eluting interfering molecule.

3.6 Wine analysis

The described method was subsequently used for the quantification of β-ionone and β-damascenone in wines made from rare autochthonous varieties from north-western Italy. The measured concentrations of these molecules, as reported in **table 8**, are similar to those measured in other varieties during previous studies (Sefton, Skouroumounis, Elsey & Taylor, 2011). The average concentrations of β-ionone varied between 18 and 165 ng L^{-1} and between 443 and 4172 ng L^{-1} for β-damascenone. The Doux Of Henry, used for the production of the *Pinerolese* denomination, was the variety with the highest average concentrations of β-ionone (104 ng L^{-1}) and β-damascenone (3376 ng L^{-1}, Table 8), while the lowest average content of β-ionone was measured in the wines made from Petit Rouge (28 ng L^{-1}), typical of Val d'Aosta. The lowest concentrations of β-damascenone were measured in Pignolo Spano wines. A weak but significant positive correlation (Pearson correlation coefficient = 0.375) was observed between the concentrations of the two examined molecules.

Considering the average content of β-ionone as a function of wine age, a close relationship can be observed between these two parameters (R^2= 0.9818); this closeness corresponds to a decrease in β-ionone concentration of approximately 13 ng L^{-1} per year. Conversely, no significant differences were observed between the vintages in regard to β-damascenone.

4. Conclusions

This work concerned the optimisation of a SPME-GC-MS method for the quantification of megastigmane norisoprenoid compounds, which are detectable in concentrations on the order of tens or hundreds of ng L^{-1} and are interesting because of their aromatic characteristics. Particular attention was paid to the choice of fibre. Among those tested, PDMS-DVB with a fused silica core was the best fibre for this type of analysis. The ethanol content of the medium had a strong depressant effect on the extraction of these molecules, while less important factors included the temperature and the duration of contact. The study of the instrumental response to variations of these three factors allowed the best operating conditions to be defined.

This method was used to quantify β-damascenone and β-ionone in wines. For the first time, wines obtained from rare autochthonous grape varieties of north-western Italy were analysed. The concentrations ranged from 432 to 4128 ng L^{-1} for β-damascenone and from 17 to 162 ng L^{-1} for β-ionone; these values are higher than the perception thresholds measured in a synthetic medium.

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7.Figure and table captions

Figure 1: Effect on the norisoprenoids volatility of the addition of sodium sulphate or sodium chloride. Data are reported as percentages compared to the trial without added salts. All these analysis were performed in triplicate.

Figure 2: Contour plot of the estimated effects of temperature and time on the instrumental response (peak area) at 4 % v/v of ethanol.

Table 1: Origin and diffusion of the studied grapes and main physical-chemical characteristics of the related wines. [1] Sweet wine of semi-dried grapes.

Table 2: List of the SPME fibres used during this experiment. Coatings: polydimethylsiloxane (PDMS), divinylbenzene/polydimethylsiloxane (DVB/PDMS), Carboxen/polydimethylsiloxane (CAR/PDMS), and divinylbenzene/Carboxen/polydimethylsiloxane (DVB/CAR/PDMS)**. .**

Table 3: Experimental plan (3 factors, 3 levels) adopted in this work.

Table 4: 1: PDMS; 2: DVB/CAR/PDMS; 3: CAR/PDMS; 4: PDMS/DVB pink; 5: PDMS/DVB blue. Extractive efficiency of the fibres, reported as a percentage of the maximum observed efficiency. The data were processed by ANOVA. Different letters indicate means significantly different at $p < 0.05$ by Tukey's post-hoc test. All analysis were performed in triplicate.

Table 5: Extraction factors. Complete 3-factors ANOVA results. Main factors: time (t), temperature (T) and alcohol (A), and first-level interactions.

Table 6: Effect of the ethanol content (reported as % v/v) on the instrumental response reported as peak area. The data were processed by ANOVA. Different letters indicate means significantly different at $p < 0.05$ by Tukey's post-hoc test.

Table 7: Main validation parameters: a is the intercept; b is the slope of the calibration curve; RDS % is the relative standard deviation of the same measure repeated for 5 times.

Table 8: Norisoprenoids content in the experimental wines. All data are in µg L^{-1}. n = number of analyzed wines. All analyses were performed in duplicate.

Wine	Grape Cultivation Area	Vintage	Ethanol	anthocyanins	flavonoids	pH
			% v/v	mg L^{-1}	mg L^{-1}	
Bonarda	Chierese, and Astigiano. Sporadically near Pinerolo, lower Susa Valley and Canavese	2006	11.6	126	1433	3.66
		2007	12.2	140	1558	3.86
Brunetta di Rivoli	lower Susa Valley	2006	11.9	68	754	3.47
		2007	14.3	152	1238	3.75
		2008	12.5	121	1016	3.59
Bubbierasco	Bronda Valley (CN)	2006	12.2	59	763	3.64
		2007	13.5	95	713	3.66
		2008	13.0	102	667	3.73
Cellerina	Sporadically near Tortona, Ovada and Asti	2006	14.2	126	1568	3.41
		2008	14.1	145	1679	3.72
Chatus	Present throughout the Piedmont Alps	2005	11.5	98	1012	3.16
Doux d'Henry	Pinerolese	2008[1]	13.6	66	1789	3.60
		2008	10.9	58	692	3.29
		2006	10.9	30	794	3.28
		2007	11.6	61	927	3.41
Freisa	Astigiano and Monferrato Casalese, Chierese, Albese	2006	12.6	135	2248	3.53
		2007	14.1	130	1997	3.31
Fumin	Medium and upper Aosta Valley	2006	12.0	317	1752	3.83
		2007	11.8	304	1261	3.89
Gamba di Pernice	Present near Calosso (At)	2006	12.4	121	1280	3.58
		2007	13.8	190	1338	3.73
		2008	12.2	99	861	3.59
Montanera	Alpine areas (Chisone Valley, Biellese, Ossola Valley, Valtellina)	2006	14.5	204	1209	3.61
		2007	14.6	256	1389	3.68
		2008	13.8	260	1312	3.66
Nebbiolo Scarlatin	Western Roero and Monregalese	2006	12.5	272	2074	3.40
		2007	12.8	315	1978	3.55
		2008	11.7	312	1639	3.46
Neretto duro	Present throughout the Piedmont	2008	12.4	190	853	3.73
Petit Rouge	Aosta Valley	2006	12.4	148	940	3.83
		2007	12.7	132	776	3.88
		2008	13.1	140	755	3.95
Pignolo Spano	Near Biella, Vercelli and Novara, fairly widespread in the Sondrio area	2006	13.2	88	2887	3.79
		2007	14.1	89	1230	3.84
		2008	11.8	74	2243	3.87
Rastajola	Sporadically near Novara	2006	12.0	72	1225	3.56
		2007	14.7	101	1223	3.70
		2008	13.9	78	1238	3.70
Rossese Dolceacqua	Imperia province	2008	12.9	62	448	3.83
Vermentino nero	Lunigiana	2008	11.6	67	416	3.41

Table 1

Fiber coating	Fiber length cm	Film thickness µm	Operating Temperature (°C)	silica core
PDMS	1	100	200-280	Fused silica
PDMS/DVB (pink hub)	1	65	200-270	Flexible
PDMS/DVB (blue hub)	1	65	200-270	Fused silica
Carboxen/PDMS	1	75	250-310	Flexible
DVB/CAR/PDMS	1	50/30	230-270	Flexible

Table 2

Experiment	T[a]	t[b]	A[c]
1	-1	-1	-1
2	1	-1	-1
3	-1	1	-1
4	1	1	-1
5	-1	-1	1
6	1	-1	1
7	-1	1	1
8	1	1	1
9	-1	0	0
10	1	0	0
11	0	-1	0
12	0	1	0
13	0	0	-1
14	0	0	1
15	0	0	0
16	0	0	0
17	0	0	0

Factors	n. of levels	-1	0	1
[a]Temperature (°C)	3	40	50	60
[b]Extraction time (s)	3	30	60	90
[c]Ethanol (% v/v)	3	4	8	12

Table 3

Hydro alcoholic solution	1	2	3	4	5	F	sig.
β-damascenone	44 c	81 b	60 c	79 b	100 a	35.5	0,000
α-ionone	49 c	80 b	56 c	78 b	100 a	33.2	0,000
β-ionone	40 b	93 a	86 a	90 a	100 a	21.6	0,000
Chardonnay wine	**1**	**2**	**3**	**4**	**5**	**F**	**sig.**
β-damascenone	63 c	100 a	95 ab	81 b	99 a	24.5	0,000
α-ionone	68 c	100 a	93 ab	81 bc	90 ab	11.4	0.001
β-ionone	78 b	99 a	79 b	89 ab	100 a	6.6	0.007

Table 4

	Main factors						Interactions					
	Temperature (T)		**Time (t)**		**Alcohol (A)**		**T x t**		**T x A**		**t x A**	
	F	**sig.**	**F**	**sig.**	**F**	**sig.**	**F**	**sig.**	**F**	**sig.**	**F**	**sig.**
β-damascenone	4.0	0.20	5.8	0.147	385	0.003	67.7	0.014	0.4	0.583	13.7	0.066
α-ionone	0.4	0.715	4.8	0.172	230	0.004	37.2	0.260	0.6	0.529	8	0.106
β-ionone	1.5	0.406	13.3	0.070	416	0.002	57.4	0.017	6.7	0.123	19.4	0.048

Table 5

	4 % v/v		8 % v/v		12 % v/v		F	Sig.
α-ionone	3.0E+06	a	1.1E+06	b	2.8E+05	c	230	0.004
β-ionone	6.4E+06	a	2.1E+06	b	4.7E+05	c	416	0.002
β-damascenone	5.2E+06	a	1.6E+06	b	4.3E+05	c	385	0.003

Table 6

	Calibration range µg L^{-1}	b	a	R^2	RSD %	LOD ng L^{-1}	LOQ ng L^{-1}
β-ionone	0-7.39	1.4819	0.0492	0.999	5.05	9	12
β-damascenone	0-10.58	0.9747	0.2495	0.998	4.29	28	29

Table 7

		β-ionone (ng L^{-1})			**β-damascenone (ng L^{-1})**		
	n	Min	Max	Mean	Min	Max	Mean
Bonarda	2	19	109	80	567	1986	1311
Brunetta di Rivoli	3	44	67	54	2475	3600	2913
Bubbierasco	3	21	106	76	443	4011	2197
Cellerina	2	52	102	75	1608	2406	1887
Chatus	1	35	36	36	1692	1748	1720
Doux D'Henry	3	79	115	104	2226	4173	3376
Doux d'Henry passito	1	159	165	162	1964	2059	2011
Freisa	2	37	54	45	1854	2086	1982
Fumin	2	18	53	33	1532	3352	2267
Gamba di Pernice	3	42	140	62	1682	3149	2303
Montanera	3	29	62	49	776	1914	1158
Nebbiolo Scarlatin	3	24	57	38	855	1415	1058
Neretto duro	1	85	92	88	2535	2589	2562
Petit Rouge	3	19	36	28	699	3043	1843
Pignolo Spano	3	43	101	72	706	1403	941
Rastajola	3	34	92	67	876	2033	1374
Rossese Dolceacqua	1	50	50	50	872	873	873
Vermentino nero	1	53	73	63	1271	1720	1496

Table 8

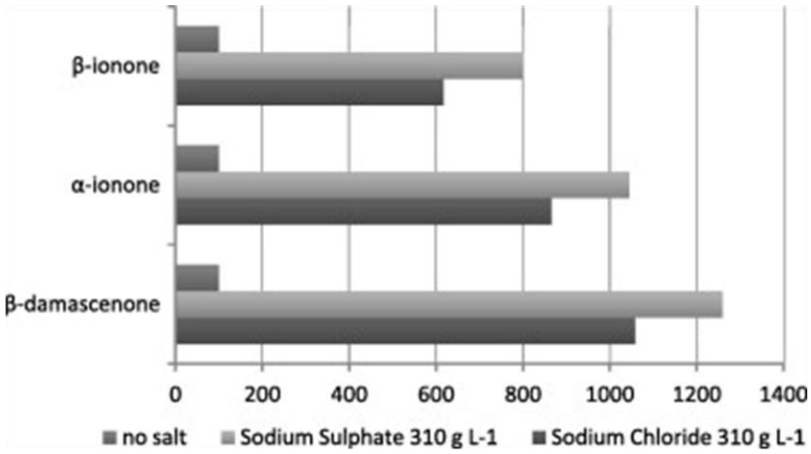

Fig. 1. Effect on the norisoprenoids volatility of the addition of sodium sulphate or sodium chloride. Data are reported as percentages compared to the trial without added salts. All these analyses were performed in triplicate.

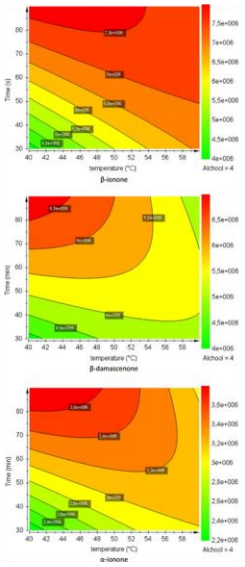

Fig. 2. Contour plot of the estimated effects of temperature and time on the instrumental response (peak area) at 4% v/v of ethanol.