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Quantitative determination of free and hydrolytically liberated β-damascenone in red grapes and wines using a stable isotope dilution assay

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Abstract

Quantitative analysis of free and hydrolytically liberated β-damascenone in grapes and wines was developed, using a stable isotope dilution assay. Free β-damascenone was isolated from grapes and wines by diethyl ether–hexane (1:1, v/v) extraction and the precursor(s) (glycosidic, polyols) of β-damascenone using Sep-Pak Plus C_{18} RP cartridges. Hydrolytically liberated β-damascenone was generated by acid hydrolysis from the precursor(s) extract. Red wines from Bordeaux (Merlot, Cabernet Sauvignon and Cabernet Franc, 1995 and 1996 vintage), Burgundy (Pinot Noir, 1995 and 1996 vintage) regions and Grenache wines from Chateauneuf du Pape and Côtes du Rhône (1995 vintage) were analysed to quantify free β-damascenone. The wines made from Grenache and Cabernet Sauvignon (1996 vintage) grapes presented the highest mean amounts of free β-damascenone, 5.4 and 5.5 $\mu g\ l^{-1}$, respectively. Merlot, Cabernet Sauvignon and Cabernet Franc grapes of Bordeaux (1996 vintage) and their corresponding wines were analysed for quantification of free and hydrolytically liberated β-damascenone. The levels of hydrolytically liberated β-damascenone in grapes could predict closely the levels of free β-damascenone in the corresponding wines after one year of ageing, i.e., almost half the levels found for the grape samples. The influence of enzyme and heat treatment of Merlot wine samples on their β-damascenone levels was studied. Heat treatment doubled the levels of this compound, but enzyme treatment generated, in the corresponding wines, half the levels of β-damascenone found in the non-enzyme treated wines.

Keywords: Grapes; Fruits; Wine; Food analysis; Damascenone

1. Introduction

The highly odorant (8*E*)-megastigma-3,5,8-trien-7-one (β-damascenone) was first identified in Bulgarian rose essential oil [1] and since has been detected in a variety of foods and stimulants: black tea, honey, coffee, beer, raspberry, apple products, grape, mandarin juice, purple passion fruit, elderberry, starfruit and tomatoes [2]. In wine, β-damascenone was first identified by Schreier and Drawert, in 1974 [3] and since then, numerous authors have reported its presence in wines of different cultivars

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[4–7] and in other alcoholic beverages (whiskey, brandy and rhum) [8].

The odour of this compound has been described as honey-like [9], flowery and ionone like [10], as well as canned apple or quince-like by gas chromatography (GC)–sniffing procedures. Buttery et al. [11] reported its odour threshold in water of 2 ng l^{-1}. Recently a higher odour threshold of 50 ng l^{-1} in 10% alcohol was reported [4], although a lot lower than 1.6 mg l^{-1} reported in white sweet wine [5].

Winterhalter et al. [12] have shown that β-damascenone was generated by acid hydrolysis from multiple precursors, in Riesling wine, after separating the precursors by DCCC (droplet counter-current chromatography). This has supported the earlier findings [13] in Concord grape skins. The precursors appear to include glycoconjugates involving different conjugating moieties and also non-glycosidic compounds (polyols). Two such polyols have been identified, in wine, the norisoprenoid enynediol [14,15] and the allenic triol [16]. Similarly, glycoconjugates of these two important polyols have been completely identified. The C3 and C9 glucopyranoside of enynediol in Riesling wine [17], the C9 glucopyranoside of enynediol in rose petals [18] and the allenic C9 glucopyranoside in *Lycium halimifolium* Mil [19]. Also other papers outlined the synthesis of precursor(s) of β-damascenone and the pathways of hydrolytic liberation of β-damascenone [20–22].

Quantification of free β-damascenone using a stable isotope dilution assay has been achieved in coffee, black tea, honey, beer and in wines from two german varieties [4,23], but the isotopically labelled internal standard used, required a multi-step synthesis. We reported recently the one-step synthesis of [$^2H_4$]β-damascenone, by equilibrating the natural analogue in a two-phase solvent system in deuterium oxide–tetrahydrofuran under basic conditions at 60°C. The four deuterium atoms were incorporated in the side chain of the megastigmane skeleton [24].

The aim of the following investigation was to develop a stable isotope dilution assay, to determine quantitatively the free and hydrolytically liberated β-damascenone in grapes and wines of different varieties, using a deuterium-labelled compound as internal standard [24]. To also develop a convenient method for the ease of isolation of the free and precursor(s) compounds of β-damascenone.

2. Experimental

2.1. Materials

Twenty-eight Merlot, Cabernet Sauvignon, Cabernet Franc, Grenache and Pinot Noir wines were analysed to determine their levels in free β-damascenone. The wines of the first three varieties were from Bordeaux region (Margaux, Pauillac, Pomerol, Fronsac, Graves and St. Emilon), the wines of Grenache were from Chateauneuf du Pape and other Côtes du Rhône regions and the wines of Pinot Noir were from Burgundy. All these samples came from wineries of the corresponding regions and were sampled after the malolactic fermentation was achieved.

The quantitative analysis of hydrolytically liberated β-damascenone was performed on Merlot, Cabernet Sauvignon, Cabernet Franc grapes and wines from Bordeaux region. The grape samples were harvested at technological maturity from mid September to the beginning of October 1996, from Margaux, Pauillac, Fronsac and St. Emilon; the grapes were stored at −20°C prior to analysis. Their corresponding wines were produced by microvinification procedure in 50-l stainless steel tanks [25] and were sampled after the malolactic fermentation was achieved, then drained and a mean quantity of 40 mg l^{-1} SO_2 was added at bottling and stored at 10°C prior to analysis. The wines were one to two years old at the time of analysis.

2.1.1. Chemicals and glassware

β-Damascenone was a gift from Firmenich, Geneva, Switzerland, and it was 77% pure by GC. The compound was further purified using silica gel 60 column chromatography (Aldrich 230–400 mesh, gradient of pentane–diethyl ether used as eluent) and by microdistillation with Buchi TO-51 drying oven. Detailed procedure for synthesis of [$^2H_4$]β-damascenone, and its purification was reported by Kotseridis et al. [24]. The organic solvents, diethyl ether, pentane, dichloromethane and hexane, were ultrapure grade, obtained from sodium dodecyl sulfate

(SDS), 13124, Peypin, France. Sep-Pak Plus C_{18} RP solid-phase extraction cartridges containing 360 mg of adsorbent (Millipore, Milford, MA, USA) were used with a 10-ml reservoir and a 12-port model vacuum manifold of solid-phase extraction, Visiprep, Supelco (Sigma–Aldrich, St. Quentin Fallavier, France). Amberlite XAD-2 (20–50 mesh) was purchased from Fluka (Buchs, Switzerland).

2.2. Isolation of free β-damascenone

For wine samples: a 250-ml volume of a wine sample was placed in a flask, then spiked with 500 μl of a [$^2H_4$]β-damascenone solution (0.58 μg ml^{-1} in ethanol) using a calibrated microliter syringe (SGE, 500 μl), closed and stirred for 10 min for equilibration of the media. This was divided into two portions of 100 ml, placed in two 200-ml flasks and each of them was extracted with 3×5 ml of diethyl ether–hexane (1:1, v/v) for 5 min on a magnetic stirrer (1100 rpm). The organic phases were separated with a separatory funnel, dried over Na_2SO_4, then filtered through glasswool and concentrated under a nitrogen stream (N_2, 5.0 quality) down to 100 μl. The final concentration factor was 1000.

For grape samples: Berries (about 1 kg each) were allowed to reach 4°C overnight, were destemmed, crushed in a fruit-juicer for 2 min, then centrifuged (7000 rpm, 15 min), while keeping the temperature at 4°C. Prior to analysis the juices were filtered through glasswool and the pH of the sample was adjusted to 8.8 by adding 10 *M* sodium hydroxide solution. A 250-ml volume of the sample was spiked with 500 μl of [$^2H_4$]β-damascenone solution (0.58 μg ml^{-1} in ethanol) and thereafter analysed as described above for the wines.

Each juice and wine sample was analysed in duplicate.

2.2.1. Isolation of β-damascenone precursors

The method used for the isolation of β-damascenone was related to methods used previously [26,27]. After the isolation of the volatiles by liquid–liquid extraction, any trace of solvent was eliminated from the samples of grape juices and wines under vacuum (ca. 35°C, 70 mmHg; 1 mmHg=133.322 Pa). The Sep-Pak Plus C_{18} RP cartridges were conditioned with 10 ml methanol, followed by 20 ml of Millipore Milli-Q water. Afterwards 10-ml aliquots of the samples of grape juices or wines were loaded onto the cartridges at a flow-rate of ca. 3 ml min^{-1} and the cartridges were washed with 3×10 ml Millipore water (at a flow-rate of ca. 3 ml min^{-1}). The precursors of β-damascenone were eluted from the cartridges with 2 ml of ethanol at a flow-rate of ca. 2 ml min^{-1}.

2.2.2. β-damascenone precursors acid hydrolysis

The ethanol extract was evaporated to dryness under vacuum (ca. 45°C, 70 mmHg). The residue was taken up in 4 ml of a citric acid buffer (pH 2.2), sealed under nitrogen atmosphere in glass ampoules and hydrolyzed at 100±2°C for 60 min to generate hydrolytically liberated β-damascenone. After cooling in an ice bath, the samples were spiked with 500 μl of [$^2H_4$]β-damascenone (0.58 μg ml^{-1} in ethanol), stirred for 5 min using a Vortex, then extracted by stirring with 2×1 ml of diethyl ether–hexane (1:1, v/v). The organic phases were blended, dried over Na_2SO_4 and concentrated under a nitrogen stream down to 100 μl.

Each juice and wine sample was analysed in duplicate.

2.3. Gas chromatography–mass spectrometry (GC–MS) conditions

GC–MS analysis was carried out using a Hewlett-Packard HP gas chromatograph 5890 series II fitted with a 50 m×0.25 mm I.D. fused-silica column, 0.2 μm film thickness, coated with Carbowax 20M. The splitless/split injection port was heated at 200°C. Injection (2 μl) of the extract was done using an automatic sampler; the split vent was opened after 30 s. The carrier gas was Helium 55 Norme Aga, and the pressure was 170 kPa with a linear velocity 40 cm s^{-1} at 40°C. The temperature program was 60°C (for 1 min), then increased at 4°C min^{-1} to 220°C and held at this temperature for a further 20 min. The GC instrument was coupled to a 5970 B mass-selective detector and a 5990 A MS chemstation (HP-UX). The temperature of the ion source [running in electron impact (EI) mode at 70 eV], of the quadrupole and of the interface was set at 250°C. The ions *m/z* 69, 175, 190 were monitored in the selected ion monitoring (SIM) mode for the de-

termination of β-damascenone and the ions *m/z* 73, 179, 193, 194 for the determination of [$^2H_4$]β-damascenone. The ions *m/z* 69, 73 were used for quantification and the ions *m/z* 175, 179, 190, 193, 194 as qualifiers.

2.3.1. Standard curves

Six solutions containing defined amounts of synthetic β-damascenone (unlabelled) and of the labelled compound in diethyl ether–hexane, in different ratios, were injected into the GC–MS system. Peak area ratios (peak area of the ion *m/z* 69/peak area of the ion *m/z* 73) were plotted against the concentration ratios (β-damascenone/0.29 μg of [$^2H_4$]β-damascenone) for the following β-damascenone concentrations: 0.06, 0.12, 0.24, 0.48, 0.96 and 1.92 μg (three replicate analyses at each concentration). The standard curve was obtained by linear regression analysis.

2.3.2. Reproducibility study

Five analyses of 100 ml of the same Merlot grape sample spiked with 2 ng of β-damascenone [by adding 2 μl of a β-damascenone solution (1 μg ml^{-1} in ethanol)] and 100 ml of a Merlot wine (without any addition of β-damascenone) were carried out to study the reproducibility of the method described above.

2.3.3. Detection limit

Volumes of 100 ml of a Merlot grape juice (1996 vintage) and 100 ml of a Merlot wine (1995 vintage), were spiked with 1.1, 1.7 and 3.5 ng, respectively of [$^2H_4$]β-damascenone {by adding 2, 3 and 6 μl of a solution of [$^2H_4$]-β-damascenone in ethanol (0.58 μg ml^{-1}), respectively}, and were submitted to the isolation procedure described above. The limit of detection was taken to be the lowest amount giving a signal-to-noise ratio of three. For an injection of 2 μl of the wine or the grape juice extract, the detection limit was chosen as the average of five determinations of the least measurable peak area added with three-times the standard deviation of this measurement [28].

2.4. Recovery of added synthetic β-damascenone from grapes

Volumes of 100 ml of a Merlot juice and 100 ml of an odour-stripped Merlot wine (by continuous extraction using dichloromethane, to eliminate any trace of β-damascenone) were spiked with 0.5 μg of synthetic β-damascenone [by adding 500 μl of a β-damascenone solution (1 μg ml^{-1} in ethanol)] and then extracted with diethyl ether–hexane (1:1, v/v) as described above. After the extraction the organic phases were spiked with 500 μl of [$^2H_4$]β-damascenone (0.58 μg ml^{-1} in ethanol), concentrated and injected into the GC–MS system, under the conditions described above. The result found in the wine or juice extract, was compared with the result found in a solution containing the same quantities of the labelled internal standard and of the synthetic compound; furthermore, after the third extraction, the aqueous phase was extracted once more by 5 ml of the solvent, totalling four extractions, concentrated and injected into the GC–MS system. The experiment was duplicated.

2.5. Recovery of β-damascenone precursors

The efficiency of the Sep-Pak Plus C_{18} RP cartridges (360 mg of adsorbent) used in this assay to extract β-damascenone precursors from 10 ml juice or 10 ml wines was assessed by acid hydrolysis of the firstly eluted fraction through the C_{18} RP cartridges, extraction and GC–MS analysis, as described above, and the hydrolytically liberated β-damascenone levels were compared to those generated by the ethanol eluate.

2.5.1. Time of hydrolysis

The β-damascenone precursors from 10 ml of juices from Merlot grapes and from Cabernet Sauvignon grapes were extracted as described above. The ethanolic eluates of each sample were divided into three aliquots of 0.5 ml and submitted to acid hydrolysis for 30, 60 and 90 min, respectively.

2.5.2. Use of regenerated C_{18} RP cartridges

Each used cartridge was regenerated by 20 ml methanol, then by 40 ml of Milli-Q water to eliminate any methanol trace. Their efficiency to extract β-damascenone precursors was assessed in duplicate using the procedure described in Sections 2.2.1 and 2.2.2.

2.6. Influence of heat and enzyme treatment on β-damascenone levels

Five litres of centrifuged juice from Merlot Pomerol grapes (1997 vintage), produced by crushing in a fruit juicer, were passed through a column of XAD-2 resin, as described by Günata et al. [29]. The methanol fraction, was evaporated to dryness under vacuum (ca. 45°C, 70 mmHg). The residue was taken up in 2 l of the same Merlot juice, then divided into two portions of 1 l. Three microvinifications were carried out, one on 1 l of the Merlot juice without addition of precursors (control sample, CWA), and two on the 1-l samples containing the precursors (PET and SP). After alcoholic fermentation, the three samples were added with 20 mg l^{-1} SO_2 and the PET sample was treated with 35 mg l^{-1} of commercial enzymes (AR 2000 R6374, Gist-Brocades) for five days at 28°C. Afterwards the three samples were divided into two portions of 500 ml, and one 500 ml portion for each sample was submitted to acid hydrolysis, by sealing them under a nitrogen atmosphere in glass ampoules which were heated at 45 °C for three weeks. Three new samples were obtained, CWAHT, PETHT and SPHT. The six samples were added with mean quantity of 40 mg l^{-1} SO_2, bottled and stored under nitrogen atmosphere at 5°C, prior to analysis. The wines were ca. one year old at the time of analysis.

3. Results and discussion

Quantification was achieved by monitoring the fragment ion m/z 69 for the natural analogue and the corresponding fragment ion m/z 73 of [$^2H_4$]β-damascenone (Fig. 1). These ions were the most abundant fragment ions in both of the compounds, which facilitate quantification of trace compounds. At length, monitoring of two fragment ions of β-damascenone m/z 175, 190 (and m/z 179, 193 and 194, respectively of [$^2H_4$]β-damascenone) allowed one to control the chromatographic purity of the peak of natural β-damascenone in the analysed samples, by comparing their relative abundances.

3.1. Extraction of free β-damascenone

The abundance ratio in the reference solution (8.2) compared with the abundance ratio in the diethyl ether–hexane juice and wine extract (7.7 and 7.6, respectively) demonstrated the efficiency of the extraction (almost 93% yield for the two samples). In addition the emulsion obtained with this solvent was moderate and in the fourth extraction β-damascenone was undetectable by GC–MS.

3.1.1. Validation of the method

The square of the correlation coefficient of the regression line, obtained from the calibration data

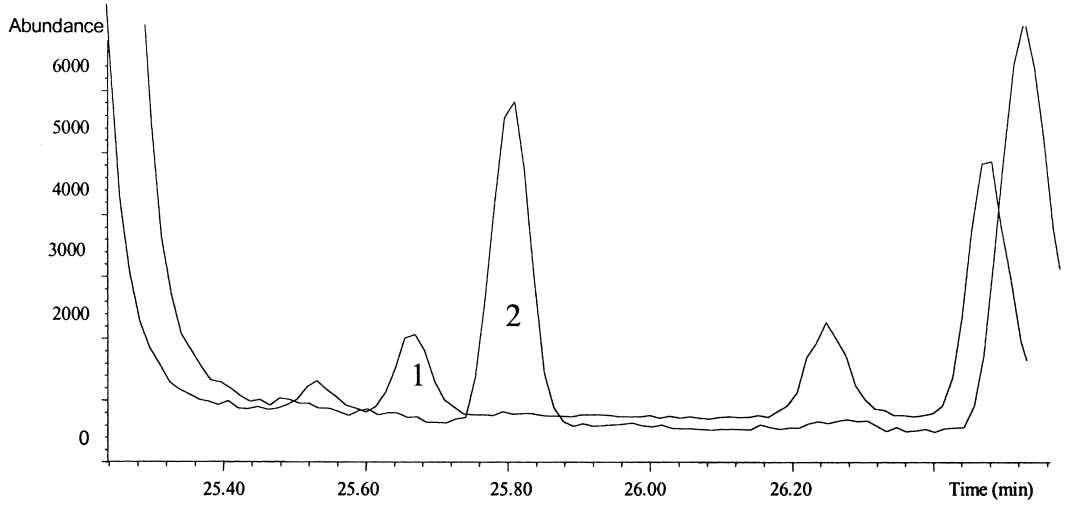

Fig. 1. Mass chromatogram of the diethyl ether–hexane (1:1, v/v) extract of a Grenache wine, from Chateauneuf du Pape, vintage 1995, containing 7.9 μg l^{-1} of β-damascenone. Peaks: 1=ion m/z 73.00 ([$^2H_4$]β-damascenone); 2=ion m/z 69.00 (β-damascenone).

was 0.999. The reproducibility was satisfactory as a relative standard deviation (RSD) of 3.4% was obtained for the grape and wine sample. The detection limit was 17 ng l^{-1} with an estimated signal-to-noise ratio of 3:1, for the Merlot juice and wine used in this study.

3.1.2. Extraction of β-damascenone precursors

The efficiency of the Sep-Pak Plus C_{18} RP cartridges to extract the precursors of β-damascenone of 10 ml juice or wine was assessed. Acid hydrolysis of the firstly eluted fraction of the juice sample generated 30 ng l^{-1} of hydrolytically liberated β-damascenone, while acid hydrolysis of the ethanol fraction liberated 12.5 μg l^{-1}. Acid hydrolysis of the firstly eluted fraction of the wine sample liberated 21 ng l^{-1} of β-damascenone, while acid hydrolysis of the ethanol fraction liberated 6.5 μg l^{-1}. These results showed the efficiency of the Sep-Pak Plus C_{18} RP cartridges to extract the precursors of β-damascenone from 10 ml of juice or wine.

In previous studies the volume of methanol to elute the precursors of β-damascenone from the C_{18} RP columns was greater, up to 300 ml [13,26], as also the quantity of C_{18} RP absorbent was greater. As it was shown recently [27], 1.5 ml of ethanol was sufficient to elute most of the glycosides of juice and wine from the Sep-Pak Plus C_{18} RP cartridges; thus 2 ml of ethanol was used to elute the β-damascenone precursors which generated after acid hydrolysis 12.5 μg l^{-1} of β-damascenone. A second elution of the cartridge with 1 ml more ethanol afforded after acid hydrolysis only 50 ng l^{-1} of β-damascenone which was negligible compared to the first elution with 2 ml of ethanol.

The kinetics of β-damascenone formation from the acid hydrolysis of its precursor(s) compounds was studied in grape samples of two varieties, Merlot and Cabernet Sauvignon. As shown in Fig. 2, acid hydrolysis of the precursors of β-damascenone from the Merlot sample was complete only after 60 min. On the contrary, the major part of β-damascenone formation was seen generated after 30 min from its precursor(s) compounds in the Cabernet Sauvignon sample. It has been recently shown that the glucoside of enynediol (one of the identified precursors of β-damascenone in grape) was hydrolysed 8–10 times more slowly than the corresponding aglycone [30] at

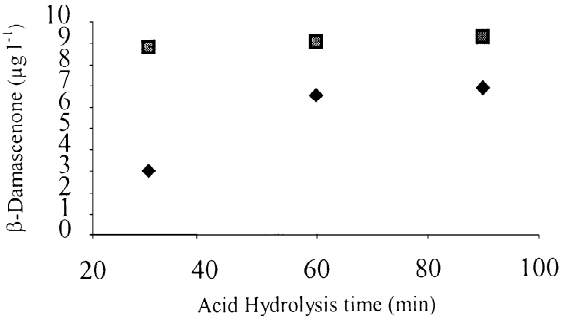

Fig. 2. Influence of the acid hydrolysis time on the levels of β-damascenone (μg l^{-1}) liberated from the precursor(s) extracts of (♦) Merlot and (■) Cabernet Sauvignon grapes.

50°C. The existence of different structures of β-damascenone precursors (be it aglycon or glycoside of different conjugating moiety) in the grape of these two varieties could explain the difference found on the kinetics of the acid hydrolysis. Thus, in the following study all the samples were submitted to acid hydrolysis during 60 min.

The use of the same Sep-Pak Plus C_{18} RP cartridge for more than one extraction was tested. As observed the cartridges could be used twice with a slight modification of the results of 10% (mean levels of 6 μg l^{-1} of hydrolytically liberated β-damascenone for the first time, and of 5.5 μg l^{-1} for the second time), but a third use of the cartridge allowed an extract of hydrolytically liberated β-damascenone which was clearly lower (4.5 μg l^{-1}).

3.2. Analysis of grape samples and their corresponding wines

As reported previously [31], free β-damascenone was not detected in grapes of *Vitis vinifera*, but multiple precursors able to generate β-damascenone were present. All the grape samples analysed during this assay contained levels of free β-damascenone (Table 1) around 30 ng l^{-1}, except the Cabernet Franc St. Emilion 2 grape sample with 84 ng l^{-1}, which were far lower than the levels of hydrolytically liberated β-damascenone (7–16 μg l^{-1}, i.e., 9.4 μg l^{-1} for the Cabernet Franc St. Emilion 2 grape sample). However all the levels of free β-damas-

Table 1
Levels of free (ng l^{-1}) and hydrolytically liberated β-damascenone (μg l^{-1}) in grape samples from Bordeaux region

	Grape samples					
	Free β-damascenone (ng l^{-1})			Hydrolytically liberated β-damascenone (μg l^{-1})		
	Sample 1	Sample 2	Mean	Sample 1	Sample 2	Mean
Merlot Fronsac	28.9	28.6	28.7	7.4	8.3	7.8
Merlot Margaux	25.9	22.7	24.3	7.9	7.0	7.4
C.S.[a] Pauillac	34.2	32.8	33.5	18.5	17.1	18.1
C.S.[a] Margaux	37.6	35.2	36.4	17.5	15.7	16.6
C.f.[b] St. Emilion 1	23.3	19.5	21.4	12.5	11.7	12.1
C.f.[b] St. Emilion 2	86.4	81.0	83.7	9.2	9.5	9.4

[a] C.S.=Cabernet Sauvignon.
[b] C.f.=Cabernet Franc.

cenone were equal to or higher than the olfactive detection threshold of this compound [4,11].

As shown in Tables 1 and 2, the Cabernet Sauvignon and Cabernet Franc samples contained higher levels of precursor(s) of β-damascenone than the Merlot samples. The levels and the structures of β-damascenone precursors could be characteristic of the cultivar [31]. The results found that one year old wines had almost half the amount of hydrolytically liberated β-damascenone precursor(s) to those of the grapes, indicative that precursor(s) compounds were hydrolytically transformed to β-damascenone in the wines (Tables 1 and 2). Thus this assay could predict approximatively the levels of free and hydrolytically liberated β-damascenone in the wines. Furthermore the free β-damascenone levels were higher in Cabernet Sauvignon and Cabernet Franc than those of Merlot wines (Table 2).

3.3. Analysis of other wine samples from different regions and vintages

The wines of five cultivars of the 1995 vintage were analysed to determine their free β-damascenone levels (Table 3). The levels were different between the cultivars but also between the wines of the same cultivar. Probably the differences between vineyards, climates, could have an impact on the levels of precursor(s) β-damascenone in grapes and consequently free β-damascenone levels in the wines. The Grenache wines from Chateauneuf du Pape presented the highest levels in free β-damascenone, followed by the Cabernet Sauvignon wines. The levels found were higher than the levels reported for two white wines [4], which could be explained by the different winemaking processes and particularly by the maceration with skins for 15–20 days in the case of the red

Table 2
Levels of free and hydrolytically liberated β-damascenone (μg l^{-1}), in wines corresponding to the grape samples of Table 1

	Wine samples					
	Free β-damascenone (μg l^{-1})			Hydrolytically liberated β-damascenone (μg l^{-1})		
	Sample 1	Sample 2	Mean	Sample 1	Sample 2	Mean
Merlot Fronsac	3.5	3.5	3.3	5.1	5.0	5.0
Merlot Margaux	4.3	4.8	4.5	4.8	4.1	4.4
C.S.[a] Pauillac	6.1	6.1	6.1	4.7	4.9	4.8
C.S.[a] Margaux	6.7	6.9	6.8	6.1	6.6	6.3
C.f.[b] St. Emilion 1	5.0	5.7	5.4	5.6	6.3	5.9
C.f.[b] St. Emilion 2	6.1	6.6	6.3	7.5	7.2	7.3

[a] C.S.=Cabernet Sauvignon.
[b] C.f.=Cabernet Franc.

Table 3
Levels of free β-damascenone (μg l^{-1}) in wines of different cultivars of the 1995 vintage

Cultivar	Region	β-Damascenone (μg l^{-1})		
		Sample 1	Sample 2	Mean value
Cabernet Franc	St. Emilion	1.70	1.70	1.70
Cabernet France	St. Emilion	2.80	2.80	2.80
Cabernet Franc	Pomerol	4.60	4.50	4.51
Mean of Cabernet Franc				3.00
Cabernet Sauvignon	St. Julien	4.80	4.56	4.68
Cabernet Sauvignon	Pauillac	3.33	3.27	3.30
Mean of Cabernet Sauvignon				4.00
Merlot	Pauillac	3.07	2.80	2.94
Merlot	Pomerol	4.06	3.89	3.97
Merlot	Pomerol	2.60	2.69	2.64
Merlot	Graves	2.88	2.96	2.92
Merlot	St. Emilion	2.57	2.62	2.59
Merlot	Moulis	3.57	3.22	3.40
Mean of Merlot				3.10
Grenache	Chateauneuf du Pape	7.04	7.01	7.02
Grenache	Chateauneuf du Pape	7.83	7.99	7.91
Grenache	Chateauneuf du Pape	5.64	5.94	5.79
Grenache	Côtes du Rhône	3.20	3.45	3.33
Grenache	Côtes du Rhône	2.76	2.98	2.87
Mean of Grenache				5.38
Pinot Noir	Pommard	2.63	2.89	2.76

wines analysed during this study. Indeed, it was shown that high levels of precursors of β-damascenone in Cabernet Sauvignon grapes were located in the skins [32]. In regards to the 1996 vintage, Merlot and Cabernet Sauvignon wines, as well as a sample of a Pinot Noir wine were analysed (Table 4). The difference of the levels of free β-damascenone between Merlot and Cabernet Sauvignon wines was higher than that observed for the same varieties of the 1995 vintage. Indeed the 1996 Cabernet Sauvignon wines showed almost twice the levels found in the 1996 Merlot wines, close to the levels found for the 1995 Grenache wines (Chateauneuf du Pape). The conditions of the 1996 vintage were very favourable to Cabernet Sauvignon grapes which reached an optimal grape maturity.

3.4. Analysis of heat- and enzyme-treated wines

The first observation is the recorded high levels of free β-damascenone found in the control sample CWA (Table 5) in comparison with the wines analysed previously (Tables 2, 3 and 4). This observation is accounted by the fact that the juice for the wines analysed in this experiment, was produced by maceration of the grapes in a fruit juicer (allowing for a better extraction of β-damascenone precursors) and therefore, higher levels of β-damascenone in the wines after fermentation. In normal conditions, as for the wines of Tables 2, 3 and 4, the juices were produced by more gentle procedures (traditional winemaking, production of juice by grape pressing at low values of pression).

Addition of 2.5-times the amount of glycosidic precursors in the Merlot juice produced 2.4-fold more free β-damascenone in the treated samples (PET and SP samples), i.e., less than what would be expected (3.5-fold). This difference showed that the XAD-2 extraction of β-damascenone precursors from grape juice was not total.

Table 4
Levels of free β-damascenone (μg l^{-1}) in wines of different cultivars of the 1996 vintage

Cultivar	Region	β-Damascenone (μg l^{-1})		
		Sample 1	Sample 2	Mean value
Merlot	Graves	1.81	1.81	1.81
Merlot	Moulis	3.31	3.41	3.35
Merlot	St. Emilion	3.00	3.00	3.00
Merlot	Pomerol	2.67	2.78	2.72
Merlot	Pomerol	2.40	2.39	2.40
Merlot	St. Emilion	2.45	2.42	2.44
Merlot	Pomerol	3.67	3.47	3.57
Mean of Merlot				2.75
Cabernet Sauvignon	Graves	7.39	7.25	7.32
Cabernet Sauvignon	Moulis	4.87	5.12	5.00
Cabernet Sauvignon	Pauillac	4.33	4.29	4.31
Mean of Cabernet Sauvignon				5.54
Pinot Noir		4.12	4.49	4.31

Enzyme treatment, as expected, did not produce more free β-damascenone (PET β-damascenone levels towards the levels found in the SP sample, Table 5). Indeed, Sefton et al. [33] showed that β-damascenone was formed by acid-catalyzed hydrolysis. Enzyme-catalyzed hydrolysis of grape glycoside fractions, produces only the aglycones (polyols) and β-damascenone can be formed only from further acid-catalysed hydrolyses of these aglycons (polyols).

Heat treatment of the control sample (CWA and CWAHT after heat treatment) and of the non enzyme treated sample (SP and SPHT after heat treatement) generated about the same proportion of free β-damascenone (1.9-fold vs. 2.3-fold), but a much higher proportion than heat treatement of the enzyme treated sample (PET and PETHT after heat treatment) (1.3-fold). As reported recently, there is a significant influence of the glycosidic linkage on the chemical reactivity of the bound aglycone [31]. Thus Skouroumounis et al. [30] showed that β-D-glucopyranoside of enynediol, one of the β-damascenone precursors identified in grapes [34], gave rise to a higher proportion of β-damascenone than its corresponding aglycone (2-fold). It was assumed that through stabilisation (by glycosylation) at the C9 position, a dehydration at C3 was favoured, thus resulting in higher yields of β-damascenone. The corresponding aglycone favoured the generation of the odourless 3-hydroxy-β-damascone (the other product of hydrolysis of enynediol).

Table 5
Influence of enzyme (ET) and heat treatment (HT) on the levels of β-damascenone formation (μg l^{-1}) in Merlot wine samples with (samples PET and SP) and without (sample CWA) addition of glycosidic extracts

	β-Damascenone (μg l^{-1})			
	Non-heat-treated samples		Heat-treated samples	
Control sample	CWA	20.2	CWAHT	38.0
Precursors added samples				
Enzyme-treated	PET	47.3	PETHT	57.7
Non-enzyme-treated	SP	48.0	SPHT	110.0

4. Conclusion

A method has been described for quantitative determination of free and of hydrolytically liberated β-damascenone, in grape juices and wines. An estimation of the levels of β-damascenone in one year old wines was established by measurement of the hydrolytically liberated β-damascenone in the grape juice. It was generally found, that half the levels measured in the grape juice were representa-

tive of the amounts of β-damascenone measured in the wine.

Many factors influenced the levels of free β-damascenone in the wines such as cultivar, region and vintage conditions. The levels of free β-damascenone was higher than the odour threshold of this compound, in all the analysed wines during this study.

Enzyme and heat treatment of Merlot wine samples influenced their β-damascenone levels. Heat treatment doubled the levels of this compound. Enzymatic treated sample, liberating aglycone precursors of β-damascenone, formed lower amounts of β-damascenone than the non-enzymatically treated sample. The importance on enzyme treatment reduced the potential levels of β-damascenone formation in the wine.

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