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Monoterpenic and norisoprenoidic glycoconjugates of *Vitis vinifera* L. cv. Melon B. as precursors of odorants in Muscadet wines

R. Schneider[a,b,*], A. Razungles[a], C. Augier[a], R. Baumes[a]

[a]*Unité Mixte de Recherche – Sciences pour l'Œnologie, 2 place Viala, 34060 Montpellier cedex 1, France*

[b]*Comité Interprofessionnel des Vins de Nantes, Maison des Vins, Bellevue, 44 190 La Haie Fouassière, France*

Abstract

The volatile monoterpenic and norisoprenoidic compounds released by glycosidase enzyme hydrolysis of C_{18} reversed-phase isolates from the juice of *Vitis vinifera* L. cv. Melon B. have been qualitatively and quantitatively determined using GC–MS and GC–FID. The components analyzed were broadly similar to those previously reported for other varieties but the level of bound *p*-menth-1-en-7,8-diol was higher in this cultivar. Then the monoterpenic and norisoprenoidic volatiles released from the same glycosidic extracts under mild acid conditions, mimicking wine aging conditions, have been analyzed using GC–Olfactometry and GC–MS. The most odorous compounds detected were *p*-cymene, terpinen-4-ol, *cis*- and *trans*-vitispiranes, 1,6,6-trimethyl-1,2-dihydronaphtalene (TDN), β-damascenone and riesling acetal. To assess their potential levels in corresponding wines after ageing, most of these odorants were generated by harsh acid hydrolysis from the precursors extracts and quantitatively determined using SPME and GC–MS/MS. For the development and application of this analysis, the odorants not commercialy available were synthesized. The total amounts of norisoprenoidic odorants generated by acid hydrolysis of the glycosidic extracts were shown to be proportional to the total amounts of these precursors.

Keywords: Vitis vinifera; Monoterpenic glycoconjugates; Norisoprenoidic glycoconjugates; C13-norisoprenoids; Monoterpenes

1. Introduction

Structural studies have allowed the identification in grapes of four groups of glycosides: β-D-glucopyranosides, 6-*O*-(α-L-rhamnopyranosyl)-β-D-glucopyranosides, 6-*O*-(α-L-arabinofuranosyl)-β-D-glucopyranosides [1,2] and 6-*O*-(β-D-apiofuranosyl)-β-D-glucopyranosides [3]. These flavourless glycosides, as well as the cysteine derivatives discovered recently [4], make up in grape a reserve of aroma compounds that can be liberated in wine [5]. These glycosides can be conveniently extracted from grape using solid phases, like XAD-2 resin [6] or C_{18}-RP adsorbent [7,8]. However, due to the complexity of these glycosidic extracts, their direct quantitative analysis was restricted only to the most abundant benzyl, 2-phenylethyl and monoterpenyl glycosides [3,9]. Therefore, the most used quantitative methods were indirect and consisted in the analysis of the volatiles liberated from the glycosidic extracts by enzymatic or acid hydrolysis [10]. The mild enzyme method was the most efficient technique to generate most aglycones unchanged. It proceeded for the diglycosides through a sequential mechanism, which required the occurrence in the enzymatic preparation used of β-D-glucopyranosidase, α-L-rhamnopyrano-

*Corresponding author. Tel.: +33-499-612-485; fax: +33-499-612-857.

E-mail address: schneidr@ensam.inra.fr (R. Schneider).

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sidase, α-L-arabinofuranosidase and β-D-apiofuranosidase [11]. However even this mild enzyme method cannot totally eliminate the formation of artifacts [12,13]. Furthermore the aglycone fraction obtained can be spoiled by traces of polar non glycosidic compounds, extracted with glycosides and not totally eliminated through the processing of the glycosidic extracts. In spite of these limitations, which must be taken into account, this method allowed to cope with the noble part of these glycosides as regards aroma generation [10].

Among the aglycones identified in grape, monoterpenes and C_{13}-norisoprenoids were the most involved in aroma generation, either directly from the glycosides or via polyhydroxylated derivatives [10]. The purpose of the present work was to make a qualitative and quantitative study of these isoprenoid aglycones in Melon B. grapes (grown mainly in the Nantes region in western France), not investigated up to now. Furthermore, the odorants generated from their glycosidic bound forms in the Muscadet wines made from these grapes were investigated.

2. Experimental

2.1. Plant material

The Melon B. grape samples, grown in five different vineyards located in different areas of the Muscadet region (Nantes region, Western France) were harvested at technological maturity from the beginning of September to mid October, in 1998 and 1999 (encoded HAI, DRA, MON, LOR and LIM in Table 1). In 1999, Melon B. grapes from six complementary vineyards of the Muscadet region were added to the study (encoded A–F in Table 2). Grapes were frozen and stored at −20°C till analysis.

2.2. Chemicals

Theaspirane and α-ionone were purchased from Fluka Chemie AG (St. Quentin Fallavier, France). β-damascenone was a gift from Firmenich (Geneva, Switzerland). Analytical grade solvents, diethyl ether, pentane, dichloromethane (pestanal quality) were purchased from Riedel de Häen GmbH (St. Quentin Fallavier, France) and silica gel 60 (230–400 mesh ASTM) from Merck (Darmstadt, Germany).

2.3. NMR Spectroscopy

^{1}H-NMR spectra were obtained at 500 MHz with a Varian UNITY INOVA 500 MHz spectrometer. Measurements were made at 25°C in $CDCl_3$ with tetramethylsilane as an internal standard in 5 mm sample probe.

2.4. Isolation of glycoconjugates from Melon B. grapes

2.4.1. Isolation of glycoconjugates using C_{18} RP cartridges

Frozen berry samples were allowed to reach +4°C overnight, then destemmed, crushed in a fruit-juicer for 2 min and centrifuged (10 000 *g*, 15 min), while keeping the temperature at 4°C. Prior to analysis, the juice was filtered through glass wool. Isolation of the glycosidic compounds from grape juice was achieved using C_{18} reversed-phase cartridges (Lichrolut RP-18, Merck-France, 0.5 g of phase) as reported previously [7,8]. Each cartridge was pre-conditioned with 20 ml of methanol, then with 20 ml of Milli-Q water. Afterwards 100 ml of grape juice were loaded onto two superimposed C_{18} RP cartridges at a flow-rate of ca. 3 ml min^{-1}, then the cartridges were washed with 150 ml Milli-Q water then with 25 ml of pentane/dichloromethane (2/1 v/v). The glycoconjugates were finally eluted from the cartridges with 25 ml of methanol. The glycosidic extract was concentrated to dryness at 40°C under vacuum.

2.4.2. Preparation of a glycoconjugates pool from Melon B. grapes using XAD-2

Grapes from the HAI which contained the highest levels in C_{13}-norisoprenoids precursors in 1998, were harvested in 1999 to carry out this experiment. A volume of 2 l of centrifuged juice from Melon B. grapes grown in the HAI vineyard in 1999, in which a sufficient quantity of grape was harvested, were prepared as above and were passed through a column of XAD-2 resin (length 32 cm, diameter 32 mm), as described by Günata et al. [6]. After adsorption of the juice, the column was eluted with (i) water (2 l), (ii) pentane/dichloromethane mixture (2/1 v/v; 500

Table 1
Monoterpenic and C_{13}-norisoprenoidic volatiles (μg/l) liberated by enzymatic hydrolysis from glycosides of Melon B. grapes grown in five Muscadet vineyards in 1998 and 1999 (mean of three repetitions±standard deviation)

RI[a]			1998				
C_{13}-norisoprenoids			HAI-98	DRA-98	MON-98	LOR-98	LIM-98
2473	9-Hydroxymegastigm-7-en-3-one		27±3	19±1	9±1	9±1	12±1
2537	3-Hydroxy-β-damascone		45±5	39±2	24±1	36±2	38±3
2553	3-Hydroxy-7,8-dihydro-β-ionone		5±0.3	5±0.3	3±0.2	4±0.3	5±0.5
2585	Megastigm-7-en-3,9-diol[b]		30±2	11±1	7±1	5±0.4	9±0.4
2635	3-Oxo-a-ionol		114±12	74±2	38±1	52±3	63±8
2640	4-Oxo-β-ionol		16±2	19±2	16±2	8±1	9±0.0
2700	3-Hydroxy-7,8-dihydro-β-ionol 3-hydroxy-β-ionone		9±1	6±0.2	4±0.2	4±0.3	6±1
2711	4-Oxo-7,8-dihydro-β-ionol		9±1	9±1	9±0.1	7±0.5	8±1
2725	3-Oxo-7,8-dihydro-α-ionol		5±0.2	5±1	7±0.4	5±0.2	5±1
2767	3-Hydroxy-7,8-dihydro-β-ionol		38±3	23±1	13±0.4	21±1	23±3
3002	4,5-Dihydrovomifoliol		43±1	25±1	17±0.4	12±1	20±3
3131	Vomifoliol		18±1	19±1	13±1	8±0.4	12±1
		Total	360±30	255±9	159±2	172±9	210±18
Monoterpenes							
1445	*trans*-Linalool oxide furan		7±0.3	5±0.3	5±0.4	4±0.2	5±1
1473	*cis*-Linalool oxide furan		4±0.1	2±0.2	2±0.1	1±0.1	2±0.1
1698	α-Terpineol		7±1	9±1	6±1	4±0.5	7±0.4
1732	*trans*-Linalool oxide pyran		15±1	14±1	10±1	11±0.4	12±1
1756	*cis*-Linalool oxide pyran		2±0.0	2±0.2	1±0.0	2±0.1	1±0.0
1788	Nerol		14±0.2	3±0.2	4±0.1	3±0.1	4±0.5
1832	Geraniol		14±2	8±1	12±0.2	8±0.3	8±1
1840	exo-2-Hydroxy-8-cineole		2±0.0	1±0.1	2±0.2	1±0.1	1±0.0
1957	3,7-Dimethyl-1,5-octadien-3,7-diol		4±0.3	4±0.3	2±0.2	2±0.2	4±0.3
2220	8-Hydroxy-6,7-dihydrolinalol		11±1	10±0.3	12±1	10±1	13±2
2275	E-8-Hydroxylinalol		78±4	71±2	67±2	70±3	73±7
2316	Z-8-Hydroxylinalol+geraniol hydrate[c]		79±5	60±2	82±2	48±2	89±8
2526	*p*-Menth-1-en-7,8-diol		102±2	95±2	119±4	56±2	80±11
		Total	329±13	283±10	322±6	218±7	298±30
RI[a]			1999				
C_{13}-norisoprenoids			HAI-99	DRA-99	MON-99	LOR-99	LIM-99
2473	9-Hydroxymegastigm-7-en-3-one		22±3	32±1	23±0.4	26±3	20±1
2537	3-Hydroxy-β-damascone		34±3	41±1	29±0.4	32±3	25±1
2553	3-Hydroxy-7,8-dihydro-β-ionone		5±0.5	5±0.1	5±1	4±1	4±0.4
2585	Megastigm-7-en-3,9-diol[b]		29±3	30±2	26±0.2	27±2	25±2
2635	3-Oxo-α-ionol		62±7	85±4	51±1	65±7	48±3
2640	4-Oxo-β-ionol		19±2	24±1	17±1	17±2	15±1
2700	3-Hydroxy-7,8-dihydro-β-ionol 3-hydroxy-β-ionone		6±1	9±0.3	5±0.1	7±1	5±0.3
2711	4-Oxo-7,8-dihydro-β-ionol		11±1	7±0.2	8±1	6±1	4±0.0
2725	3-Oxo-7,8-dihydro-α-ionol		12±1	8±1	10±1	10±1	6±1
2767	3-Hydroxy-7,8-dihydro-β-ionol		32±2	32±2	20±1	24±3	23±2
3002	4,5-Dihydrovomifoliol		27±1	37±3	26±2	27±2	27±3
3131	Vomifoliol		19±2	21±2	25±3	13±2	14±1
		Total	277±22	332±12	244±0.1	259±25	216±14

Table 1. Continued

RI[a]		1998				
Monoterpenes						
1445	*trans*-Linalool oxide furan	5±0.2	9±1	7±1	6±1	4±1
1473	*cis*-Linalool oxide furan	2±0.0	3±0.4	4±0.5	3±0.1	2±0.2
1698	α-Terpineol	6±0.5	8±1	8±0.3	7±1	6±0.4
1732	*trans*-Linalool oxide pyran	8±1	19±2	13±2	10±1	9±0.5
1756	*cis*-Linalool oxide pyran	3±0.4	6±1	7±1	4±0.2	3±0.2
1788	Nerol	5±1	6±1	6±1	4±0.3	4±0.4
1832	Geraniol	14±1	13±1	12±0.5	10±1	9±1
1840	exo-2-Hydroxy-8-cineole	1±0.1	2±0.1	3±0.4	1±0.1	2±0.3
1957	3,7-Dimethyl-1,5-octadien-3,7-diol	3±0.3	3±0.3	4±0.4	1±0.1	3±0.3
2220	8-Hydroxy-6,7-dihydrolinalol	11±0.3	17±2	16±2	10±1	10±1
2275	E-8-Hydroxylinalol	77±7	79±3	81±9	67±5	75±4
2316	Z-8-Hydroxylinalol+geraniol hydrate[c]	65±6	82±5	74±10	39±3	83±5
2526	*p*-Menth-1-en-7,8-diol	102±4	132±9	163±22	84±9	130±11
	Total	301±21	377±18	399±45	246±23	343±23

[a] Retention Index on DB-WAX.
[b] Tentatively identified.
[c] The mean relative abundance based on the ratio of mass fragments m/z 71 and m/z 59=90:10.

ml) and finally (iii) methanol (2 l). The methanol fraction was evaporated to dryness at 40°C under vacuum to yield the glycoconjugates pool used for the aging experiment (see Section 2.6).

2.5. Hydrolysis of glycosidic extracts and extraction of volatiles released

2.5.1. Enzymatic hydrolysis

An enzymatic solution containing 300 μl of phosphate (0.1 *M*)–citrate (0.2 *M*) buffer (pH 5.0) and 7 mg of a hydrolase enzyme AR2000 (Gist-Brocades, Seclin, France) was initially extracted with pentane/dichloromethane (2/1 v/v; 2×1 ml) and final traces of solvent were removed under a stream of nitrogen. This solution was then added to a C_{18}-RP glycosidic extract from 100 ml of grape juice and extracted with pentane/dichloromethane (2/1 v/v; 3×1 ml). The reaction mixture was incubated at 40°C for 16 h. Then, it was cooled at room temperature and was extracted with pentane/dichloromethane (2/1 v/v, 5×2 ml). The organic layer was dried, internal standard, 4-nonanol (16.15 μg), added, and then concentrated at 35°C (Vigreux column then Dufton column) to an approximative final volume of 400 μl. The aglycones extract was kept at −20°C till analyzed by GC–MS.

2.5.2. Acid hydrolysis

A glycosidic extract from 100 ml of grape juice using C_{18}-RP cartridges was added with 5 ml of an aqueous solution of citric acid (0.2 *M*; pH 2.5) then heated in a sealed vial at 100°C for 1 h as described by Kotséridis et al., [14]. The ampoule was cooled at room temperature, then refrigerated to 0°C. The acid hydrolysate was then transferred into a 10 ml septum-sealed glass vial used for its Solid Phase Micro-Extraction (SPME) as described below.

A 100 μm polydimethylsiloxane (PDMS) fiber (Supelco inc., USA) was used. Before each extraction, the SPME fiber was conditioned at 250°C for 1 h as prescribed by the supplier. The acid hydrolysate was added with 0.3 g of NaCl and 228 ng of $[^2H]_4$-β-damascenone as internal standard and then stirred (750 rpm) at room temperature for 10 min. Then the SPME fiber was introduced through the septum into the vial and exposed to the headspace for 20 min. The desorption was carried out immediately after in the heated chromatograph injector of the gas chromatograph–ion trap mass spectrometer system (GC–ITMS) (see Section 2.7.2).

2.6. Accelerated aging experiment

The glycoconjugates pool prepared above was added with 0.5 l of a synthetic wine (ethanol/water

Table 2
Monoterpenic and C_{13}-norisoprenoidic volatiles (μg/l) liberated by enzymatic hydrolysis from glycosides of Melon B. grapes grown in other Muscadet vineyards in 1999 (mean of three repetitions±standard deviation)

RI[a]			A	B	C	D	E	F
C_{13}-norisoprenoids								
2473	9-Hydroxymegastigm-7-en-3-one		24±1	22±1	28±0.4	21±1	37±1	15±0.3
2537	3-Hydroxy-β-damascone		32±1	32±0.4	39±1	28±2	35±1	27±1
2553	3-Hydroxy-7,8-dihydro-β-ionone		4±0.5	4±0.1	5±0.3	4±0.2	4±0.3	3±0.2
2585	Megastigm-7-en-3,9-diol[b]		30±1	14±0.5	28±2	25±0.4	24±1	16±1
2635	3-Oxo-a-ionol		70±3	50±2	70±3	49±1	89±4	45±1
2640	4-Oxo-β-ionol		25±2	15±1	20±1	20±1	28±1	15±2
2700	3-Hydroxy-7,8-dihydro-β-ionol		6±0.5	6±0.5	7±0.4	6±0.2	11±0.3	4±0.1
2711	4-Oxo-7,8-dihydro-β-ionol +3-hydroxy-β-ionone		6±0.7	6±1	8±0.4	6±1	8±1	6±0.1
2725	3-Oxo-7,8-dihydro-α-ionol		6±0.1	8±0.4	15±1	8±1	12±2	9±1
2767	3-Hydroxy-7,8-dihydro-β-ionol		25±1	24±2	32±0.3	23±1	26±0.3	22±1
3002	4,5-Dihydrovomifoliol		32±0.4	13±1	27±2	30±2	23±1	18±1
3131	Vomifoliol		30±1	11±1	21±3	13±2	19±2	12±1
		Total	292±11	205±4	299±10	232±7	315±10	192±2
Monoterpenes								
1445	*trans*-Linalool oxide furan		6.68±0.5	3±0.2	6±1	5±1	11±1	2±0.1
1473	*cis*-Linalool oxide furan		3.43±0.3	1±0.2	2±0.3	2±0.1	5±0.3	6±0.1
1698	α-Terpineol		7.10±0.1	5±0.4	6±1	6±0.4	10±1	4±0.2
1732	*trans*-Linalool oxide pyran		11.65±1	8±1	8±1	9±0.4	23±1	6±1
1756	*cis*-Linalool oxide pyran		6.76±1	2±0.2	3±0.3	4±0.3	7±1	2±0.1
1788	Nerol		4.13±0.3	5±0.4	5±1	4±0.4	7±1	4±0.1
1832	Geraniol		9.31±0.3	17±2	12±1	13±2	17±0.2	11±0.1
1840	exo-2-Hydroxy-8-cineole		1.46±0.0	1±0.0	1±0.1	2±0.1	1±0.1	1±0.0
1957	3,7-Dimethyl-1,5-octadien-3,7-diol		1.57±0.1	3±0.1	11±0.4	3±0.4	11±0.4	2±0.1
2220	8-Hydroxy-6,7-dihydrolinalol		14.68±0.5	8±0.3	15±2	10±1	15±1	4±0.1
2275	E-8-Hydroxylinalol		82.06±1	63±7	74±2	85±11	96±1	54±1
2316	Z-8-Hydroxylinalol+geraniol hydrate[c]		69.66±2	68±8	79±1	85±10	75±0.2	47±1
2526	*p*-Menth-1-en-7,8-diol		124.75±0.5	60±2	97±0.3	94±8	76±4	78±4
		Total	343.22±5	245±26	320±6	323±35	354±2	220±6

[a] Retention Index on DB-WAX.
[b] Tentatively identified.
[c] The mean relative abundance based on the ratio of mass fragments *m*/*z* 71 and *m*/*z* 59=90:10.

12/88 v/v containing 4 g of tartaric acid and adjusted to pH 3 with sodium hydroxide) and heated at 45°C under nitrogen during 3 weeks [15].

Afterwards, 500 ml of the model wine were poured into a 1 l Erlenmeyer and cooled to 0°C in an ice bath under nitrogen. Dichloromethane (150 ml) was added and the mixture was stirred during 20 min at 700 rpm as described by Moio et al., [16]. The mixture was centrifuged for 15 min at 10 000 *g* (4°C) and the organic phase was separated in a separatory funnel. The aqueous phase was poured in the initial Erlenmeyer for a second extraction with 150 ml of dichloromethane as described above. The organic phases were dried over sodium sulfate and then concentrated by distillation through a Vigreux column at 45°C to 1 ml. It was kept at −20°C till analyzed by GC–MS and GC–O.

2.7. GC-Analysis

2.7.1. Operating conditions for the aglycones extracts

Identification: A Hewlett-Packard 6890 Series chromatograph coupled to a Hewlett-Packard 5989

mass spectrometer was equipped with a DB-WAX capillary column (30 m×0.25 mm, 0.5 μm, J&W Scientific Inc., USA) and an ''on column'' injector (30 to 250°C at 180°C min^{-1}). The helium gas vector flow was maintained at 1.4 ml min^{-1} throughout the analysis. Oven temperature followed the program: 30 to 60°C at a ramp rate of 70°C min^{-1}, constant for 3 min, 60°C to 245°C at a rate of 3°C min^{-1} and then constant for 20 min. Injections of about 1 μl were performed on column. Ionization was achieved under electron impact mode (ionization energy of 70 eV), the source, transfer line and quadrupole temperatures were 250, 250 and 120°C respectively. Detection was carried out in scan mode (1 scan s^{-1}) covering a mass range (m/z) of 29 to 350 amu. The identification of the compounds was confirmed by injection of pure standards or comparison of their retention index and MS reported in the literature.

Quantification: A Varian 3300 GC equipped with a DB-WAX capillary column of the same characteristics as above, a SPI injector (20 to 250°C at 180°C min^{-1}) and a flame ionization detector (250°C) was used. The carrier gas was hydrogen at 1.6 ml min^{-1}. Oven temperature followed the program: 30 to 60°C at a rate of 70°C min^{-1}, constant for 3 min, 60°C to 245°C at a rate of 3°C min^{-1} and then constant for 20 min. Injections of about 1.5 μl were performed.

Gas Chromatography–Olfactometry analysis (GC–O): GC–O analysis was carried out using a Hewlett-Packard HP gas chromatograph 5890 series II fitted with a DB-WAX capillary column (30 m× 0.25 mm, 0.5 μm, J&W Scientific Inc., USA). The injection (1μl) of the extract was splitless/split (split ratio 1/10; 250°C). The carrier gas was hydrogen (Linde gaz, Marseille), with a flow-rate of 2 ml min^{-1}. The oven temperature program was the same as above (quantification). The GC effluents were split to a sniffing port and a flame ionization detector (3/1). The sniffing tests were performed alternatively by two trained judges.

2.7.2. Operating conditions for hydrolytically released volatiles

GC–Ion Trap Mass Spectrometry (GC–ITMS): A Varian 3800 GC was coupled to a Varian Saturn 2000 mass spectrometer (ITMS). Chromatographic conditions were as previously described (see Section 2.7.1), except for the injector temperature which was kept at 250°C. Transfer of the sample to the GC–ITMS column was accomplished by keeping the SPME fiber for 5 min in the heated chromatograph injector.

Deuterated and natural β-damascenone were detected using MS/MS and multiple reaction monitoring mode (MRM) with the following parameters: scan channel 1, parent ion mass m/z 190 with an isolation window of 3 amu in non resonant mode, excitation storage 62 m/z, excitation amplitude of 40 V; scan channel 2, parent ion mass m/z 194 with an isolation window of 3 amu in non resonant mode, excitation storage 64 m/z, excitation amplitude of 40 V. Natural and deuterated β-damascenone were quantified using respectively the ions m/z 175 and m/z 176 and 179 for quantification and the ions m/z 157 and m/z 161 as qualifiers.

Vitispiranes, 1,1,6-trimethyl-1,2,3,4-tetrahydronaphtalene (TTN), 1,1,6-trimethyl-1,2-dihydronaphtalene (TDN) and Riesling acetal were detected in Electronic Impact mode (70 eV) and quantified using the following ions: for vitispiranes m/z 192 for quantification and m/z 177, 136, 121 as qualifiers, for TTN m/z 159 and m/z 174, and for TDN m/z 157 and m/z 172, 142. Riesling acetal was determined using the ratio of its Total Ion Current and the TIC of vitispiranes.

Calibrations curves: Aliquots of synthetic vitispiranes, TDN/TTN and β-damascenone, were separately dissolved in ethanol. Increasing amounts of these solutions were added with a solution of 228 ng of $[^2H]_4$-β-damascenone in 5 ml of the citric acid solution (pH 2.5) used in acid hydrolysis (see Section 2.5).

β-damascenone 62-124-620-1240-3100 ng; vitispiranes 18-36-180-360-900 ng; TDN 17.3-34.6-173-346-865 ng; TTN 12-24-120-240-600 ng. Analysis was performed by SPME and GC–ITMS as described above. Concentration ratios between each compound and the internal standard $[^2H]_4$-β-damascenone were plotted against the corresponding area ratios using linear regression (Table 4).

2.8. Chemical syntheses

$[^2H]_4$-β-damascenone: Starting from 90 mg (0.48 mmol) of β-damascenone, the labelling procedure

described by Kotséridis et al. [17] was repeated once more on the crude product (for 1 day only) to yield after purification 10 mg of $[^2H]_4$-β-damascenone, showing MS and ^{1}H-NMR data similar to those reported.

1,1,6-trimethyl-1,2-dihydronaphtalene (TDN) and 1,1,6-trimethyl-1,2,3,4-tetrahydronaphtalene (TTN): 5.6 g of α-ionone added with 32.5 mg of iodine were distilled in a claisen at 270°C according to the procedure described by Bogert and Fourman [18]. The initial distillate was eliminated and 3.7 g of a mixture of TDN and TTN was obtained after flash-chromatography on silica gel (elution with pentane). 1.8 g of the purified mixture, dissolved in 40 ml of anhydrous ethanol, were hydrogenated using 90 mg of palladium on activated carbon (10%). When the consumption of hydrogen stopped (about 30 min), the mixture was filtered on celite and concentrated under vacuum. The colorless residue (1.6 g), dissolved in 30 ml of CCl_4 under nitrogen, was added with 1.6 g of *N*-bromosuccinimide (9.3 mmol.) and the mixture was stirred at room temperature for 10 min. After addition of 15 mg of benzoyl peroxide, the mixture was stirred and irradiated for 4 h with a 100 W tungsten lamp. Then 2 g of anhydrous Na_2CO_3 and 3.5 ml of *N,N*-dimethylformamide were added. After 30 min, the mixture was filtered and concentrated under vacuum. After purification by flash-chromatography on silica gel (elution with pentane), 0.8 g of an oily mixture of TDN and TTN was recovered, which was analyzed both by GC–ITMS and by ^{1}H-NMR (500 MHz, $CDCl_3$): δ 1.25 (s, $(CH_3)_2$–C-1 TDN), 1.27 (s, $(CH_3)_2$–C-1 TTN), 1.64 (m, H-2 TTN), 1.79 (m, H-3 TTN), 2.22 (br.d, J=4.4 Hz, H-2 TDN), 2.27 (s, CH_3–C-6 TTN), 2.29 (s, CH_3–C-6 TDN), 2.72 (br.t, J=6.3 Hz, H-4 TTN), 5.92 (dt, J=9.8, 4.4 Hz, H-3 TDN), 6.41 (d, J=9.8 Hz, H-4 TDN), 6.86 (s, H-5 TDN+TTN), 6.98 (m, H-7 TDN+TTN), 7.22 (m, H-8 TDN+TTN), to give the TDN/TTN ratio 60/40 w/w. These data were similar to those reported previously [19].

Cis- and trans-vitispiranes: 970 mg (4.6 mmol) of the four diastereoisomeric theaspirane-epoxides were synthesized starting from 970 mg (5 mmol) of diastereoisomeric theaspirane according to the procedure described by Schulte-Elte et al. [20].

All the crude epoxide obtained was dissolved in a mixture of 10 ml of THF and 10 ml of sulfuric acid 0.1 N under nitrogen. The mixture was stirred at room temperature for 5 days. After extraction with diethyl ether, the crude product was purified by flash-chromatography on silica gel (elution with pentane/diethyl ether 95/5, then 70/30) and the fractions containing the 2,6,10,10-tetramethyl-1-oxaspiro[4.5]decan-6,7-diols were gathered to yield 388 mg (1.6 mmol) of a mixture giving four peaks in GC–ITMS with practically identical EIMS, m/z (rel. int.): 126 (100), 111 (20), 211 (19), 43 (19), 127 (15), 128 (12), 125 (10), 144 (9), 195 (5), 69 (5), 193 (4).

250 mg of this mixture were dissolved in 7 ml of triethylamine and a few drops of $POCl_3$ were added. The mixture was stirred at room temperature for 15 h then hydrolyzed with ice-water. After diethyl ether extraction and purification by flash-chromatography on silica gel (elution with pentane/diethyl ether 95/5 v/v), 21 mg of a mixture of *cis*- and *trans*-vitispiranes (0.11 mmol) was obtained which was analysed by GC–ITMS and ^{1}H-NMR (500 MHz, $CDCl_3$): δ 0.90, 0.99 (2×s, $(CH_3)_2$–C-10 *trans*), 0.89, 0.95 (2×s, $(CH_3)_2$–C-10 *cis*), 1.22 (d, J=6.3 Hz, CH_3–C-2 *cis*), 1.34 (d, J=6.2 Hz, CH_3–C-2 *trans*), 1.2–2.2 (m, H-3, H-4, H-9 *cis*+*trans*), 4.04 (m, H-2 *trans*), 4.31 (m, H-2 *cis*), 4.85, 5.07 (2×s, CH_2–C6 *cis*), 4.88, 5.19 (2×s, CH_2–C6 *trans*), 5.60 (m, H-8 *cis*+*trans*), 6.07 (m, H-7 *cis*+*trans*), to give the *trans*/*cis* ratio of 78/22 w/w. These data were similar to those reported previously [20].

3. Results and discussion

3.1. Glycoconjugates composition of Melon B. grapes

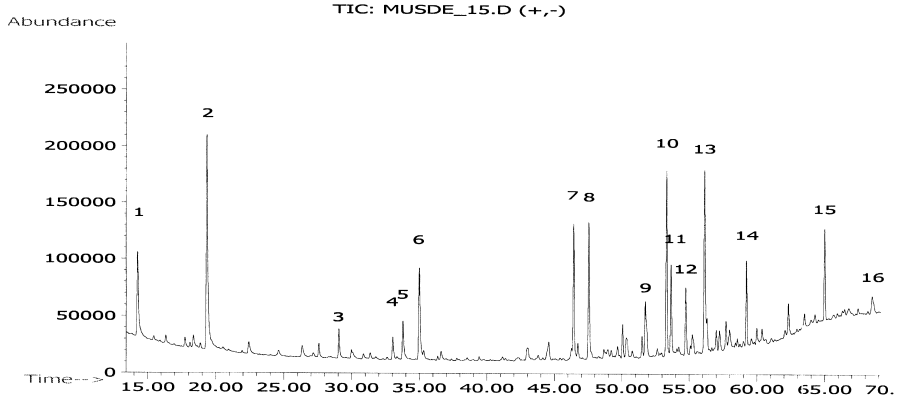
Glycoconjugates from Melon B. grapes were extracted using reversed-phase C_{18} cartridges [7,8]. The enzymatic preparation used for the liberation of aglycones moieties from these glycosidic extracts contains the glycosidase activities required to hydrolyze the grape glycosides [5]: β-D-apiosidase (564.6 nkat/g), α-L-rhamnosidase (236.7 nkat/g), α-L-arabinofuranosidase (13 500 nkat/g) and β-D-glucopyranosidase (4380 nkat/g). As this enzymatic preparation was used in low amount, it was not

expected to generate artifacts or only in very low levels, e.g. no 3-oxo-β-damascone was detected [12,13]. However the glycosidase activities contained in this low amount could release much higher levels of aglycones than those liberated from the glycosidic extracts analyzed.

The volatiles extracted with pentane/dichloromethane (2/1 v/v) from the hydrolysates of these glycosidic extracts were analyzed using GC–MS (a chromatogram is shown Fig. 1). The levels of the C_{13}-norisoprenoidic and monoterpenic volatiles released in these conditions from the Melon B. grape samples studied are shown in Tables 1 and 2. These compounds were previously identified in other grape varieties; they made up about 70% of the total level of the volatiles released. The other volatiles were alcohols (15–20%), C_6 alcohols (5%) and benzenoid compounds (10%). Coefficients of variation of these quantitative determinations were ranging from 1 to 20% for all the volatiles released.

The levels of the monoterpenic compounds, particularly mono-oxygenated monoterpenes, were characteristic of grapes of neutral cultivars [21,22]. The variations of their total levels observed for the five vineyards, and those observed for the two vintages, were in the same order of magnitude (Table 1). The most abundant compounds were monoterpendiols, less odorous, but aroma precursors themselves [23]. One of them, *p*-menth-1-en-7,8-diol, reached high levels in these grapes, similar to those occurring in Sylvaner [24] and Sauvignon [24,25]. Higher levels were reported only in Rose Muscat grapes (a red variety of Northern Italy) [24]. This compound had been also reported as a product of α-terpineol metabolization by *Botrytis cinerea* [26]. The other bound *p*-menthane derivatives identified in grapes by Strauss et al. [27], Versini et al. [24], and Sefton et al. [25] were not found in Melon B. grapes, except α-terpineol and 2-*exo*-hydroxy-8-cineole.

The C_{13}-norisoprenoidic pattern was similar to those found in other varieties [21]. As observed for monoterpenic compounds, the variations of the total levels of C_{13}-norisoprenoidic compounds observed for the five vineyards and for the two vintages were in the same order of magnitude. However, in 1998, the C_{13}-norisoprenoid level in grapes from the HAI vineyard was higher than those in grapes from other vineyards, whereas, in 1999, differences between vineyards were less important. The most abundant C_{13}-norisoprenoid was 3-oxo-α-ionol, followed by 3-hydroxy-β-damascone and 4,5-dihydrovomifoliol. These norisoprenoidic glycoconjugates are consid-



(1) - Hexanol
(2) - 4-Nonanol (standard)
(3) - α-Terpineol
(4) – Geraniol
(5) – Benzylic alcohol
(6) – 2-Phenylethanol
(7) – *E*-8-Hydroxylinalool
(8) – *Z*-8-Hydroxylinalool
(9) – 9-Hydroxymegastigm-7-en-3-one
(10) – *p*-Menth-1-en-7,8-diol
(11) – 3-Hydroxy-ß-damascone
(12) – Megastigm-7-en-3,9-diol
(13) – 3-oxo-α-ionol
(14) – 3-Hydroxy-7,8-dehydro-ß-ionol
(15) – 4,5-Dihydrovomifoliol
(16) - Vomifoliol

Fig. 1. Chromatogram in GC–MS of the volatiles liberated by enzymatic hydrolysis of glycosidic extracts from Melon B. grapes.

ered as degradation products of carotenoids in grape [10]. They give rise during wine aging to potent odorants like β-damascenone [28].

3.2. Generation of odorants from glycoconjugates

To assess the sensory impact of the acid hydrolysates of the Melon B. grape glycoconjugates on wine aroma, an accelerated aging experiment, similar to that reported by Francis et al. [15], was carried out. This procedure, using a model wine enriched in grape glycoconjugates, has been described by Ormières et al. [29] and Kotséridis [30] to study the sensory impact of odorants released from grape glycosidic extracts on Grenache and Merlot wine aroma.

The simple and rapid extraction method previously described by Moio et al., [16] was used to obtain a representative extract of the model wine aroma [31,32]. GC–olfactometry of this extract, performed alternatively by two trained judges, allowed the detection of 17 monoterpenic and C_{13}-norisoprenoidic odorants (Table 3). Fourteen out of these 17 compounds were identified using GC–MS.

Terpinen-4-ol was reported as trace component in musts and wines of some *Vitis vinifera* cultivars [33], but it reached higher levels in Northern italian *passiti*-wines [34] and in the products of the Japanese Koshu variety, in which it is the key aroma compound [35]. *p*-Cymene has been also identified with other terpenic hydrocarbons as free component of Traminer wine [36].

p-Menth-1-en-3-ol, terpinen-4-ol, *p*-cymene, and the two isomers of anhydrolinalool oxide are known to be generated by harsh acid hydrolysis of grape glycosidic extracts [37]. *p*-Cymen-8-ol and *p*-cymene, considered as off-flavor of lemon oil [38], are known as acid catalyzed cyclization products of citral [39,40].

3,9-epoxy-*p*-menth-1-ene, the character impact compound of fresh dill herb [41] could be formed by cyclization under acidic conditions of 8-hydroxylinalool [42], aglycone present in Melon B. glycosides.

Table 3
Monoterpenic and C_{13}-norisoprenoidic odorants identified by GC–olfactometry and GC–MS

RI	Identified odorants	Odors
Monoterpenes		
1204	Anhydrolinalool oxide	fresh, minty
1275	*p*-Cymene	citrus fruit
1288	4-Heptanol+1,2,4-trimethylbenzene +unknown terpene[a]	underwood
1450	Unknown hydrocarbure[b]+dehydro-*p*-cymene	spicy, curry
1545	Benzaldehyde+3,9-epoxy-*p*-menth-1-ene	wisteria flower
1555	Linalool	bergamot
1600	*p*-Menth-1-en-3-ol	lavender
1617	Terpinen-4-ol	floral
1833	*p*-Cymen-8-ol	floral
1877	Unknown terpenoid[c]	nutmeg
C_{13}-norisoprenoids		
1553	*cis*- and *trans*-Vitispiranes	woody, spicy
1656	Riesling acetal	floral, raspberry
1706	Actinidol ethyl ether (isomer I)[d]	citrus fruit
1732	Actinidol ethyl ether (isomer II)[d]	grassy, eucalyptus
	1,6,6-Trimethyl-1,2-dihydronaphtalene	pharmaceutical
1820	β-Damascenone	floral, fruity, tobacco
2140	2-(3-Hydroxybut-1-enyl)-2,6,6-trimethylcyclohex-3-en-1-one	liquorice, smoke

[a] m/z=136, 121, 93, 79.
[b] m/z=57, 69, 70, 97, 43, 85.
[c] m/z=93, 121, 43, 95, 136.
[d] m/z=163, 45, 121, 73, 145, 91.

The C_{13}-norisoprenoidic compounds shown in Table 3 are known to be formed during wine maturation and conservation. β-Damascenone, a potent odorant first isolated in Bulgarian rose oil [43] was first identified in wine by Schreier and Drawert [44]. It has been found in many grape varieties and wines and is generated by acid hydrolysis from multiple precursors, as reviewed recently [30]. TDN, vitispiranes and Riesling acetal have been reported as key aroma of bottle-aged Riesling wines [23,45,46]. They are generated from multiple precursors [19,23,47–49]. The actinidols ethyl ethers (4,4,7a-trimethyl-2-(1-ethoxyethyl)-1-oxobicyclo[4.3.0]-nona-3,6-dienes) had mass spectra similar to the corresponding actinidols and were tentatively identified by Sefton [13] who showed that they were not produced by acid hydrolysis in absence of ethanol. The corresponding actinidols were not perceived in the GC–O experiment, although they were detected by GC–MS in concentrations higher than those of their odorous ethyl ethers. These actinidols, TDN and the ''pinacol like'' rearrangement product, 2-(3-hydroxybut-1-enyl)-2,6,6-trimethylcyclohex-3-en-1-one, have common precursors, megastigma-4,7-dien-3,6,9-triol and related derivatives [19,47,23]. These triols, as well as Riesling acetal precursors and megastigma-6,7-dien-3,5,9-triol reported as precursor of β-damascenone, 3-hydroxy-β-damascone and 3-hydroxy-7,8-dehydro-β-ionol are not amenable to analysis in the conditions used in this study [13,19] and are missing in Table 1. Thus, it was obvious that the levels of the C_{13}-norioprenoidic compounds identified in the enzyme hydrolysates could not be indicative of the aroma potential of the grapes analyzed. Therefore, it was necessary to develop a simple analytical procedure to assess the levels of the C_{13}-norisoprenoidic odorants generated in wine from the glycosidic extracts of these grapes. The synthesis of some of these odorants was needed to achieve this goal.

3.3. Chemical synthesis of C_{13}-norisoprenoidic odorants

$[^2H]_4$-β-damascenone: $[^2H]_4$-β-damascenone was prepared as described by Kotséridis et al., [17] by equilibrating β-damascenone in deuterium oxide/tetrahydrofuran under basic conditions.

TDN and TTN: Cyclization of α-ionone using the procedure of Bogert and Fourman [18] afforded not only TTN but a mixture of TTN and TDN (2:1), which was catalytically hydrogenated to TTN. Bromination of TTN and elimination of hydrogenbromide in the basic conditions reported by Strauss et al., [23], afforded a mixture of TDN and TTN, the proportion of which was determined by ^{1}H-NMR: 60% of TDN and 40% of TTN. This proportion did not change in the presence of oxygen. As these two products were well separated in the chromatographic conditions used in the quantitative determinations, this mixture was used in the calibration experiments.

Cis- and trans-vitispiranes: They were prepared from theaspirane using the synthesis reported by Shulte-Elte et al., [20], which was slightly modified. Epoxidation of a mixture of diastereoisomers of theaspirane and cleavage of the epoxides under mild aqueous conditions gave the four corresponding *trans*-diols without undesirable by-products. Their dehydration afforded a mixture of *cis*- and *trans*-vitispiranes which was used in the calibration experiments. Its ^{1}H-NMR spectrum compared to the data reported by Schulte-Elte et al., [20] showed that the major diastereoisomer was *trans*-vitispirane (78%), easily distinguished from the minor *cis*-vitispirane (22%) by the chemical shifts of the proton H-2 (4.04 vs. 4.31 ppm), one of the geminal olefinic protons (5.19 vs. 5.07 ppm) and the methyl group on carbon C-2 (1.34 vs. 1.22 ppm).

3.4. Generation of norisoprenoidic odorants from Melon B. grape precursors under harsh conditions

The conditions reported previously to release β-damascenone from its grape precursors [14] were used for the fast generation of the odorants released under the hydrolytic conditions mimicking wine aging. Those conditions allowed to form in 1 h most norisoprenoidic odorants released under mild conditions, but 2-(3-hydroxybuten-1-enyl)-2,6,6-trimethylcyclohex-3-enone was hardly generated.

On the other hand, six odorous monoterpenes were detected using GC–O in the accelerated aging experiment. However, only one, *p*-cymene, was generated using harsh acidic conditions, which showed

that these conditions were not representative of the aging conditions for these monoterpenic compounds.

As the odorants released were volatile and apolar compounds, SPME [50,51] was a simple and fast technique to extract them from the headspace of the vial in which they were hydrolytically released. This technique has been originally used for the analysis of aqueous environmental samples by direct immersion of the fiber into the liquid sample [52], but is now applied to the headspace in food analysis [53–56]. As the analytes studied were similar, weakly polar C_{13}-norisoprenoidic compounds, they should have liquid–gas coefficients in the same magnitude order. Thus $[^2H_4]$-β-damascenone synthesized and used previously in a stable isotope assay to determine quantitatively the free and hydrolytically liberated β-damascenone in grapes and wines [14], was chosen as internal standard. However, to enhance the selectivity in measuring the low levels of the hydrolytically released β-damascenone, a GC–MS/MS method, using a benchtop ion trap mass spectrometer (ITMS), was developed. A comprehensive study on the theoretical aspects of IT–MS/MS analytical method was reported previously [57]. Re-fragmentation of the molecular ions generated by electronic impact from natural and deuterated β-damascenone gave respectively the unlabelled and labelled fragments m/z 175 and m/z 176 and 179 (loss of CD_3 and CH_3), which were used for quantification, whereas m/z 157 and 161 fragment ions (cyclization and dehydration of the previous fragment ions) were respectively used as qualifiers. Higher ejection amplitude values gave more fragmentation and a lower signal-to-noise ratio. The IT–MS/MS method allowed to detect selectively the labelled and natural β-damascenone even at low levels, without interference from matrix.

The sensitivity and selectivity in measuring the levels of the other norisoprenoidic odorants was satisfactory using a single GC–ITMS method in electron impact and extracting the characteristic fragment ions from the full scan signal (Table 4). As *cis*- and *trans*-vitispiranes were not well separated on DB-WAX [58], quantifications were performed by integrating both peaks of these diastereoisomers. On the other hand, as synthesis of Riesling acetal was cumbersome [19], this compound was quantitatively determined relatively to vitispiranes, the structure of which is similar to that of Riesling acetal.

Calibration curves for β-damascenone, vitispiranes, TDN and TTN were linear on a large range of concentrations. The repeatability of SPME analysis was satisfactory with coefficients of variation for all compounds lower than 9% (Table 4). The estimated detection limits for these compounds (Table 4), were lower than the respective odor threshold of the most potent odorant, β-damascenone (10 ng/l in wine [30]).

Table 5 shows the levels of the odorants released using this method from the samples of Melon B. grapes analyzed above. Other Melon B. grape samples from other vineyards of the same region (noted A–F) were also analyzed (Tables 2 and 5). In all these samples, the more abundant C_{13}-norisoprenoidic odorant released was vitispirane, with levels ranging from 2.5 to 7.5 μg/l, much lower than its odor threshold [45]. However this threshold was determined on a mixture of isomeric vitispiranes and must be considered carefully. The levels of all the others odorants were very low (≤1.1 μg/l). β-

Table 4
Parameters of the method used for the quantification of the C_{13}-norisoprenoidic odorants released by acid hydrolysis

Compounds	Fragment ion used for quantification	Internal Standard	R^2 values[a]	Estimated detection limit	Coefficient of variation ($n=6$)
TDN	157	$[^2H]_4$-β-damascenone	0.997	1 ng/l	9%
Vitispiranes	192	$[^2H]_4$-β-damascenone	0.994	3 ng/l	7%
TTN	159	$[^2H]_4$-β-damascenone	0.992	6 ng/l	8%
Riesling acetal	total ion	vitispiranes	–	6 ng/l	8%
β-Damascenone	175 (MS/MS)	$[^2H]_4$-β-damascenone	0.998	10 ng/l	6%

[a] R^2 values of the regression lines with equations: TTN: $y=22.7x+0.7$ vitispiranes: $y=7.9x+0.9$; TDN: $y=20.5x-1.3$ β-damascenone: $y=1.1x+0.3$ where y is the areas ratio and x the concentrations ratio

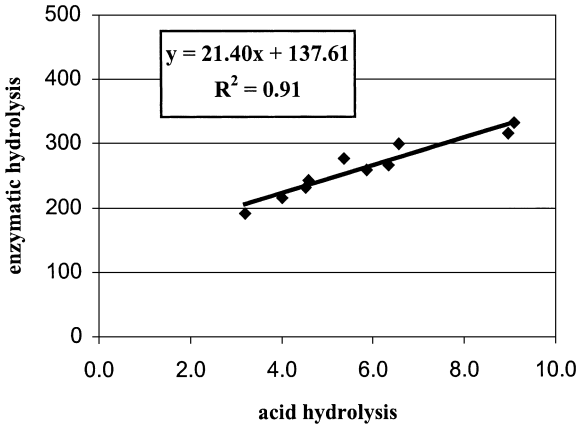
Table 5
Amounts of the C_{13}-norisoprenoidic compounds released by acid hydrolysis from Melon B. grape glycosidic extracts (μg/l)

Vineyards[a]	Vitispiranes	TTN	TDN	β-Damascenone	Riesling acetal	*Total*
MON-99	3.51	0.17	0.04	0.90	0.04	*4.65*
LIM-99	3.04	0.13	0.12	0.74	0.04	*4.6*
DRA-99	6.87	0.14	0.74	1.29	0.08	*9.13*
LOR-99	4.80	0.12	0.23	0.73	0.09	*5.97*
HAI-99	4.23	0.16	0.22	0.77	0.07	*5.45*
A	5.10	0.15	0.27	0.85	0.09	*6.46*
B	3.86	0.12	0.18	0.37	0.07	*4.41*
C	5.32	0.16	0.30	0.80	0.06	*6.65*
D	3.18	0.12	0.24	0.82	0.05	*4.41*
E	7.50	0.12	0.64	0.72	0.05	*9.03*
F	2.49	0.13	0.12	0.47	0.02	*3.23*

[a] The same codes were used in Tables 1 and 2.

damascenone was the only one to reach levels higher than its odor threshold (10 ng/l in wines [30]). Even if β-damascenone precursors are not totally hydrolyzed during wine aging, this compound should be a key odorant of aged Muscadet wines.

Finally, Fig. 2 shows the linear relationship found between the total levels of the norisoprenoidic volatiles released by enzymatic hydrolysis and those released by acid hydrolysis [$F_{(0.1\%,\ 2,\ 8)}<94.5$]. This relationship was surprising, as most volatiles shown in Tables 1 and 2 were not precursors of the hydrolytically released volatiles (see above). It showed that the biogenesis in grape of the different C_{13}-norisoprenoidic precursors were closely correlated. Thus, the levels of the C_{13}-norisoprenoidic volatiles enzymatically released from these grapes were indicative of their aroma potential in norisoprenoidic odorants and could be used to assess this potential.



[a] the samples studied were those of table 5

Fig. 2. Linear regression between the total C13-norisoprenoid levels released by enzymatic hydrolysis and acid hydrolysis (μg L^{-1}) of Melon B. grape glycosidic extracts (the samples studied were those of Table 5).

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