

Effects of Different β -D-Glycosidases on Bound Aroma Compounds in Muscat Grape Determined by HS-SPME and GC-MS

Wenhui Kang³, Yan Xu^{1,2,3,*}, Ling Qin⁴ and Yuxia Wang³

ABSTRACT

J. Inst. Brew. 116(1), 70–77, 2010

Important “floral” aromas naturally occur in grapes predominantly as flavourless glycoconjugate precursors. Since these aroma compounds can be released by hydrolysis, different glycosidase enzymes can potentially contribute different aromas to wines. In this paper, we first established a procedure for profiling the free and bound volatile compounds in grape using GC-MS combined with headspace solid-phase microextraction (HS-SPME). Comparison of the free and bound aroma compounds revealed that non-volatile glycosides, known as aroma precursors, occur in high concentrations in musts. Among all compounds identified, 11 were fully quantified according to established standard calibration curves, while others were semi-quantified. Using three different glycosidase enzymes, a total of 38 bound volatile compounds were identified in Muscat grape, including terpenes, higher alcohols, C-6 compounds, and phenols, among others. The different enzymes had significant effects on the varietal aroma. Principal component analysis indicated that the characteristic aroma hydrolyzed by the commercial enzyme AR2000 was clearly different from that produced by other enzymes.

Key words: Bound aroma compound, flavour, GC-MS, β -D-glycosidase, HS-SPME, Muscat grape.

INTRODUCTION

Winemaking is a biotechnological process. Its flavour is influenced by many factors, such as the variety of grape, the yeast, enzymes and other enological parameters^{3,10}. With the development of modern scientific technology, increasing attention is now being paid to the enhancement of varietal flavours^{1,33}. The varietal flavour of a wine is mainly derived from glycosylated aroma precursors³³. In wine grapes, the major precursors include structures such as β -D-glucopyranoside, β -apiosyl- β -D-glucopyranoside, α -L-arabinofuranosyl- β -D-glucopyranoside and

α -L-rhamno-pyranosyl- β -D-glucopyranoside^{16,36,40}. Numerous aglycones have been identified following hydrolysis of wine and grape juice glycosides, including terpenes, norisoprenoids, aliphatics and phenolic compounds³⁹. It is widely accepted that β -D-glycosidase (β G, EC 3.2.1.21) is the key enzyme responsible for the hydrolysis of aroma precursors⁹.

For aroma precursor analysis, many methods have been proposed and generally involve extraction and detection of the glycoside^{6,7,11,13,24,29,32}. A modified method was developed by Arevalo et al., which involved extraction on a C₁₈ reversed-phase (RP) cartridge and detection based on quantification of the glucose released through hydrolysis of precursors^{1,38}. Another method, used by Lopez et al., was based on quantification of the volatile compounds, released following mild acid hydrolysis, by gas chromatography-mass spectrometry (GC-MS)¹⁹. However, because acid hydrolysis promoted by heating causes the rearrangement of monoterpene aglycones²⁰, enzymatic hydrolysis methods have generally been recommended for analysis of bound aromas. Previously, flavour analysis generally relied on distillation and solvent extraction (e.g., pentane/diethyl ether, dichloromethane)⁸. Currently, headspace solid-phase microextraction (HS-SPME) is widely used. This method uses no solvent, is more rapid and is also highly reproducible and sensitive^{5,8,12,22,27,30,42}. For these reasons, the current study on bound aroma analysis used methods based on extraction on a C₁₈ RP cartridge, enzymatic hydrolysis, HS-SPME and GC-MS.

Addition of commercial glycosidic enzymes to wine can greatly increase desirable volatiles^{4,6,9,17,24}. When musts were fermented using a fungal glycosidase enzyme, the enzyme-treated wines had different sensorial attributes from control, unhydrolyzed wines, and were judged as being more floral and fruity with sweet, ripened fruit notes²⁴. Use of *Saccharomyces* and non-*Saccharomyces* wine yeasts, with high glycosidase activity, can also exert a critical influence on the levels of most varietal aroma compounds, affecting all families including nor-isoprenoids, terpenols, benzenoids, volatile phenols, vanillins and lactones¹⁷. Van Rensburg and Pretorius³⁴ mentioned that enzymes important in winemaking would include pectinase, proteinases, glycosidases and glucose oxidase, among others. Of these, the β -D-glycosidases (β Gs) are one of the key factors that contribute to wine varietal aroma and flavour³⁴. The use of different sources of β G enzymes has been proposed as a potential method for improving wine flavour. Although many papers have ex-

¹Jiangnan Univ, State Key Lab Food Sci & Technol, Wuxi 214122, P.R. China.

²Jiangnan Univ, Minist Educ, Key Lab Ind Biotechnol, Wuxi 214122, P.R. China.

³Jiangnan Univ, Sch Biotechnol, Wuxi 214122, P.R. China.

⁴Department of Life Science, Hebei Normal University of Science and Technology, 066004 Changli, Hebei, P. R. China.

* Corresponding author. E-mail: biosean@yahoo.com.cn.

aminated changes in bound compounds in many varieties^{17,23,28}, little research to date has been carried out on commercial enzymes containing different β G enzymes and their effects on the aromatic flavours in wines.

The main objective of the present study was to compare the changes in aroma compounds using different forms of β G enzymes and a profiling method to analyze the free or released aroma compounds in grapes. Principal component analysis (PCA) was used for these comparisons.

MATERIALS AND METHODS

Isolation of the glycosides

After de-stemming, crushing and pressing, the must from the Muscat grape (vintage 2008) provided by a winery in Changyu, Shandong Province, China was employed. Samples were centrifuged at 4,500 rpm for 5 min and stored at -20°C prior to analysis. The glycoside fractions were isolated using C_{18} RP cartridges (0.5 g, Waters, Milford, MA, USA) according to the method proposed by Villena et al., with a few modifications¹. The cartridges were pretreated with 10 mL methanol (HPLC grade) followed by 10 mL Milli-Q water. An appropriate volume of each sample (2 mL for must) was loaded onto the cartridge. These volumes were considered optimal for column retention capacity. The cartridge was then washed with 30 mL water. The free aroma fraction was eluted with 10 mL of pentane and discarded, and the column was washed with 10 mL water. The glycoside fraction was eluted with 10 mL of methanol. The methanolic extracts were evaporated to dryness under vacuum and resuspended in 2 mL of 0.1 M citrate phosphate buffer (pH 5.0). About 40 samples were collected together prior to hydrolysis.

Experimental treatments

Three enzyme preparations were tested. The commercial enzyme preparation AR2000 (DSM, France) contains several glycosidase activities, including β -D-glycosidase, α -L-arabinofuranosidase, α -L-arabinopyranosidase, and α -L-rhamnosidase, all of which can hydrolyze different glycosides to yield free flavour aglycones². The second enzyme, β -D-glycosidase (EC 3.2.1.21, from *Aspergillus niger*), was purchased from Sigma. The third enzyme was a generous donation (Wei-rong Yao, Jiangnan University, China) from a microbial population that was screened personally and identified as *Aspergillus niger*¹⁸.

A typical enzymatic treatment consisted of first transferring 8 mL of glycosidic mixture into 20 mL sealed glass headspace vials. To the mixture, either AR2000 (AR), purchased β -D-glycosidase (BGA) or donated β -D-glycosidase (BGY) was added (5 U/mL). β -D-glycosidase activity was assayed by measuring the amount of *p*-nitrophenol (pNP) liberated from *p*-nitrophenyl- β -D-glycoside (pNPG) (Sigma-Aldrich, USA) as substrate. A glycosidic sample without added enzyme (NE) served as a control. To analyze free aroma compounds, the original grape juice sample (FS) was used. Referencing the method proposed by Schneider et al.,²⁹ glycosidic mixtures were incubated at 40°C for 48 h, then cooled to room temperature. All treatments were carried out in duplicate.

Chemicals and reagents

Aroma standards β -myrcene, limonene, linalool, α -terpineol, citronellol, geraniol, 2-octanol, 3-methyl-1-butanol, 2-ethyl-1-hexanol, benzyl alcohol, phenylethylalcohol and 2-methyl-4-vinylphenol were purchased from Sigma. A C6–C30 hydrocarbon mixture, used for determination of Kovats retention indices, was obtained from Fluka (Sigma-Aldrich).

HS-SPME

A 50/30 μm DVB/CAR/PDMS fibre (Supelco, Inc., Bellefonte, PA) was used for aroma extraction. The sample (8 mL), transferred to a 20 mL vial (item S126-0020, I-CHEM), was saturated with sodium chloride (2.4 g), then 5 μL of a 2-octanol internal standard solution was added to each vial to give a final 2-octanol concentration of $79.06 \mu\text{g L}^{-1}$. Each sample was equilibrated at 45°C in a thermostatic bath for 45 min, then extracted for 30 min at the same temperature, with stirring. After extraction, the fibre was inserted into the injection port of the GC (250°C) to desorb the analytes.

GC-MS analysis

A Gerstel MPS2 auto sampler (Gerstel, Baltimore, MD, USA) mounted to an Agilent 6890N gas chromatograph (Little Falls, DE, USA), paired with an Agilent 5975 mass selective detector, constituted the analytical system. The software used was MSD ChemStation.

A DB-Wax column (30 m \times 0.32 mm I.D., 0.25 μm film thickness) (J&W Scientific, Folsom, CA, USA) was used for all analyses. Helium carrier gas was used with a total flow of 2 mL min^{-1} . The oven parameters were as follows: initial temperature was 50°C held for 2.0 min, followed by an increase to 230°C at a rate of $6^{\circ}\text{C min}^{-1}$, the oven was then held at 230°C for 25 min before returning to the initial temperature (50°C). The MS detector was operated in scan mode (mass range 50–200) and the transfer line to the MS system was maintained at 250°C .

Identification and quantification

The identification of the volatile standard compounds was based on the comparison of their GC mass spectra and retention times with those of authentic standards from Sigma-Aldrich. The tentative identification of compounds for which it was not possible to find reference volatiles, was carried out by comparison of their mass spectra with spectral data from the Wiley G 1035 A library. For quantification purposes, calibration curves were used when standards were available; otherwise, semi-quantitative analyses were done, assuming a response factor equal to one.

Volatile compound content was calculated from the GC-peak areas relative to the GC-peak area of the internal standard.

Chemical aroma standard mixtures were prepared in a model solution similar to the extracted grape solution (5 g L^{-1} of tartaric acid dissolved in Milli-Q water, pH adjusted to 5.0 with NaOH). Standard concentrations ranged from $0.01 \mu\text{g L}^{-1}$ to 2 mg L^{-1} and were selected to bracket the concentrations of each individual compound found in the grape samples.

Standard curves were created using the optimized headspace SPME sampling method (45°C for 45 min, DVB/CAR/PDMS fibre) with subsequent injection into the GC-MS using the conditions described below.

The peak area of each standard target ion relative to the peak area of the 2-octanol internal standard (target ion) was plotted against the ratio of the standard to 2-octanol to create a standard curve. The linear regression equations obtained were used to calculate the concentration (g L^{-1}) of each compound in every sample.

Identification of compounds detected by GC-MS analysis was done by comparing mass spectra and retention indices (RI) with the authentic standards and published data, as well as by comparing their mass spectra with the MS library of Wiley7.0 and Nist05. Retention indices were calculated using a mixture of n-paraffin C6–C30 as standards.

Semi-quantitative determinations were obtained using 2-octanol as an internal standard. Volatile compound content was calculated from the GC-peak areas relating to the GC-peak area of the internal standard.

Statistical analysis

Means, relative standard deviation (RSD) and linear regressions (standard curves) were calculated in Excel (Microsoft, Redmond, WA, USA). The statistical significance of the free and bound volatile compounds was determined by one-way ANOVA. Principal component analysis (PCA) was performed, using the SPSS 17.0 for Windows statistical software package.

RESULTS AND DISCUSSION

HS-SPME sampling parameters

Extractions were carried out at 40, 45 and 50°C for 30, 45 and 60 min, based on previously published methods^{8,37}. As shown in Fig. 1, there were no significant differences between samples extracted at different temperatures and times. A sample temperature of 45°C and SPME extraction time of 45 min were finally selected as a compromise between the appropriate temperature and time.

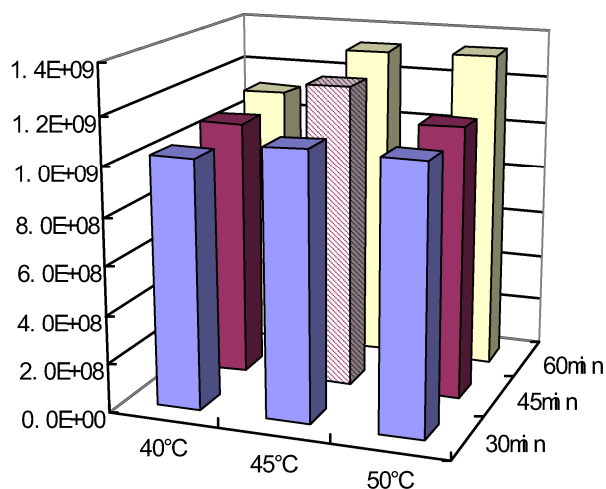


Fig. 1. Comparison of extraction temperature and time on total peak area of volatiles extracted from the grape homogenates.

Standard calibration curves

Calibration curves for 11 volatile compounds were obtained using the optimized HS-SPME sampling method (Table I). In nearly all cases, R^2 was >0.99 . The limit of quantification was dependent on the volatile compound. Calibration in a model matrix is useful as a screening protocol for monitoring overall changes in wide variety of matrices. For the most accurate quantification, calibration curves in matrices matched to the grape samples were used (e.g., standard addition calibrations) for all analytes.

Identification of the purpose of the enzymatic hydrolysis

To verify the results of the enzymatic hydrolysis, the sample hydrolyzed by AR was analyzed and compared to grape juice (FS) and the no-enzyme sample (NE) as controls. Three typical chromatograms obtained from three typical samples are shown in Fig. 2.

As shown in Tables II and III, the levels of aromatic compounds in the AR sample (36 compounds) released by enzymatic hydrolysis were dramatically increased compared to the FS (29 compounds) and NE (7 compounds). Eleven of these compounds were fully quantified with authentic standards using calibration curves (Table II). For the remaining compounds, relative concentrations were calculated based on the response of 2-octanol I.S. (Table III).

Only a few volatile compounds (e.g. linalool) were present in NE. After enzymatic hydrolysis, many compounds were liberated from the matrix. As shown in Tables II and III, the category and quantities of the AR sample were much higher than in the NE, similar to other reports⁴¹. The total terpene content ($4665.83 \mu\text{g/L}$) of AR (Table II) was notably higher than in the NE ($214.60 \mu\text{g/L}$). This result suggested that the method might be successfully used to analyze the bound compounds.

Free (FS) and bound (AR) compounds were compared. As shown for terpenes in Table II, which are very important compounds in Muscat grape, the total concentration of AR and FS was 4665.83 and $378.48 \mu\text{g/L}$, respectively. The former was 12.2 fold higher than the latter. Among the semi-quantified compounds (Table III), the total peak areas of terpenes in AR accounted for more than 62%. The terpene concentrations in AR and FS were 963.0 and $31.45 \mu\text{g/L}$, respectively. Similar patterns were seen for the higher alcohols, ketones, etc., in the AR and FS. The results showed that non-volatile glycosides, known as aroma precursors, occur in high concentrations in musts, in agreement with previous work^{24,32}.

Table I. Calibration curves for free and bound aroma compounds in grape. Volatile compounds identified were expressed as $\mu\text{g/L}$.

Compounds	Calibration curve	
β -myrcene	$y = 0.0393x + 0.0004$	$R^2 = 0.995$
limonene	$y = 0.0125x - 0.0002$	$R^2 = 0.990$
linalool	$y = 0.1909x + 0.0054$	$R^2 = 0.999$
α -terpineol	$y = 0.1304x + 0.0074$	$R^2 = 0.994$
citronellol	$y = 0.1246x - 0.0071$	$R^2 = 0.999$
geraniol	$y = 0.1241x - 0.0068$	$R^2 = 0.992$
3-methyl-1-butanol	$y = 0.0191x + 0.0031$	$R^2 = 0.991$
2-ethyl-1-hexanol	$y = 0.4488x + 0.0056$	$R^2 = 0.999$
benzyl alcohol	$y = 0.0033x - 0.0008$	$R^2 = 0.997$
Phenylethyl alcohol	$y = 0.009x + 0.0029$	$R^2 = 0.995$
2-methoxy-4-vinylphenol	$y = 0.0052x - 0.0004$	$R^2 = 0.996$

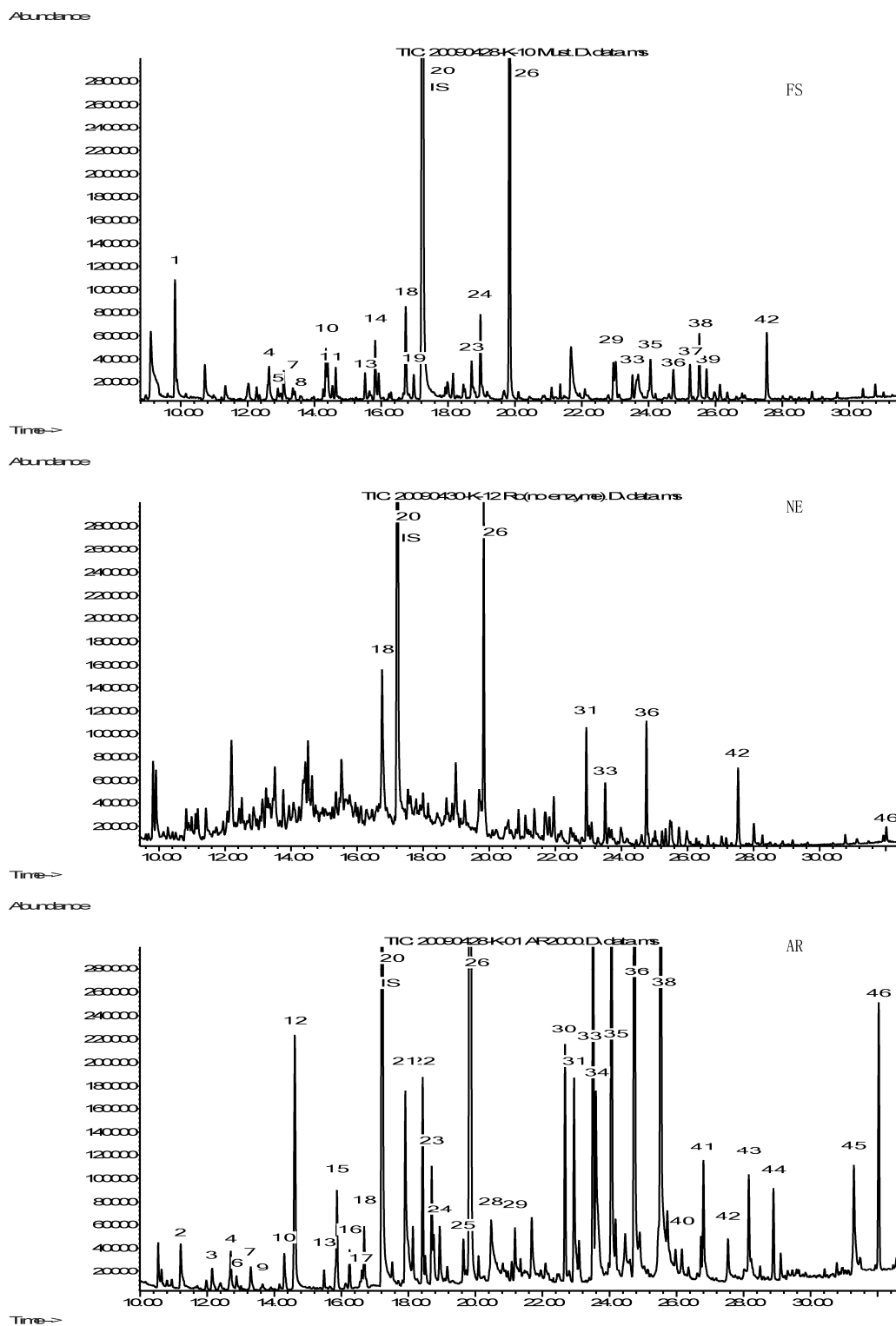


Fig. 2. Total ion chromatogram of three typical samples (FS, NE and AR) obtained by HS-SPME at the optimal sampling condition. Time scale in minutes on *x*-axis; ion abundance (mV) on *y*-axis. Peak identification: 1, hexanal; 2, β -myrcene; 3, limonene; 4, 3-methyl-1-butanol; 5, (E)-2-hexenal; 6, (E)-3,7-dimethyl-1,3,6-octatriene; 7, (Z)-3,7-dimethyl-1,3,6-octatriene; 8, 5-methyl-1,3,6-heptatriene; 9, styrene; 10, 2-octanone; 11, octanal; 12, 3-hydroxy-2-butanone; 13, 6-methyl-5-hepten-2-one; 14, 1-hexanol; 15, cis-rose oxide; 16, trans-rose oxide; 17, 2-nonanone; 18, nonanal; 19, (E)-2-hexen-1-ol; 20, (IS) 2-octanol; 21, *cis*-linaloloxide; 22, 4-methyl-2-(2-methyl-1-propenyl)-3,6-dihydro-2H-pyran; 23, 2-ethyl-1-hexanol; 24, decanal; 25, benzaldehyde; 26, linalool; 27, 3,7-dimethyl-1,5,7-octatrien-3-ol; 28, 2,3-butanediol; 29, p-menth-1-en-4-ol; 30, (Z)-3,7-dimethyl-2,6-octadienal; 31, α -terpineol; 32, (2,6,6-trimethyl-2-cyclohexen-1-yl)methanol; 33, N-ethyl-benzenamine; 34, geraniol; 35, citronellol; 36, (E)-2,7-dimethyl-2,6-octadien-1-ol; 37, 3,4-dimethyl-benzaldehyde; 38, nerol; 39, *cis*-geranylacetone; 40, benzyl alcohol; 41, phenylethyl alcohol; 42, 1-dodecanol; 43, 2-methyl-phenol; 44, ethyl tetradecanoate; 45, 4-vinylguaiaacol; 46, ethyl palmitate.

Table II. Effect of different enzymes on the fully quantified compounds ($\mu\text{g/L}$).

No.	RT	Compounds	AR ^a	BGA ^b	BGY ^c	FS ^d	NE ^e
1	11.210	β -myrcene	325.72 \pm 4.46	276.33 \pm 4.09	259.69 \pm 3.51	-	-
2	12.147	limonene	344.03 \pm 4.54	231.01 \pm 3.47	301.74 \pm 3.28	-	-
3	19.852	linalool	1659.47 \pm 38.55	905.42 \pm 89.50	721.36 \pm 2.30	245.24 \pm 19.24	127.16 \pm 10.50
4	22.943	α -terpineol	227.67 \pm 1.96	81.10 \pm 1.76	67.20 \pm 0.31	13.70 \pm 0.77	87.44 \pm 2.50
5	24.061	citronellol	486.86 \pm 1.02	302.29 \pm 5.57	233.19 \pm 5.75	16.27 \pm 1.19	-
6	12.702	3-methyl-1-butanol	517.545 \pm 117.27	4104.40 \pm 198.95	848.17 \pm 3.21	149.98 \pm 0.81	-
7	18.697	2-ethyl-1-hexanol	79.60 \pm 3.54	15.09 \pm 0.32	23.41 \pm 5.57	7.61 \pm 0.14	-
8	26.160	benzyl alcohol	1364.96 \pm 24.09	1040.94 \pm 106.93	535.94 \pm 37.44	-	-
9	26.801	phenylethyl alcohol	3437.00 \pm 184.52	8112.64 \pm 98.28	2593.92 \pm 156.55	60.18 \pm 0.45	-
10	31.290	4-vinylguaiaicol	5883.84 \pm 20.95	826.54 \pm 1.92	1045.48 \pm 19.56	-	-
11	23.593	geraniol	1622.08 \pm 18.16	1224.37 \pm 36.63	880.06 \pm 26.39	103.27 \pm 8.95	-

^a AR2000 enzyme preparation.^b Sigma β -D-glycosidase.^c Donated β -D-glycosidase.^d Grape juice.^e No-enzyme sample.**Table III.** Effect of different enzymes on the semi-quantified compounds ($\mu\text{g/L}$).

No.	RT	Compound name	AR ^a	BGA ^b	BGY ^c	FS ^d	NE ^e
1	9.826	hexanal	-	-	-	19.69 \pm 0.80	23.42 \pm 0.44
5	12.904	(E)-2-hexenal	-	8.43 \pm 1.66	-	2.06 \pm 0.05	-
6	12.875	(E)-3,7-dimethyl-1,3,6-octatriene	4.65 \pm 0.13	10.26 \pm 1.69	4.52 \pm 0.74	-	-
7	13.299	(Z)-3,7-dimethyl-1,3,6-octatriene	9.97 \pm 1.21	-	8.18 \pm 0.38	4.17 \pm 0.80	-
8	13.408	5-methyl-1,3,6-heptatriene	-	-	-	1.63 \pm 0.04	-
9	13.654	styrene	2.00 \pm 0.25	-	-	-	-
10	14.298	2-octanone	12.25 \pm 2.25	11.60 \pm 2.12	10.93 \pm 1.68	7.77 \pm 1.13	-
11	14.396	octanal	-	-	-	6.48 \pm 1.12	-
12	14.613	3-hydroxy-2-butanone	103.68 \pm 2.09	3.83 \pm 0.83	-	2.42 \pm 0.45	-
13	15.486	6-methyl-5-hepten-2-one	5.50 \pm 0.70	3.63 \pm 0.63	4.11 \pm 0.27	4.20 \pm 0.36	-
14	15.828	1-hexanol	-	11.29 \pm 0.79	6.75 \pm 0.75	10.19 \pm 0.38	-
15	15.871	cis-rose oxide	32.40 \pm 3.40	31.64 \pm 7.03	15.92 \pm 0.23	4.85 \pm 0.33	-
16	16.246	trans-Rose oxide	11.66 \pm 1.80	15.72 \pm 1.51	5.66 \pm 0.21	-	-
17	16.616	2-nonanone	5.98 \pm 0.63	3.13 \pm 0.63	-	-	-
18	16.683	nonanal	18.54 \pm 3.36	10.18 \pm 1.68	11.68 \pm 1.83	16.74 \pm 0.83	52.78 \pm 0.87
19	16.971	(E)-2-hexen-1-ol	-	-	-	5.30 \pm 0.01	-
21	17.909	cis-linaloloxide	92.52 \pm 5.04	59.45 \pm 9.20	81.92 \pm 4.79	1.37 \pm 0.18	-
22	18.427	4-methyl-2-(2-methyl-1-propenyl)-3,6-dihydro-2H-pyran	60.61 \pm 4.47	39.82 \pm 6.51	20.37 \pm 1.94	-	-
24	18.935	decanal	22.76 \pm 5.58	19.68 \pm 0.16	16.73 \pm 2.73	13.21 \pm 1.50	-
25	19.642	benzaldehyde	14.38 \pm 7.83	7.76 \pm 0.11	3.30 \pm 0.63	-	-
27	21.095	3,7-dimethyl-1,5,7-octatrien-3-ol	-	-	-	2.11 \pm 0.10	-
28	20.471	2,3-butanediol	41.53 \pm 4.36	-	-	-	-
29	21.18	p-menth-1-en-4-ol	18.74 \pm 5.52	-	-	-	-
30	22.673	(Z)-3,7-dimethyl-2,6-octadienal	78.91 \pm 6.03	116.66 \pm 25.79	-	-	-
32	23.092	(2,6,6-trimethyl-2-cyclohexen-1-yl) methanol	17.72 \pm 2.10	-	-	-	-
33	23.509	N-ethyl-benzenamine	133.51 \pm 70.36	172.10 \pm 29.46	156.44 \pm 33.34	3.00 \pm 0.60	18.69 \pm 0.56
36	24.744	(E)-2,7-dimethyl-2,6-octadien-1-ol	345.91 \pm 7.79	191.50 \pm 44.15	285.55 \pm 30.64	6.11 \pm 0.47	-
37	25.237	3,4-dimethyl-benzaldehyde	-	-	-	7.03 \pm 0.84	-
38	25.529	nerol	350.52 \pm 24.83	312.77 \pm 59.76	183.74 \pm 5.59	12.84 \pm 1.30	-
39	25.733	cis-geranylacetone	-	-	-	4.27 \pm 0.27	-
42	27.536	1-dodecanol	12.16 \pm 2.05	19.23 \pm 2.24	10.58 \pm 2.13	9.58 \pm 1.68	25.34 \pm 0.68
43	28.156	2-methyl-phenol	32.70 \pm 1.68	-	-	-	-
44	28.89	ethyl tetradecanoate	27.34 \pm 3.28	-	6.16 \pm 0.52	-	-
46	32.031	ethyl palmitate	82.31 \pm 17.24	17.22 \pm 3.19	10.68 \pm 0.01	3.75 \pm 0.55	4.86 \pm 0.50

^a AR2000 enzyme preparation.^b Sigma β -D-glycosidase.^c Donated β -D-glycosidase.^d Grape juice.^e No-enzyme sample.

Effect of different enzymes on bound aroma compounds

During vinification, different enzyme preparations that can impart different characteristic aromas should be investigated. In the present study, three enzymes containing β -D-glycosidase activity were selected for investigation of their effects on wine aroma. Enzymatic treatment dramati-

cally increased the total concentration of aroma compounds released (Table II and III), as previously reported by other researchers^{6,36}. A total of 45 volatile compounds were identified in Muscat, including terpenes, higher alcohols, C-6 compounds, esters and phenols.

Eleven of the compounds shown in Table II were quantified with authentic standards using calibration curves. Total concentration of identified volatiles in AR, BGA and

BGY were 15948.80, 17120.14 and 7510.15 $\mu\text{g/L}$, respectively. The statistical analysis results showed that different enzymatic treatment had a significant effect on the varietal aromas.

Monoterpenes are important odour and flavour compounds in grapes, and include geraniol, linalool, citronellol, α -terpineol and nerol among others^{3,10}. Linalool has a typical floral odour, which is a typical varietal aroma of Muscat. In this experiment, AR treatment enhanced the release of linalool. The compound α -terpineol has a characteristic lilac odour, with a sweet taste reminiscent of peach on dilution. Limonene has a pleasant, lemon-like odour. Limonene released by BGA was lower than by BGY or AR. β -Myrcene has a pleasant, sweet, balsamic, plastic odour. The amount of β -myrcene released by AR was significantly higher than by BGA and BGY. Citronellol and geraniol have a characteristic rose-like odour. Similar to other terpenes, AR can significantly enhanced the release of α -terpineol, citronellol and geraniol from the bound form.

Alcohols were also prominently influenced by enzyme type. The compound 3-methyl-1-butanol (isoamyl alcohol) has a fuel oil, whiskey-characteristic pungent odour. The concentration in the BGA treated samples was 4104.40 $\mu\text{g/L}$, greater than other enzymatic treatments. The minimal detection threshold value is 250 ppb, so it has a prominent effect on the aroma characteristics. The average concentration of 2-ethyl-1-hexanol was lower than isoamyl alcohol. The concentration in the BGA treated samples was lower than in those treated with AR and BGY.

With regard to higher alcohols, benzyl alcohol and 2-phenylethanol were identified. Benzyl alcohol has a characteristic pleasant, fruity odour and a slightly pungent, sweet taste, with aroma recognition threshold values of 1.2 ppm²⁵. BGA and AR released higher levels of benzyl alcohol. Phenylethyl alcohol has a characteristic rose-like odour and an initially slightly bitter taste, then sweet and reminiscent of peach. The concentration of 2-phenylethanol in the BGA sample (8112.64 $\mu\text{g/L}$) was significantly higher than in the BGY (2593.92 $\mu\text{g/L}$) and AR (3437.00 $\mu\text{g/L}$) samples. The compound 2-phenylethanol has been reported as the most important bound compound in Albarino grapes³¹.

4-Vinylguaiacol (PVG) was also detected. It has a curry-like odour with a low volatile threshold value (10 ppb), and it can be formed by thermal degradation of ferulic acid. The concentration of 4-vinylguaiacol in AR, BGA and BGY was 5883.84, 826.54 and 1781.98 $\mu\text{g/L}$ respectively, therefore it might be a substantial contributor to the overall aroma of samples.

For the other semi-quantified compounds shown in Table III, a total of 34 compounds were identified including 11 terpenes, 4 alcohols, 5 aldehydes, 5 ketones, 1 phenol, 2 esters, 4 benzene derivatives and others.

Terpenes dominated the odour and flavour compounds in grapes, accounting for more than 62% of the total concentration. These results agreed with other studies³¹. Terpene levels in AR, BGA and BGY were 963, 738 and 503.57 $\mu\text{g/L}$, respectively. In the case of alcohols, 2,3-butanediol was only found in AR samples, not in BAG and BGY samples. However, 1-hexanol was only found in

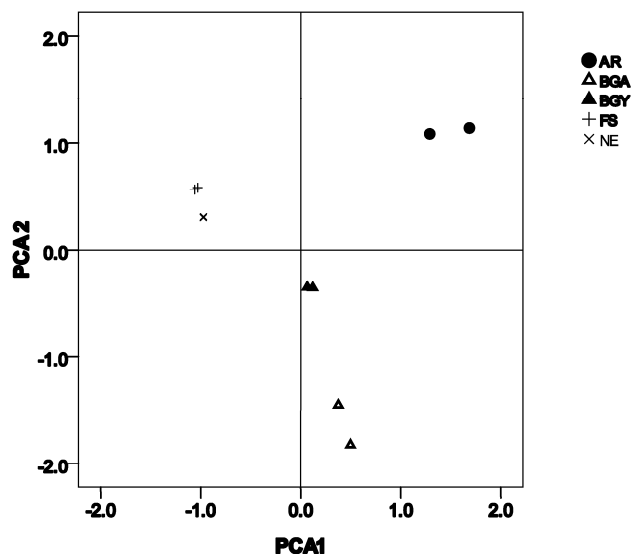


Fig. 3. Principal component analysis of different samples. Legend: (●) AR-2000 enzyme preparation; (△) BGA, Sigma β -D-glycosidase; (▲) BGY, donated β -D-glycosidase; (+) FS, grape juice; (x) NE, no-enzyme sample.

BGA and BGY samples, not in AR samples. Aldehyde concentrations in AR, BGA and BGY samples were 41.3, 38.29, 28.41 $\mu\text{g/L}$, respectively. The different enzymatic treatments showed great differences in ketone levels. The 3-hydroxy-2-butanone levels in the AR samples were significantly higher than in the BGA samples, but were not detected in the BGY samples. For esters, two compounds were identified in Muscat grape, and showed levels of 109.65, 17.22, 16.84 $\mu\text{g/L}$ in AR, BGA and BGY respectively. The compound 2-methyl phenol was detected only in the AR samples. Benzene derivatives and others were also identified.

The above results indicated there were significant differences in the typical characteristic aroma compounds after hydrolyzing with different enzymes containing β -D-glycosidase. Similar to our results, Ferreira et al.¹⁴ and Rodríguez et al.²⁶ noted that selected species of non-*Saccharomyces* yeasts (*Candida*, *Kloeckera*, *Pichia*, and *Metschnikowia*) had high glycosidase activities that could potentially contribute aromas to wines by releasing flavour compounds from glycosidically-bound, non-volatile precursors.

Principal component analysis

Pre-fermentation processing enzymes including pectinase, β -D-glycosidase and protease etc. have been used for a long time by the wine and juice industries^{15,21}. In recent years, increasing attention has been paid to β -D-glycosidase, and its contribution to the formation of varietal character of wines^{21,35}. For comparing the changes in aroma, principal component analysis (PCA) was employed in the present study. PCA indicated that different samples can generally be distinguished based on their volatile composition. The first principal component (x-axis; explaining 58.35% of the variability in the data) was influenced by most of compounds. The second principal component (y-axis; explaining 18.40% of the variability in the data) was mainly influenced by the terpenes (such

as linalool, *cis/trans*-rose oxide, (E/Z)-3,7-dimethyl-1,3,6-octatriene, (Z)-3,7-dimethyl-2,6-octadienal) and higher alcohols (benzyl alcohol and phenylethyl alcohol). As shown in Fig. 3, the aromatic character of AR was clearly different from that of the BGA and BGY. There was a relatively close relationship between BGA and BGY.

CONCLUSIONS

A combined HS-SPME/GC-MS method has been developed that can be used to profile and quantify free and enzymatically released volatile compounds in Muscat grapes. According to the established analytical method, a total of 36, 29 and 7 volatile compounds were identified in the samples AR, FS and NE, respectively. The results showed that substantial aroma flavours were released by enzymatic hydrolysis, indicating that these important "floral" aromas are naturally present in grapes as non-volatile bound glycosides.

Using our established method, we were able to observe significant changes in released aroma compounds by using different enzyme preparations containing β -D-glycosidase. A total of 38 released aroma compounds were identified in Muscat, including terpenes, higher alcohols, C-6 compounds, esters, and phenols, among others. Eleven of the compounds were quantified with authentic standards, and the other compounds were semi-quantified based on response of the 2-octanol. Moreover, evaluation by principal component analysis of three enzyme treatments showed great differences in the aroma characteristics of the samples, indicating that different typical wine aromas can be enhanced during vinification by selecting appropriate enzyme preparations containing β -D-glycosidase.

ACKNOWLEDGEMENTS

This project was partially funded with support from the Ministry of Science and Technology, P. R. China under No. 2001BA501B07 and from the Program for Changjiang Scholars and Innovative Research Team in the University (PCSIRT) under IRT0532. We thank Ji-ming Li and Wei-rong Yao for the donation of the grapes and the BGY enzyme, respectively.

REFERENCES

1. Arevalo Villena, M., Diez Perez, J., Ubeda, J. F., Navascues, E. and Briones, A. I., A rapid method for quantifying aroma precursors: Application to grape extract, musts and wines made from several varieties. *Food Chem.*, 2006, **99**, 183-190.
2. Baek, H. H. and Cadwallader, K. R., Contribution of free and glycosidically bound volatile compounds to the aroma of muscadine grape juice. *J. Food Sci.*, 1999, **64**, 441-444.
3. Berger, R. G., *Flavours and Fragrances*. Springer Verlag: Berlin, 2007.
4. Bloem, A., Lonvaud, A., Bertrand, A., de Revel, G., Wender, L. P. B. and Mikael Agerlin, P., Ability of *Oenococcus oeni* to influence vanillin levels. *Dev. Food Sci.*, 2006, **43**, 137-140.
5. Bonino, M., Schellino, R., Rizzi, C., Aigotti, R., Delfini, C. and Baiocchi, C., Aroma compounds of an Italian wine (Ruche) by HS-SPME analysis coupled with GC-ITMS. *Food Chem.*, 2003, **80**, 125-133.
6. Cabaroglu, T., Selli, S., Canbas, A., Lepoutre, J.-P. and Gunata, Z., Wine flavor enhancement through the use of exogenous fungal glycosidases. *Enzyme Microb. Technol.*, 2003, **33**, 581-587.
7. Cabrita, M. J., Freitas, A. M. C., Laureano, O., Borsa, D. and Di Stefano, R., Aroma compounds in varietal wines from Alentejo, Portugal. *J. Food Comp. Anal.*, 2007, **20**, 375-390.
8. Canuti, V., Conversano, M., Calzi, M. L., Heymann, H., Matthews, M. A. and Ebeler, S. E., Headspace solid-phase microextraction-gas chromatography-mass spectrometry for profiling free volatile compounds in Cabernet Sauvignon grapes and wines. *J. Chromatogr. A* 2009, **1216**, 3012-3022.
9. Castro Vázquez, L., Pérez-Coello, M. S. and Cabezudo, M. D., Effects of enzyme treatment and skin extraction on varietal volatiles in Spanish wines made from chardonnay, muscat, airén, and macabeo grapes. *Anal. Chim. Acta*, 2002, **458**, 39-44.
10. Clarke, R. J. and Bakker, J., *Wine flavour chemistry*. Blackwell Pub: Oxford, 2004.
11. D'Incecco, N., Bartowsky, E., Kassara, S., Lante, A., Spetolli, P. and Henschke, P., Release of glycosidically bound flavour compounds of chardonnay by *Oenococcus oeni* during malolactic fermentation. *Food Microbiol.*, 2004, **21**, 257-265.
12. Fan, W. L., Xu, Y. and Yu, A. M., Influence of oak chips geographical origin, toast level, dosage and aging time on volatile compounds of apple cider. *J. Inst. Brew.*, 2006, **112**, 255-263.
13. Fang, Y., *Development of volatile compounds in pinot noir grapes and their contributions to wine aroma*. PhD thesis, Oregon State University, 2006.
14. Ferreira, A. M., Clímaco, M. C. and Faia, A. M., The role of non-*Saccharomyces* species in releasing glycosidic bound fraction of grape aroma components. *J. Appl. Microbiol.*, 2001, **91**, 67-71.
15. Fugelsang, K. C. and Edwards, C. G., *Wine Microbiology: Practical Applications and Procedures*. Springer: New York, 2007.
16. Gunata, Y. Z., Bayonove, C. L., Tapiero, C. and Cordonnier, R. E., Hydrolysis of grape monoterpenyl β -D-glucosides by various β -glucosidases. *J. Agr. Food Chem.*, 1990, **38**, 1232-1236.
17. Hernandez-Orte, P., Cersosimo, M., Loscos, N., Cacho, J., Garcia-Moruno, E. and Ferreira, V., The development of varietal aroma from non-floral grapes by yeasts of different genera. *Food Chem.*, 2008, **107**, 1064-1077.
18. Liu, L., Zhu, S., Zhu, T., Zhang, M., Wu, J. and Chen, J., Production of gentiooligosaccharide by recombinant β -glucosidase. *Wei Sheng Wu Xue Bao*, 2009, **49**, 597-602.
19. Lopez, R., Ezpeleta, E., Sanchez, I., Cacho, J. and Ferreira, V., Analysis of the aroma intensities of volatile compounds released from mild acid hydrolysates of odourless precursors extracted from tempranillo and grenache grapes using gas chromatography-olfactometry. *Food Chem.*, 2004, **88**, 95-103.
20. Mateo, J. J. and Di Stefano, R., Description of the β -glucosidase activity of wine yeasts. *Food Microbiol.*, 1997, **14**, 583-591.
21. Moreno-Arribas, M. V. and Polo, C., *Wine Chemistry and Biochemistry*. Springer New York: New York, 2008.
22. Ong, P. K., The flavor chemistry of rambutan (*Nephelium lappaceum* L.) and lychee (*Litchi chinensis* Sonn.). PhD thesis, Cornell University, 1998.
23. Orlic, S., Huic, K., Urlic, B. and Redzepovic, S., Screening for production of extracellular hydrolytic enzymes by *Saccharomyces* wine yeasts isolated from Croatian vineyards. *Periodicum Biologorum*, 2007, **109**, 201-204.
24. Palomo, E. S., Hidalgo, M. C. D., Gonzalez-Vinas, M. A. and Perez-Coello, M. S., Aroma enhancement in wines from different grape varieties using exogenous glycosidases. *Food Chem.*, 2005, **92**, 627-635.
25. Rapp, A. and Mandery, H., Wine aroma. *Cell. Molec. Life Sci.*, 1986, **42**, 873-884.
26. Rodríguez, M. E., Lopes, C. A., Broock, M., Valles, S., Ramón, D. and Caballero, A. C., Screening and typing of Patagonian wine yeasts for glycosidase activities. *J. Appl. Microbiol.*, 2004, **96**, 84-95.
27. Rodríguez-Bencomo, J. J., Conde, J. E., Garcia-Montelongo, F. and Perez-Trujillo, J. P., Determination of major compounds in sweet wines by headspace solid-phase microextraction and gas chromatography. *J. Chromatogr. A*, 2003, **991**, 13-22.

28. Rodriguez-Bencomo, J. J., Mendez-Siverio, J. J., Perez-Trujillo, J. P. and Cacho, J., Effect of skin contact on bound aroma and free volatiles of Listan Blanco wine. *Food Chem.*, 2008, **110**, 214-225.
29. Schneider, R., Charrier, F., Moutounet, M. and Baumes, R., Rapid analysis of grape aroma glycoconjugates using Fourier-transform infrared spectrometry and chemometric techniques. *Anal. Chim. Acta*, 2004, **513**, 91-96.
30. Schneider, R., Razungles, A., Augier, C. and Baumes, R., Monoterpenic and norisoprenoid glycoconjugates of *Vitis vinifera* l. Cv. Melon b. As precursors of odorants in muscadet wines. *J. Chromatogr. A*, 2001, **936**, 145-157.
31. Selli, S., Cabaroglu, T., Canbas, A., Erten, H. and Nurgel, C., Effect of skin contact on the aroma composition of the musts of *Vitis vinifera* l. Cv. Muscat of bornova and narince grown in Turkey. *Food Chem.*, 2003, **81**, 341-347.
32. Ugliano, M., Bartowsky, E. J., McCarthy, J., Moio, L. and Henschke, P. A., Hydrolysis and transformation of grape glycosidically bound volatile compounds during fermentation with three *Saccharomyces* yeast strains. *J. Agr. Food Chem.*, 2006, **54**, 6322-6331.
33. Ugliano, M., Genovese, A. and Moio, L., Hydrolysis of wine aroma precursors during malolactic fermentation with four commercial starter cultures of *Oenococcus oeni*. *J. Agr. Food Chem.*, 2003, **51**, 5073-5078.
34. van Rensburg, P. and Pretorius, I. S., Enzymes in winemaking: Harnessing natural catalysts for efficient biotransformations - a review. *S. Afr. J. Enol. Viticult.*, 2000, **21** (Special issue), 52-70.
35. Villena, M. A., Iranzo, J. F. U. and Perez, A. I. B., β -Glucosidase activity in wine yeasts: Application in enology. *Enzyme Microb. Technol.*, 2007, **40**, 420-425.
36. Voirin, S. G., Baumes, R. L., Bitteur, S. M., Gunata, Z. Y. and Bayonove, C. L., Novel monoterpene disaccharide glycosides of *Vitis vinifera* grapes. *J. Agr. Food Chem.*, 1990, **38**, 1373-1378.
37. Wang, L., Xu, Y., Zhao, G. and Li, J., Rapid analysis of flavor volatiles in apple wine using headspace solid-phase microextraction. *J. Inst. Brew.*, 2004, **110**, 57-65.
38. Williams, P. J., Cynkar, W., Francis, I. L., Gray, J. D., Iland, P. G. and Coombe, B. G., Quantification of glycosides in grapes, juices, and wines through a determination of glycosyl glucose. *J. Agr. Food Chem.*, 1995, **43**, 121-128.
39. Williams, P. J., Sefton, M. A. and Marinos, V. A., Hydrolytic flavor release from non-volatile precursors in fruits, wines and some other plant-derived foods. In: Recent Developments in Flavor and Fragrance Chemistry. R. Hopp and K. Mori, Eds., 1993, pp. 283-290.
40. Williams, P. J., Strauss, C. R., Wilson, B. and Massywestropp, R. A., Studies on the hydrolysis of *Vitis vinifera* monoterpene precursor compounds and model monoterpene β -D-glucosides rationalizing the monoterpene composition of grapes. *J. Agr. Food Chem.*, 1982, **30**, 1219-1223.
41. Wilson, B., Strauss, C. R. and Williams, P. J., Changes in free and glycosidically bound monoterpenes in developing muscat grapes. *J. Agr. Food Chem.*, 1984, **32**, 919-924.
42. Xu, Y., Fan, W. L. and Qian, M. C., Characterization of aroma compounds in apple cider using solvent-assisted flavor evaporation and headspace solid-phase microextraction. *J. Agr. Food Chem.*, 2007, **55**, 3051-3057.

(Manuscript accepted for publication February 2010)