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ScienceDirect
Food Chemistry 107 (2008) 1064–1077
Food Chemistry
www.elsevier.com/locate/foodchem

The development of varietal aroma from non-floral grapes by yeasts of different genera

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Received 26 April 2007; received in revised form 27 July 2007; accepted 10 September 2007

Abstract

A fraction of glycosidic precursors extracted from different non-floral grapes has been reconstituted with a synthetic must and the must has been fermented in duplicate by yeasts belonging to different genera previously selected by their high glycosidase activity (*Saccharomyces cerivisiae*, *Saccharomyces bayanus*; *S. cerevisiae x S. bayanus*, *Brettanomyces bruxellensis*, *Hanseniaspora uvarum*, *Kloeckera apiculata*, *Torulaspora delbrueckii* and *Debaryomyces carsonii*). Fermentation was allowed to take place for 3 weeks, but only was complete for *Saccharomyces* yeasts. The wines obtained were analyzed by sensory analysis and by gas chromatography and gas chromatography–mass spectrometry to determine the sensory descriptors and the aroma composition. The results have shown that the yeast genus exerts a critical influence on the levels of most varietal aroma compounds, affecting to all families coming from precursors, including norisoprenoids, terpenols, benzenoids, volatile phenols, vanillins and lactones. Leaving aside ethylphenols and vinylphenols, most aroma compounds are produced at relatively low concentrations, but in numbers enough to likely cause a sensory effect.

Keywords: Yeast; Wine; Fermentation; Aroma; Variety

1. Introduction

It is a fact that the aroma of young, non aged, wine is formed mainly by fermentation. Although the grapes and the musts from non-floral grape varietals do not show intense or explicit flavors, the wines obtained after their fermentation often show pleasant aromas which can be related to the varietal origin (Delfini et al., 2001). The nature of these varietal aromas is only partly known because only in a limited group of varieties are we able to clearly assign the chemicals responsible for the varietal aroma. This is clearly the case of Muscat grapes, which even in the unfermented must show their specific terpenic character (Ribéreau-Gayon, Boidron, & Terrier, 1975). In the cases of Sauvignon Blanc or Verdejo grapes, the varietal character has been successfully attributed to some polyfunctional mercaptans which are released by the yeast during fermentation (Campo, Cacho, & Ferreira, 2005; Tominaga, Darriet, & Dubourdieu, 1996; Tominaga, Murat, & Dubourdieu, 1998). In many other cases such as those of Chardonnay or Macabeo, however, the compounds causing the varietal impression have not been clearly identified (Escudero et al., 2004; Lee & Noble, 2006; Lorrain et al., 2006). Although a part of the varietal impression is related to the amino acid profile of the variety (Hernández-Orte, Cacho, & Ferreira, 2002), a significant part of it is assumed to come from specific odorless precursors (Francis, Kassara, Noble, & Williams, 1999; Williams & Francis, 1996; Williams, Sefton, & Wilson, 1989). These precursors can

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doi:10.1016/j.foodchem.2007.09.032

be glycosides, polyhydroxilated molecules (Williams, Strauss, & Wilson, 1980) or cysteinil-derivatives (Tominaga, Peyrot des Gachons, & Dubourdieu, 1998).

The action of yeasts on the glycosidic and polyhydroxilated precursors to form aroma molecules related to the variety is not well known. Although much research has been conducted (Delcroix, Gunata, Sapis, Salmon, & Bayonove, 1994; Fernandez-Gonzalez & Di Stefano, 2004; Fernandez-Gonzalez, Di Stefano, & Briones, 2003; Hernandez, Espinosa, Fernandez-Gonzalez, & Briones, 2003; Mateo & Di Stefano, 1997; Spagna, Barbagallo, Palmeri, Restuccia, & Giudici, 2002; Ugliano, Bartowsky, McCarthy, Moio, & Henschke, 2006), a large part has focused exclusively on the formation of terpene molecules. These molecules are, no doubt, important aroma contributors but they are not key constituents in many wines made from non-floral grapes (Culleré, Escudero, Cacho, & Ferreira, 2004; Escudero et al., 2004; Lopez, Ortin, Perez-Trujillo, Cacho, & Ferreira, 2003). A recent report has shown, so far, that more than 40 different aroma chemicals belonging to different chemical classes are formed or released from precursors during fermentation by *Saccharomyces cerevisiae* (Loscos, Hernandez-Orte, Cacho, & Ferreira, 2007). Differences between yeast strains were not very important and were of a mere quantitative character. On the other hand, it is a fact that some other non-*Saccharomyces* yeasts and bacteria can take part in the fermentation of grapes (Domizio et al., 2007; Egli, Edinger, Mitrakul, & Henick-Kling, 1998; Lema, Garcia-Jares, Orriols, & Angulo, 1996). Grapes arriving to the cellar tend to have variable proportions of some yeasts with a low ethanol tolerance, such as species of *Hanseniaspora, Kloeckera, and Candida* and some of these non-*Saccharomyces* yeasts can proliferate in the first steps of fermentation (Belancic, Gunata, Vallier, & Agosin, 2003; Mansfield, Zoecklein, & Whiton, 2002; Mendes Ferreira, Climaco, & Mendes Faia, 2001). Some reports have demonstrated that those yeasts produce and excrete to the media several enzymes which can interact with precursors to form aroma compounds. However, the type of aroma compounds really formed or released from precursors by these yeasts is not really known. The main goal of the present work is, therefore, to study the differential abilities of yeasts belonging to different genus to form aroma molecules from grape precursors during fermentation.

2. Materials and methods

2.1. Microorganisms and growing conditions

Five *Saccharomyces cerevisiae* strains (ISE 1; ISE 4; ISE 40; ISE 196) and type strain, CBS 1171 (ISE 1450); three *Saccharomyces bayanus* (ISE 250; ISE 949) and type strain CBS 4309 (ISE 1449); one natural hybrid of *S. cerevisiae* and *S. uvarum* selected by Ciolfi (Velletri, Italy) S6U (Ciolfi, 1992, 1994); four *Brettanomyces* (ISE 371; ISE 372; ISE 373; ISE 374); three *Hanseniaspora uvarum*, ISE 1342; ISE 1336; and type strain CBS 479 (ISE 1456); three *Kloeckera apiculata* (ISE 308; ISE 345; ISE 346), one *Torulaspora delbrueckii*, type strain CBS 1146 (ISE 1448) and one *Debaryomyces carsonii* (ISE 302) strain were considered in the initial study.

The strains belong to the collection of the CRA Istituto Sperimentale per l'Enologia (ISE) at Asti, and they were originally isolated from musts or wines from various wine-producing areas in Italy and other countries (Table 1).

All the yeasts were grown in YEPG medium (yeast extract 1%; peptone 1%; glucose 2%) at 25 °C. The cellular growth was controlled by absorbance at 610 nm (one absorbance unit corresponds to 2.4×10^7 CFU/ml). When the inocula reached an optic density equivalent to 50×10^6 cells/ml, they were centrifuged at 2795*g* for 10 min and the supernatant was discarded.

2.2. Determination of β-glucosidase activity

Such activity was carried out after the method proposed by Mateo and Di Stefano (Mateo & Di Stefano, 1997) with some slight modifications. The pellet obtained in the preparation of the inoculum was washed with 10 ml of NaCl 0.9% (w/v), was centrifuged and was re-suspended in 10 ml of buffer citrate/phosphate 0.2 M pH 5. After shaking it in the vortex, it was incubated at 30 °C for 24 h.

Table 1
Yeast strains considered in this study

Yeasts	Strains	Codes	Relative activity[a] pH 5
Saccharomyces			
Saccharomyces cerevisiae	ISE 1		+
	ISE 40	L40	+++
	ISE 196		+
	ISE 1450	L1450	+++
	ISE 4		+
S. cerevisiae x S.uvarum	**ISE S6u**	LS6u	+++
S. bayanus	**ISE 1449**	L1449	+++
	ISE 250	L250	+++
	ISE 949		+
Non-saccharomyces			
B. bruxellensis	ISE 371		+
	ISE 374	L374	+++
	ISE 373		+
	ISE372	L372	+++
Hanseniaspora uvarum	ISE 1342		+
	ISE 1336		++
	ISE 1456	L1456	+++
Kloeckera apiculata	**ISE 346**	L346	+++
	ISE 345		++
	IS 308	L308	+++
Torulaspora delbrueckii	**ISE 1448**	L1448	+++
Debaryomyces carsonii	**ISE 302**	L302	+++

Distribution of β-glycosidase activity among yeast strains *Saccharomyces* and non-*Saccharomyces* and codes (in bold) for the yeasts selected for further work.

[a] Activities (+, trace; ++, medium; +++ strong) against *p*-NPG (see Section 2 for details).

All these operations were carried out under strict sterile conditions. After 24 h, the inoculum was centrifuged again, and was again re-suspended in 2 ml of a citrate/phosphate buffer 0.2 M pH 5 containing 7 mM of *p*-nitrophenyl-β-D-glucopyranoside (*p*-PNG, Fluka, Buchs, Switzerland) and incubated at 25 °C for 2 h. The samples were spiked with 2 ml of Na_2CO_3 0.1 M (pH 10.2), filtered (0.22 μm) and their absorbance at 400 nm was finally measured.

2.3. Preparation of the precursor extract

The precursors were extracted from four different non-floral grape varieties (Verdejo, Chardonnay, Garnacha and Tempranillo) in order to obtain a complex "multivarietal" pool of precursors. Grapes were treated in batches of 500 g of grapes of a single variety, and were destemmed by hand and homogenized with a mixer Ultra Turrax T25 Basic (Ika, Labortechnik) in presence of 0.13 M NaF and 50 mg/l ascorbic acid. The triturate was centrifuged at 2264*g* for 15 min at 5 °C to separate the must from the skins, followed by a filtration through filter paper. The mashes of skins obtained (around 80 g per batch) were suspended in 380 ml of a buffer solution (0.1 M Na_2HPO_4/NaH_2PO_4) at pH 7 and 13% ethanol and allowed to macerate in the dark (36 h, 20 °C, nitrogen atmosphere) to extract the precursors. This solution was centrifuged at 2264*g* for 15 min at 20 °C, and the supernatant was filtered through filter paper. Ethanol was then removed at room temperature by vacuum distillation in a rotary evaporator. This solution (ca. 260 ml per batch) is the "macerate". The must (ca. 300 ml per batch) and the macerate were percolated through two LiChrolut EN (1300 mg) resin beds (previously pre-conditioned with 32 ml of dichloromethane, 32 ml of methanol and 65 ml of water). In both cases the column was washed with 26 ml of water, and then with 40 ml of a pentane:dichloromethane (2:1, v/v) mixture. The retained precursors were finally eluted with 50 ml of an ethyl acetate:methanol (9:1, v/v) mixture (ethyl acetate extract). Three batches per variety were processed, and the corresponding ethyl acetate extracts were mixed and evaporated under vacuum to dryness. These dry extracts were reconstituted in 20 ml of a 50% ethanol solution. Finally, the macerate and must extracts for the four varieties were mixed to form the multivarietal mix used to spike the musts.

2.4. Alcoholic fermentation

Fermentation medium. Synthetic nutrient medium (SNM) prepared as described by Wickerham (1951) was supplemented with glucose (200 g/l) and buffered to pH 3.5 with KOH. Before yeast inoculation, the medium was sterilized by filtration (0.45 μm Schleicher & Schull, Postfch, Germany).

Fermentation conditions. Cells were pre-cultured in YEPG. Fermentations were carried out in 250 ml sterile Erlenmeyer flasks kept in an incubator regulated at 20 °C. Fermentations were monitored by CO_2 release: the amount of CO_2 released was determined by measuring weight loss at least every 24 h.

Experimental treatments. The 12 yeast strains with a strong β-glucosidase activity (Table 1) were selected for the alcoholic fermentation. This was carried out in duplicate in Erlenmeyers. Each flask was filled with 150 ml of the SNM, 3.9 ml of the glycosidic precursors extract (which approximately corresponds to the original precursor concentration in the grapes) and was inoculated with yeasts at 10^6 cells/ml.

When the weight of the samples became constant, but never before 3 weeks, wines were centrifuged at 2795*g* for 10 min, were stored 2 days at 4 °C for sensory evaluation and were finally kept frozen until the analysis of aroma compounds.

2.5. Extraction and analysis of minor volatile compounds (SPE and GC–ion trap–MS analysis)

This analysis was carried out using the method proposed and validated by López, Aznar, Cacho, and Ferreira (2002). The method was modified to use a smaller quantity of sample and also incorporates a new washing step in order to improve the chromatographic resolution. In accordance with this method, 15 ml of wine, containing 10 μl of a surrogate standards solution (isopropyl propanoate, 3-octanone, heptanoic acid and β-damascone, 2000 μg/g in ethanol), was passed through a 50 mg LiChrolut EN cartridge at about 2 ml min^{-1}. The sorbent was washed with 5 ml of 40% methanol solution and dried by letting air pass through (−0.6 bar, 10 min). Analytes were recovered by elution with 600 μl of dichloromethane. An internal standard solution (4-methyl-4-pentanol, 4-hydroxy-4-methyl-2-pentanone and 2-octanol, at a concentration of 350, 450 and 500 μg/g, respectively, in dichloromethane) was added to the eluted sample. The extract was then analyzed by GC with ion trap–MS detection under the conditions described below.

2.6. Extraction and analysis the volatiles liberated by acid hydrolysis

The determination of the volatiles liberated by harsh acid hydrolysis of the aroma precursors in the pool of precursors (sample B2HAH) was carried out using the method proposed by Ibarz, Ferreira, Hernandez-Orte, Loscos, and Cacho (2006).

2.7. Gas chromatography–mass spectrometry conditions

Gas chromatographic analysis was performed with a CP-3800 chromatograph coupled to a Saturn 2200 ion trap mass spectrometric detection system from Varian (Sunnyvale, CA, USA). A DB-WAXETR capillary column (J&W Scientific, Folsom, CA, USA) (60 m × 0.25 mm I.D., film thickness 0.5 μm) preceded by a 3 m × 0.25 mm

uncoated (deactivated, intermediate polarity) precolumn from Supelco (Bellefonte, PA, USA) was used. Helium was the carrier gas at a flow rate of 1 ml min^{-1}. The oven temperature program was 3 min at 40 °C, 10 °C min^{-1} up to 90 °C, 2 °C min^{-1} up to 230 °C and finally held at this temperature for 37 min. Initially the injector was kept at 35 °C during 0.3 min and a pressure pulse of 25 psi during 2.60 min was applied. The injector was then heated to 250 °C at rate of 200 °C min^{-1}. The splitless time was 2.60 min. Silanized glass wood was used as a packing material in the insert. The injection volume was 4 μl. The global run time was recorded in full scan mode (40–220m/z mass range). The chromatographic data were analyzed by Varian Saturn GC–MS Version 6.3 software.

2.8. Major compounds (microextraction and GC–FID analysis)

Quantitative analysis of major compounds was carried out using the method proposed and validated by Ortega, Lopez, Cacho, and Ferreira (2001). In accordance with this method, 3 ml of wine and 7 ml of water were salted with 4.5 g of ammonium sulphate and extracted with 200 μl of dichloromethane. The extract was then analyzed by GC with FID detection using the conditions described elsewhere (Ortega et al., 2001). Quantitative data were obtained by interpolation of relative peak areas in the calibration graphs built by the analysis of synthetic wines containing known amounts of the analytes. 2-Butanol, 4-methyl-2-pentanol, 4-hydroxy-4-methyl-2-pentanone, and 2-octanol, at a concentration of 200 μg/g in dichloromethane, were used as internal standards. The extract was analyzed by GC with FID detection under the conditions described in the reference (Ortega et al., 2001).

2.9. Sensory analysis

Descriptive analysis has been carried out using a structured scale (62 cm long) developed in the Istituto Sperimentale per l'Enologia, Asti (Vaudano et al., 2005). The sensory panel consisted of six females and two males, with ages ranging from 25 to 50, all of them belonging to the laboratory staff and with considerable experience in sensory analysis. Results were processed by ANOVA tests at 95%.

2.10. Statistical treatment

The analysis of variance was carried out with the statistical programme Stat View (SAS Institute, Cary, NC, USA). Principal component analysis (PCA) was carried out with SPSS release 11.2 for Windows (SPSS Inc., Chicago, IL).

3. Results

The glycosidase activities of 21 yeasts belonging to different genera were screened by using p-NPG as substrate at pH 5. Results of this assay are presented in Table 1. As can be seen, 12 strains showed a high hydrolytical activity and were selected for the subsequent study. The table also suggests that the hydrolytical activity is more frequent among non-*Saccharomyces* yeasts.

3.1. Aroma compounds formed from precursors

Table 2 gives the increment of aroma compounds as a consequence of the presence of precursors in the fermentation media. This comparison has been carried out with just two of the selected yeasts, a *Saccharomyces* and a *Brettanomyces*, since the major focus of the present work was to study the differential action of yeasts on the formation of varietal aroma compounds and not to assess the origin of all the possible compounds released or formed from the precursors. Nevertheless, the results were in complete agreement with those recently reported (Loscos et al., 2007) and showed that more than 40 compounds belonging to different classes, such as lactones, cinnamates, volatile phenols, vanillin-derivatives, nor-isoprenoids and terpenols are formed from precursors, independently of the genus of the yeast used. Data in the table also may suggest that yeasts were able to form some aroma compounds from the unspecific precursors present in the synthetic media (see Section 2), such as linalool (Carrau et al., 2005; Hock, Benda, & Schreier, 1984), vanillin, or even β-ionone. While the ability of yeast to synthesize the novo terpenes has been clearly documented in the literature (Carrau et al., 2005; Hock et al., 1984), the ability to form some nor-isoprenoids, such as β-ionone, is less clear, even although some yeasts are able to synthesize carotenoids (Madhour, Anke, Mucci, Davoli, & Weber, 2005). The same can be said of vanillin (Priefert, Rabenhorst, & Steinbuchel, 2001). However, the levels of these compounds in those blank samples were so low that we cannot rule out the possibility that their presence may arise from some impurities present in the reagents used to prepare the synthetic media or even in the inoculum. Results in the table also show that the presence of precursors in the fermenting media brings about some changes in the levels of some fermentative compounds, such as isoamyl alcohol, isobutyric or isovaleric acids, in accordance with results of a previous report (Loscos et al., 2007).

These results are in good agreement with the sensory characteristics of the samples, as shown in Fig. 1. The presence of precursors draws on a significant increase of some sensory nuances of the wines, such as violet, exotic fruit, white flower, peach, roast or dry fruit, in the case of the *Saccharomyces* yeast. These increments in the sensory scores were consistent with the increments observed in the levels of some important aroma compounds, such as vanillin-derivatives, cinnamates, γ and δ-lactones, volatile phenols, terpenols and nor-isoprenoids. In the case of *Brettanomyces*, the most intense effect was a decrease of the cheese note (consistent with the decrease observed in the levels of isovaleric and 2-methylbutyric acids), an increase

Table 2
Effect of the presence of precursors in the fermentation media on the volatile composition of the wines obtained with L372 *Brettanomyces bruxelensis* and the L1450 *Saccharomyces cerevisiae* yeast strains

	Brettanomyces 372			*Saccharomyces cerevisiae* 1450		
	Inc[a]	%	$p(t)$[b]	Inc[a]	%	$p(t)$[b]
Lipids derivatives						
Z-3-Hexen-1-ol	0.30	100	0.010	0.11	100	0.010
δ-Octalactone	0.15	70	0.011	0.39	24	0.102
γ-Nonalactone	0.55	46	0.011	0.63	52	0.011
γ-Decalactone	0.43	29	0.147	0.32	22	0.265
E-Whiskylactone	0.00			0.08	27	0.286
δ-Decalactone	2.29	48	0.011	2.96	14	0.359
2-Ethylhexanoic acid	0.31	5	0.045	0.35	6	0.034
Shikimic derivatives						
Benzenoids						
Benzoic acid	48.3	52	0.012	−57.4	−55	0.111
Benzaldehyde	0.33	15	0.252	0.03	1	0.489
Phenylacetaldehyde	0.83	39	0.252	−0.25	−17	0.118
Ethyl dihydrocinnamate	0.02	100	0.026	0.08	20	0.064
Ethyl cinnamate	0.00			0.33	100	0.010
2-Phenoxyethanol	5.34	36	0.029	2.27	16	0.355
Ethylparaben	0.00			1.37	100	0.010
Volatile phenols						
Guaiacol	0.08	21	0.011	−0.04	−12	0.137
4-Ethylguaiacol	519	100	0.010	0.00		
Eugenol	0.22	100	0.011	0.04	100	0.051
4-Vinylguaiacol	1.08	99	0.011	5.38	97	0.010
E-Isoeugenol	0.72	100	0.011	1.09	100	0.010
4-Ethylphenol	584	100	0.010	0.00		
4-Vinylphenol	2.96	100	0.010	2.79	59	0.010
4-Allyl-2,6-dimethoxyphenol	0.00			0.22	100	0.010
Dihydromethyleugenol[c]	12.3	100	0.010	5.05	100	0.010
Vanillins						
Vanillin	6.29	100	0.010	0.54	8	0.116
Methyl vanillate	15.3	100	0.010	5.41	100	0.010
Ethyl vanillate	2.09	100	0.011	2.98	100	0.011
Acetovanillone	37.4	93	0.010	12.3	80	0.010
Zingerone	1.62	100	0.011	2.87	100	0.010
Homovanillyl alcohol	3.15	100	0.010	1.21	100	0.011
Homovanillic acid[c]	58.0	100	0.010	17.3	100	0.010
Syringaldehyde	7.51	100	0.010	6.92	100	0.025
Acetosyringone	5.75	100	0.010	1.77	100	0.010
Nor-isoprenoids						
β-Damascenone	0.47	100	0.010	0.54	100	0.011
α-Isomethyl-ionone	0.03	5	0.359	0.48	100	0.016
β-Ionone	−0.05	−25	0.269	0.09	34	0.022
3-Oxo-β-ionone[c]	0.25	100	0.010	0.57	100	0.010
Actinidiols[c]	0.47	100	0.011	0.85	100	0.010
3-Oxo-α-ionol[c]	3.65	100	0.010	1.72	100	0.010
3-Hydroxy-7,8-dihydro-β-ionol[c]	0.20	100	0.013	0.18	100	0.012
Terpenes						
Linalool	0.58	90	0.020	0.74	41	0.013
α-Terpineol	0.34	63	0.028	0.39	36	0.016
β-Citronellol	0.62	21	0.315	1.10	31	0.070
Nerol	0.21	100	0.026	0.02	2	0.472
Farnesol (2*E*, 6*E*)	1.13	21	0.146	20.4	57	0.010
Linalool acetate[c]	0.03	16	0.315	0.03	4	0.351
2,6-Dimethyl-1,7-octadien-3,6-diol[c]	0.00			0.09	15	0.151
Terpinyl acetate[c]	0.00			0.12	100	0.010
3,7-Dimethyl-1,5-octadien-3,7-diol[c]	0.52	100	0.010	0.52	100	0.010
Neric acid[c]	9.55	100	0.011	7.09	100	0.012

(*continued on next page*)

Table 2 (*continued*)

	Brettanomyces 372			*Saccharomyces cerevisiae* 1450		
	Inc[a]	%	$p(t)$[b]	Inc[a]	%	$p(t)$[b]
Miscellaneous						
Furfural	0.41	36	0.154	0.10	10	0.345
Pantolactone	0.06	3	0.426	0.76	25	0.025
Fermentative compounds						
Acetaldehyde[d]	0.16	10	0.473	−14.7	−38	0.290
Acetoin[d]	0.00			−4.71	−52	0.246
Isobutanol[d]	0.00			−1.37	−9	0.250
Isoamyl alcohol[d]	0.90	20	0.014	11.7	14	0.010
β-Phenylethanol[d]	1.46	100	0.211	0.14	1	0.470
Isobutyric acid[d]	0.00	0	0.494	−0.22	−107	0.033
Isovaleric acid	−132	−110	0.012	1.70	8	0.281
2-Methylbutyric acid	−84.3	−150	0.017	0.73	6	0.355
Isoamyl acetate[d]	0.00			0.03	30	0.171
Phenylethyl acetate	0.39	100	0.110	45.9	23	0.265
γ-Butyrolactone[d]	−0.02	−9	0.328	0.03	15	0.101
Ethyl decanoate	1.59	43	0.055	0.49	5	0.347
Ethyl 3-hydroxybutyrate[d]	0.00			0.02	100	0.211
Ethyl lactate[d]	0.00			0.40	22	0.251
Diethyl succinate[d]	−0.36	−55	0.028	0.02	10	0.416
Butyric acid[d]	0.09	37	0.306	−0.10	−33	0.207
Hexanoic acid[d]	−0.02	−1	0.483	−0.32	−17	0.104
Octanoic acid[d]	−1.03	−100	0.039	−0.27	−18	0.145
Decanoic acid[d]	−0.13	−242	0.087	0.06	18	0.108

Except were indicated, concentration data are in $\mu g\,l^{-1}$. The compound identification has based on the work of Ibarz et al. (2006).

[a] Increment of aroma compound as a consequence of the presence of precursors.

[b] Significance of the increment as given by a *t*-test.

[c] Data are area normalized to the internal standard.

[d] Data in $mg\,l^{-1}$.

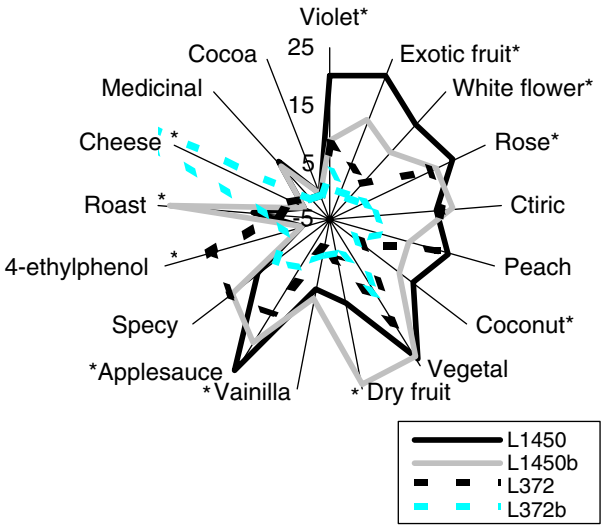

Fig. 1. Sensory descriptive analysis. Effect of the presence of precursors in the aroma of the corresponding wines for a *Saccharomyces cerevisiae* (L1450) and (a) *Brettanomyces bruxellensis* (L372) yeast strain. The (b) denotes blank sample without precursor addition. Data are averages of two replicate samples.*Difference significant at $P < 0.05$.

of the 4-ethylphenol note (explained by the high levels of 4-ethylphenol and 4-ethylguaicol) and a slight increase of the different fruity and flowery notes.

3.2. Role of yeast genus on varietal aroma formation

The subsequent study will focus on the 45 aroma compounds that, according to a previous report and to data in Table 2, are more clearly related to the presence of precursors. Quantitative results for these compounds are given in Tables 3 and 4, while Fig. 2 shows the principal component plots obtained with these data. The plot reveals a very interesting thing: the six blank samples plus the two samples inoculated with *Debaryomyces*, whose fermentative activity was insignificant, are all grouped together in the left part of the plot. Cluster analysis confirmed the existence of this clustering (data not shown). Blank samples include both fermentations of synthetic media (without presence of precursors) and non-fermented synthetic media with precursors kept at room temperature the whole experiment. For the latter, the presence of aroma compounds should be attributed exclusively to the natural acid hydrolysis of glycosides. As the variable loadings plot reveals, this group of eight samples contained minima levels of nearly all the aroma compounds, since most aroma compounds have positive loadings in the first principal component. This clearly indicates that the formation of the varietal aroma compounds requires strictly both the existence of fermentation and the presence of precursors, confirming previous results (Loscos et al., 2007) and in agreement with the everyday experience of winemakers.

Table 3
Aroma composition of the wines obtained by fermenting a SNM containing a precursor extract with different yeasts

Yeasts	L1450	L40	L1449	L250	S6u	L1448	L1456	L308	L346	L372	L374	L302
Lipids derivatives												
Z-3-Hexen-1-ol	0.11 ± 0.01	0.07 ± 0.03	0.08 ± 0.02	0.09 ± 0.01	0.09 ± 0.01	0.10 ± 0.04	0.05 ± 0.04	0.06 ± 0.03	0.05 ± 0.02	0.30 ± 0.01	0.30 ± 0.01	0.32 ± 0.02
γ-Octalactone	nd	nd	nd	nd	nd	nd	0.62 ± 0.02	0.50 ± 0.19	0.38 ± 0.10	nd	0.48 ± 0.03	nd
δ-Octalactone	1.64 ± 0.44	1.79 ± 0.14	1.21 ± 0.13	1.42 ± 0.01	1.26 ± 0.49	0.83 ± 0.02	0.48 ± 0.01	0.49 ± 0.08	0.49 ± 0.05	0.21 ± 0.01	0.24 ± 0.02	0.67 ± 0.70
γ-Nonalactone	1.21 ± 0.04	1.46 ± 0.10	1.14 ± 0.05	1.12 ± 0.01	1.12 ± 0.07	2.05 ± 0.06	1.52 ± 0.01	1.20 ± 0.20	1.30 ± 0.01	1.18 ± 0.13	0.92 ± 1.31	0.75 ± 0.71
γ-Decalactone	1.47 ± 0.03	1.10 ± 0.26	1.47 ± 0.80	0.70 ± 0.15	1.29 ± 0.16	8.60 ± 0.36	0.91 ± 0.09	0.99 ± 0.21	0.84 ± 0.26	1.46 ± 0.37	1.44 ± 0.06	8.37 ± 0.71
E-Whiskylactone	0.31 ± 0.16	0.42 ± 0.12	1.29 ± 0.42	2.24 ± 0.20	0.72 ± 0.20	nd	0.65 ± 0.25	3.0 ± 2.63	2.08 ± 0.48	nd	nd	nd
δ-Decalactone	20.5 ± 7.91	28.1 ± 1.78	18.4 ± 0.27	20.4 ± 2.90	24.2 ± 3.60	11.9 ± 0.79	8.39 ± 0.77	6.5 ± 9.29	12.3 ± 3.92	4.78 ± 0.12	4.49 ± 0.12	14.7 ± 2.12
δ-Nonalactone	nd	nd	nd	nd	nd	nd	0.07 ± 0.03	nd	nd	nd	nd	nd
2-Ethylhexanoic acid	5.78 ± 0.03	6.76 ± 0.68	5.83 ± 0.19	5.48 ± 0.10	5.90 ± 0.21	5.88 ± 0.22	6.21 ± 0.29	5.83 ± 0.26	5.92 ± 0.10	6.25 ± 0.14	6.27 ± 0.05	7.18 ± 0.14
Shikimic derivatives												
Benzenoids												
Benzoic acid	104 ± 1.18	84.6 ± 6.97	185 ± 19.5	134 ± 19.5	152 ± 20.9	32.0 ± 5.99	130 ± 8.82	114 ± 21.6	116 ± 2.54	92.7 ± 0.42	108 ± 0.64	8.46 ± 0.57
Benzaldehyde	3.22 ± 0.47	3.99 ± 0.59	4.83 ± 0.39	2.38 ± 0.35	3.37 ± 0.36	2.36 ± 0.03	2.81 ± 0.43	5.12 ± 0.80	4.16 ± 0.26	2.25 ± 0.47	2.75 ± 0.48	2.12 ± 0.14
Phenylacetaldehyde	1.47 ± 0.20	2.87 ± 0.83	6.68 ± 2.58	6.00 ± 0.16	2.27 ± 0.07	2.51 ± 0.29	5.09 ± 0.01	15.1 ± 3.30	8.32 ± 0.52	2.16 ± 1.46	1.55 ± 0.29	0.86 ± 0.14
Ethyl dihydrocinnamate	0.38 ± 0.08	0.24 ± 0.02	0.36 ± 0.02	0.81 ± 0.03	0.37 ± 0.08	1.09 ± 0.01	0.71 ± 0.27	0.08 ± 0.01	0.12 ± 0.04	0.02 ± 0.01	0.04 ± 0.01	nd
Ethyl cinnamate	0.33 ± 0.03	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
2-Phenoxyethanol	13.7 ± 6.98	17.4 ± 0.03	13.3 ± 2.55	12.3 ± 1.81	17.4 ± 3.06	23.2 ± 2.93	19.2 ± 4.19	11.7 ± 1.61	15.0 ± 3.83	14.7 ± 0.73	13.4 ± 1.83	14.5 ± 1.41
Ethylparaben	1.37 ± 0.26	1.82 ± 0.43	1.52 ± 0.01	1.52 ± 0.30	1.70 ± 0.05	nd	nd	nd	nd	nd	nd	nd
Volatile phenols												
Guaiacol	0.34 ± 0.01	0.43 ± 0.01	0.40 ± 0.05	0.42 ± 0.01	0.42 ± 0.02	0.45 ± 0.01	0.36 ± 0.01	0.36 ± 0.02	0.35 ± 0.01	0.39 ± 0.02	0.41 ± 0.02	1.34 ± 0.07
4-Ethylguaiacol	nd	nd	nd	nd	nd	nd	0.07 ± 0.06	nd	nd	519 ± 78.0	473 ± 23.4	nd
m-Cresol	nd	nd	nd	nd	nd	nd	0.15 ± 0.01	nd	nd	nd	0.15 ± 0.01	nd
Eugenol	0.04 ± 0.02	0.19 ± 0.06	0.26 ± 0.10	nd	0.19 ± 0.08	0.53 ± 0.08	0.04 ± 0.04	nd	nd	0.22 ± 0.06	0.13 ± 0.01	0.40 ± 0.07
4-Vinylguaiacol	5.57 ± 0.24	111 ± 5.73	59.8 ± 4.14	67.2 ± 4.07	80.0 ± 0.05	2.57 ± 0.25	6.99 ± 0.37	13.9 ± 8.79	10.5 ± 3.62	1.09 ± 0.14	1.03 ± 0.13	4.60 ± 0.71
E-Isoeugenol	1.09 ± 0.14	1.60 ± 0.37	2.07 ± 0.60	1.52 ± 0.28	2.01 ± 0.36	2.89 ± 0.33	1.07 ± 0.25	0.84 ± 0.01	0.74 ± 1.04	0.72 ± 0.15	0.56 ± 0.01	1.74 ± 0.35
4-Ethylphenol	nd	nd	nd	nd	nd	0.05 ± 0.01	nd	nd	nd	585 ± 77.5	525 ± 30.0	0.04 ± 0.01
4-Vinylphenol	4.70 ± 0.01	178 ± 12.8	117 ± 24.6	136 ± 6.06	149 ± 16.9	4.22 ± 0.02	8.39 ± 1.79	13.7 ± 3.90	21.3 ± 5.59	2.96 ± 0.12	2.69 ± 0.10	12.0 ± 1.41
4-Allyl-2,6-dimethoxyphenol	0.22 ± 0.03	0.48 ± 0.02	0.28 ± 0.07	0.51 ± 0.03	0.72 ± 0.09	nd	0.14 ± 0.01	0.24 ± 0.01	nd	nd	0.21 ± 0.01	nd
Dihydromethyleugenol[a]	5.05 ± 0.22	7.74 ± 0.43	7.28 ± 0.48	7.68 ± 0.55	6.97 ± 0.80	5.24 ± 0.11	7.98 ± 0.10	9.72 ± 4.47	8.24 ± 1.23	12.2 ± 0.94	10.5 ± 0.61	5.24 ± 0.11
Vainillins												
Vanillin	6.99 ± 0.41	7.47 ± 0.36	7.43 ± 0.72	7.66 ± 0.94	8.03 ± 1.54	8.98 ± 0.78	7.80 ± 0.75	7.09 ± 0.24	6.77 ± 0.78	6.29 ± 0.02	6.24 ± 0.01	6.33 ± 0.07
Methyl vanillate	5.41 ± 0.19	9.02 ± 0.25	9.11 ± 0.51	9.89 ± 0.01	8.93 ± 1.60	1.90 ± 0.18	9.31 ± 0.37	8.53 ± 1.22	9.55 ± 0.25	15.3 ± 1.86	13.7 ± 0.59	0.57 ± 0.07
Ethyl vanillate	2.98 ± 0.62	1.80 ± 0.12	2.23 ± 0.48	1.87 ± 0.08	1.98 ± 0.22	12.4 ± 1.61	2.67 ± 0.19	2.32 ± 0.17	3.26 ± 0.83	2.09 ± 0.15	2.34 ± 0.29	4.19 ± 0.71
Acetovanillone	15.3 ± 0.06	24.8 ± 1.26	25.4 ± 0.59	25.3 ± 0.44	24.7 ± 4.01	12.3 ± 0.30	23.4 ± 0.25	23.6 ± 3.13	25.8 ± 1.72	40.3 ± 4.42	35.1 ± 1.92	11.3 ± 0.71
Zingerone	2.87 ± 0.11	5.75 ± 0.48	5.31 ± 0.02	4.26 ± 0.04	5.06 ± 0.89	0.94 ± 0.02	4.11 ± 0.52	2.83 ± 0.77	2.80 ± 0.98	1.62 ± 0.40	1.65 ± 0.42	0.58 ± 0.07
Homovanillyl alcohol	1.21 ± 0.05	0.91 ± 0.11	1.0 ± 0.01	1.06 ± 0.12	0.90 ± 0.07	nd	nd	1.15 ± 0.12	1.06 ± 0.12	3.15 ± 0.36	5.86 ± 0.69	2.31 ± 0.21
Homovanillic acid[a]	17.2 ± 1.11	29.1 ± 5.49	31.7 ± 3.73	25.4 ± 1.96	35.8 ± 3.92	23.1 ± 1.67	19.1 ± 2.20	26.0 ± 4.65	16 ± 16.5	57.9 ± 1.24	58.6 ± 3.43	23.1 ± 1.67
Syringaldehyde	6.92 ± 0.95	6.16 ± 0.08	7.19 ± 0.63	6.30 ± 0.11	6.23 ± 0.19	13.3 ± 1.13	nd	6.80 ± 0.16	6.68 ± 0.94	7.51 ± 0.68	6.15 ± 0.06	6.02 ± 0.14
Acetosyringone	1.77 ± 0.02	2.96 ± 0.04	3.04 ± 0.12	3.20 ± 0.01	2.65 ± 0.37	1.40 ± 0.04	2.12 ± 0.09	2.42 ± 0.24	2.55 ± 0.22	5.75 ± 0.36	5.18 ± 0.33	1.81 ± 0.21
Nor-isoprenoids												
β-Damascenone	0.54 ± 0.03	0.60 ± 0.04	0.55 ± 0.03	0.65 ± 0.05	0.63 ± 0.03	0.21 ± 0.02	0.45 ± 0.04	0.40 ± 0.05	0.42 ± 0.03	0.47 ± 0.10	0.39 ± 0.01	0.41 ± 0.07
α-Isomethyl-ionone	0.48 ± 0.03	0.75 ± 0.09	0.68 ± 0.03	0.43 ± 0.02	0.56 ± 0.02	0.44 ± 0.01	0.56 ± 0.05	0.59 ± 0.09	0.55 ± 0.01	0.57 ± 0.07	0.57 ± 0.07	0.56 ± 0.02
β-Ionone	0.27 ± 0.03	0.37 ± 0.04	0.32 ± 0.02	0.22 ± 0.02	0.29 ± 0.05	nd	0.08 ± 0.01	0.14 ± 0.07	0.11 ± 0.01	0.18 ± 0.05	0.17 ± 0.07	0.17 ± 0.07
Riesling acetal[a]	nd	nd	nd	nd	nd	0.35 ± 0.12	nd	nd	nd	nd	nd	nd
3-Oxo-β-ionone[a]	0.57 ± 0.12	0.61 ± 0.01	0.58 ± 0.11	0.66 ± 0.02	0.57 ± 0.09	0.95 ± 0.08	0.45 ± 0.01	0.36 ± 0.10	0.37 ± 0.03	0.25 ± 0.06	0.21 ± 0.01	0.95 ± 0.08

Actinidiols[a]	0.85 ± 0.15	0.99 ± 0.09	0.86 ± 0.25	1.10 ± 0.05	0.94 ± 0.26	1.68 ± 0.17	0.84 ± 0.06	0.69 ± 0.12	0.70 ± 0.01	0.47 ± 0.03	0.39 ± 0.01	1.68 ± 0.17
Norisoprenoide[a]	nd	0.01 ± 0.02	nd	nd	0.01 ± 0.02	nd	nd	nd	nd	nd	nd	
3-oxo-α-ionol[a]	1.72 ± 0.17	3.08 ± 0.31	2.47 ± 0.03	3.02 ± 0.25	2.79 ± 0.69	2.32 ± 0.28	1.79 ± 0.22	1.2 ± 1.70	3.31 ± 0.74	3.65 ± 0.01	3.08 ± 0.25	2.32 ± 0.28
3-Hydroxy-7,8-dihydro-β-ionol[a]	0.18 ± 0.01	0.21 ± 0.02	0.24 ± 0.03	0.17 ± 0.03	0.18 ± 0.02	nd	0.22 ± 0.01	0.24 ± 0.04	0.22 ± 0.05	0.20 ± 0.07	0.18 ± 0.02	nd
2,3-Dehidro-4-oxo-β-ionol[a]	nd	0.28 ± 0.08	nd	nd	0.19 ± 0.02	nd	0.18 ± 0.01	0.16 ± 0.01	0.21 ± 0.06	nd	0.14 ± 0.05	nd
Terpenes												
Linalool	1.80 ± 0.20	2.51 ± 0.11	1.60 ± 0.08	1.87 ± 0.04	2.09 ± 0.05	1.18 ± 0.04	0.87 ± 0.09	0.72 ± 0.15	0.72 ± 0.04	0.64 ± 0.16	0.54 ± 0.04	0.83 ± 0.07
α-Terpineol	1.07 ± 0.09	1.14 ± 0.03	1.03 ± 0.06	0.89 ± 0.02	1.08 ± 0.14	1.39 ± 0.17	0.63 ± 0.07	0.56 ± 0.02	0.56 ± 0.05	0.53 ± 0.11	0.51 ± 0.01	0.67 ± 0.07
β-Citronellol	3.61 ± 0.59	4.05 ± 0.23	4.82 ± 0.36	3.15 ± 0.38	3.65 ± 0.76	2.49 ± 0.15	2.94 ± 0.17	4.07 ± 0.75	3.12 ± 0.33	2.96 ± 1.20	2.74 ± 0.40	1.75 ± 0.28
Nerol	1.27 ± 0.39	0.73 ± 0.21	0.73 ± 0.05	0.53 ± 0.08	0.62 ± 0.24	1.81 ± 0.01	0.38 ± 0.16	0.64 ± 0.47	0.53 ± 0.15	0.21 ± 0.07	0.07 ± 0.02	2.25 ± 0.21
Farnesol (2*E*, 6*E*)	35.8 ± 0.98	35.4 ± 8.03	45.8 ± 10.0	32.4 ± 21.0	44.3 ± 5.00	89.6 ± 4.53	13.1 ± 1.10	7.37 ± 0.98	16.1 ± 5.02	5.35 ± 0.88	4.81 ± 2.15	nd
Linalool acetate[a]	0.62 ± 0.06	0.98 ± 0.16	1.09 ± 0.04	0.52 ± 0.14	0.79 ± 0.09	nd	0.07 ± 0.01	0.11 ± 0.03	0.07 ± 0.01	nd	0.20 ± 0.04	nd
Terpinen-4-ol[a]	0.16 ± 0.03	0.17 ± 0.02	0.17 ± 0.03	0.14 ± 0.01	0.13 ± 0.01	nd	0.11 ± 0.03	0.11 ± 0.03	0.09 ± 0.01	0.17 ± 0.06	0.07 ± 0.01	nd
2,6-Dimethyl-1,7-octadien-3,6-diol[a]	0.60 ± 0.07	0.75 ± 0.06	0.86 ± 0.08	0.50 ± 0.04	0.81 ± 0.11	nd	nd	nd	nd	0.08 ± 0.01	nd	nd
Terpinyl acetate[a]	0.12 ± 0.02	0.17 ± 0.01	0.26 ± 0.11	0.16 ± 0.01	0.17 ± 0.01	0.62 ± 0.11	0.14 ± 0.03	0.16 ± 0.04	0.13 ± 0.04	nd	nd	0.62 ± 0.11
3,7-Dimethyl-1,5-octadien-3,7-diol[a]	0.52 ± 0.03	0.38 ± 0.08	0.42 ± 0.03	0.45 ± 0.01	0.17 ± 0.24	0.43 ± 0.01	0.47 ± 0.07	0.49 ± 0.13	0.41 ± 0.04	nd	0.59 ± 0.02	0.43 ± 0.01
Terpin[a]	nd	nd	nd	nd	nd	nd	0.27 ± 0.01	nd	nd	0.52 ± 0.06	nd	nd
Neric acid[a]	7.09 ± 1.15	9.18 ± 0.81	6.32 ± 0.42	6.19 ± 0.74	8.10 ± 0.68	nd	8.58 ± 0.01	6.52 ± 1.07	6.26 ± 0.24	9.55 ± 2.22	8.73 ± 0.46	nd
Miscellaneous												
Furfural	0.96 ± 0.21	1.45 ± 0.26	1.62 ± 0.01	0.61 ± 0.20	1.23 ± 0.11	0.51 ± 0.01	0.89 ± 0.19	1.05 ± 0.05	0.95 ± 0.09	1.13 ± 0.37	1.26 ± 0.32	nd
Pantolactone	3.00 ± 0.29	3.24 ± 0.91	3.76 ± 0.05	4.27 ± 0.50	4.26 ± 0.08	nd	2.45 ± 0.34	3.47 ± 0.25	2.86 ± 0.03	2.09 ± 0.40	2.50 ± 0.12	nd
Fermentative compounds												
Acetaldehyde[b]	38 ± 31.5	10.8 ± 0.01	27.9 ± 7.74	9.49 ± 0.47	49.7 ± 24.2	65.9 ± 5.97	33.5 ± 3.58	169 ± 30.6	105 ± 9.00	3.07 ± 0.01	2.76 ± 0.01	3.05 ± 0.01
Diacetyl[b]	nd	nd	nd	nd	nd	5.33 ± 0.11	nd	0.50 ± 0.17	0.40 ± 0.13	nd	nd	nd
Acetoin[b]	9.1 ± 7.61	2.11 ± 0.08	26 ± 17.5	4.07 ± 0.60	3.34 ± 0.69	40.8 ± 2.53	40.1 ± 4.93	419 ± 44.8	340 ± 31.2	nd	nd	nd
1-Butanol[b]	nd	nd	3.18 ± 0.36	nd	nd	nd	nd	nd	nd	nd	nd	nd
Isobutanol[b]	14.7 ± 1.18	16.6 ± 0.19	8.05 ± 0.30	10.1 ± 1.92	22.5 ± 0.91	14.5 ± 0.89	13.5 ± 1.13	13.8 ± 0.05	16.0 ± 0.66	nd	nd	4.33 ± 0.63
Isoamyl alcohol[b]	85.9 ± 1.18	77.0 ± 3.62	64.4 ± 6.09	83 ± 18.2	97.2 ± 9.75	58.5 ± 3.30	65.5 ± 2.99	57.3 ± 3.52	62.1 ± 0.89	4.43 ± 0.10	5.78 ± 0.17	nd
β-Phenylethanol[b]	14.5 ± 2.22	20.3 ± 1.04	29.4 ± 5.56	78.4 ± 0.80	16.7 ± 0.68	21.6 ± 0.59	32.8 ± 4.97	17.8 ± 2.69	29.6 ± 0.36	2.91 ± 0.01	nd	nd
Isobutyric acid[b]	0.21 ± 0.01	0.23 ± 0.01	0.22 ± 0.03	0.25 ± 0.01	0.28 ± 0.01	0.72 ± 0.04	0.58 ± 0.12	0.24 ± 0.01	0.22 ± 0.02	0.41 ± 0.01	0.48 ± 0.07	0.60 ± 0.01
Isovaleric acid	21.4 ± 2.65	10.5 ± 0.83	18.6 ± 0.65	24.2 ± 5.40	13.7 ± 1.54	10.7 ± 1.27	16 ±9.04	12.2 ± 2.45	7.34 ± 1.60	120 ± 11.3	249 ± 4.58	14.4 ± 1.41
2-Methylbutyric acid	12.3 ± 1.86	6.24 ± 0.49	18.0 ± 0.92	26.4 ± 0.09	11.2 ± 0.05	14.6 ± 1.85	14 ± 8.34	14.6 ± 3.51	6.86 ± 1.83	56.3 ± 7.64	128 ± 2.22	41.7 ± 2.12
Isoamyl acetate[b]	0.10 ± 0.03	0.04 ± 0.01	0.04 ± 0.01	0.06 ± 0.03	0.07 ± 0.03	nd	nd	nd	nd	nd	nd	nd
Phenylethyl acetate	199 ± 85.6	103 ± 4.90	118 ± 1.36	1049 ± 499	218 ± 84.9	30.5 ± 6.30	5876 ± 2324	117 ± 11.3	201 ± 44.5	0.39 ± 0.32	0.57 ± 0.14	nd
γ-Butyrolactone[b]	0.24 ± 0.03	1.02 ± 0.01	0.25 ± 0.07	1.62 ± 0.14	0.86 ± 0.11	0.62 ± 0.05	0.27 ± 0.01	0.21 ± 0.01	nd	0.23 ± 0.02	0.25 ± 0.01	0.20 ± 0.01
Ethyl decanoat	9.06 ± 0.87	9.22 ± 0.29	8.67 ± 0.76	10.8 ± 3.12	9.20 ± 0.53	nd	1.83 ± 0.61	1.86 ± 0.08	2.66 ± 0.19	3.73 ± 0.68	3.72 ± 0.84	nd
Ethyl 3-hydroxybutyrate[b]	0.04 ± 0.01	0.07 ± 0.01	nd	nd	0.06 ± 0.01	nd	nd	nd	nd	nd	nd	nd
Ethyl lactate[b]	1.79 ± 0.69	1.88 ± 0.01	2.77 ± 0.70	2.80 ± 0.46	2.00 ± 0.89	nd	nd	nd	nd	nd	nd	nd
Diethyl succinate[b]	0.26 ± 0.06	0.31 ± 0.01	0.27 ± 0.19	0.47 ± 0.02	0.39 ± 0.02	0.36 ± 0.03	0.37 ± 0.02	0.18 ± 0.01	0.32 ± 0.06	0.65 ± 0.04	0.64 ± 0.09	nd
Butyric acid[b]	0.31 ± 0.04	0.31 ± 0.01	0.27 ± 0.07	0.21 ± 0.01	0.24 ± 0.01	0.24 ± 0.04	nd	nd	nd	0.23 ± 0.01	0.38 ± 0.04	nd
Hexanoic acid[b]	1.93 ± 0.06	3.35 ± 0.17	0.70 ± 0.02	1.20 ± 0.02	1.55 ± 0.20	0.15 ± 0.01	0.47 ± 0.05	0.52 ± 0.06	0.58 ± 0.24	1.31 ± 0.07	1.71 ± 0.25	nd
Octanoic acid[b]	1.47 ± 0.17	1.53 ± 0.01	0.88 ± 0.02	1.43 ± 0.04	1.04 ± 0.18	nd	0.39 ± 0.11	0.49 ± 0.06	0.42 ± 0.27	1.03 ± 0.02	1.23 ± 0.04	nd
Decanoic acid[b]	0.32 ± 0.02	0.43 ± 0.06	0.43 ± 0.02	0.79 ± 0.25	0.46 ± 0.16	nd	0.38 ± 0.14	0.23 ± 0.02	0.34 ± 0.04	0.10 ± 0.01	0.09 ± 0.01	nd

Data are average of two replicates ± standard deviation. Except where indicated all data are in $\mu g\ l^{-1}$. For the codes of samples see Table 1.
nd: not detected.
[a] Data are area normalized to the internal standard.
[b] Data in $mg\ l^{-1}$.

Table 4
Aroma composition of the different control samples

Yeasts	L1450B	L372B	B1time	B2HAH
Lipids derivatives				
Z-3-Hexen-1-ol	nd	nd	0.16 ± 0.05	0.06 ± 0.03
γ-Octalactone	nd	0.63 ± 0.03	nd	nd
δ-Octalactone	1.24 ± 0.28	0.06 ± 0.01	nd	nd
γ-Nonalactone	0.58 ± 0.01	0.63 ± 0.06	1.00 ± 0.11	0.28 ± 0.01
γ-Decalactone	1.15 ± 0.60	1.04 ± 0.22	nd	0.05 ± 0.01
E-Whiskylactone	0.23 ± 0.07	nd	nd	nd
δ-Decalactone	17.5 ± 6.24	2.49 ± 0.07	nd	0.29 ± 0.01
δ-Nonalactone	nd	nd	nd	0.03 ± 0.01
2-Ethylhexanoic acid	5.43 ± 0.23	5.93 ± 0.08	5.46 ± 0.07	1.86 ± 0.09
Shikimic derivatives				
Benzenoids				
Benzoic acid	162 ± 46.3	44.4 ± 4.02	123 ± 108	21.8 ± 7.19
Benzaldehyde	3.19 ± 1.23	1.92 ± 0.34	5.79 ± 0.93	2.14 ± 0.71
Phenylacetaldehyde	1.72 ± 0.08	1.32 ± 0.04	nd	0.95 ± 0.02
Ethyl dihydrocinnamate	0.31 ± 0.01	nd	nd	nd
Ethyl cinnamate	nd	nd	0.14 ± 0.04	nd
2-Phenoxyethanol	11.5 ± 2.77	9.36 ± 1.76	10.4 ± 0.13	2.1 ± 1.75
Ethylparaben	nd	nd	5.90 ± 0.49	nd
Volatile phenols				
Guaiacol	0.38 ± 0.04	0.31 ± 0.01	0.31 ± 0.01	0.20 ± 0.04
4-Ethylguaiacol	nd	0.27 ± 0.03	nd	0.04 ± 0.01
Eugenol	nd	nd	0.45 ± 0.05	0.27 ± 0.04
4-Vinylguaiacol	0.19 ± 0.02	0.01 ± 0.01	6.2 ± 5.33	16.5 ± 5.80
E-Isoeugenol	nd	nd	5.02 ± 1.76	0.51 ± 0.03
4-Ethylphenol	nd	0.56 ± 0.09	nd	nd
4-Vinylphenol	1.91 ± 0.1	nd	12.7 ± 5.51	11.8 ± 4.81
2,6-Dimethylphenol	nd	nd	nd	1.21 ± 0.20
4-Allyl-2,6-dimethoxyphenol	nd	nd	nd	3.01 ± 0.90
Dihydromethyleugenol[a]	nd	nd	nd	16.1 ± 1.46
Vainillins				
Vanillin	6.45 ± 0.64	nd	12.9 ± 2.20	3.70 ± 0.01
Methyl vanillate	nd	nd	1.26 ± 0.03	1.39 ± 0.25
Ethyl vanillate	nd	nd	2.13 ± 0.17	1.48 ± 0.21
Acetovanillone	3.01 ± 0.02	2.94 ± 0.02	3.43 ± 0.20	1.97 ± 0.22
Zingerone	nd	nd	1.17 ± 0.18	1.93 ± 0.53
Homovanillyl alcohol	nd	nd	nd	10.3 ± 1.47
Homovanillic acid[a]	nd	nd	33. ± 7.74	nd
Syringaldehyde	nd	nd	16.0 ± 1.04	8.57 ± 0.61
Acetosyringone	nd	nd	nd	1.52 ± 0.31
Nor-isoprenoids				
β-Damascenone	nd	nd	nd	4.19 ± 0.76
α-Isomethyl-ionone	nd	0.54 ± 0.05	nd	0.20 ± 0.12
β-Ionone	0.18 ± 0.01	0.23 ± 0.07	0.08 ± 0.01	0.04 ± 0.01
Vitispirano A[a]	nd	nd	nd	10.0 ± 3.42
Vitispirano B[a]	nd	nd	nd	2.57 ± 0.39
Riesling acetal[a]	nd	nd	nd	4.61 ± 0.67
TDN[a]	nd	nd	nd	0.61 ± 0.19
3-Oxo-β-ionone[a]	nd	nd	nd	0.22 ± 0.04
Actinidiols[a]	nd	nd	nd	2.43 ± 0.59
Norisoprenoide[a]	0.02 ± 0.03	nd	nd	1.21 ± 0.07
3-Oxo-α-ionol[a]	nd	nd	0.73 ± 0.07	0.50 ± 0.21
Terpenes				
Linalool	1.05 ± 0.34	0.07 ± 0.04	0.59 ± 0.06	0.70 ± 0.11
α-Terpineol	0.69 ± 0.10	0.20 ± 0.04	0.35 ± 0.10	4.29 ± 0.83
β-Citronellol	2.51 ± 0.29	2.34 ± 1.00	nd	0.33 ± 0.08
Nerol	1.25 ± 0.22	nd	nd	0.22 ± 0.01
Farnesol (2*E*, 6*E*)	15.1 ± 0.47	4.22 ± 0.71	nd	0.43 ± 0.01
Z-Linalool oxide[a]	nd	nd	nd	4.01 ± 0.94
E-Linalool oxide[a]	nd	nd	nd	26.4 ± 4.77
Linalool acetate[a]	0.60 ± 0.05	0.15 ± 0.04	0.66 ± 0.04	6.03 ± 1.00

Table 4 (*continued*)

Yeasts	L1450B	L372B	B1time	B2HAH
Terpinen-4-ol[a]	0.16 ± 0.08	0.09 ± 0.03	nd	0.37 ± 0.07
2,6-Dimethyl-1,7-octadien-3,6-diol[a]	0.51 ± 0.13	nd	0.49 ± 0.04	0.66 ± 0.11
δ-Terpineol[a]	nd	nd	nd	4.38 ± 1.31
Nerol oxide[a]	nd	nd	nd	2.68 ± 0.68
Terpinyl acetate[a]	nd	nd	nd	0.93 ± 0.29
Ocimenol[a]	nd	nd	0.30 ± 0.03	1.03 ± 0.28
3,7-Dimethyl-1,5-octadien-3,7-diol[a]	nd	nd	0.52 ± 0.02	nd
Neric acid[a]	nd	nd	0.26 ± 0.37	nd
Miscellaneous				
Furfural	0.86 ± 0.22	0.72 ± 0.20	1.78 ± 0.30	nd
Pantolactone	2.23 ± 0.16	2.02 ± 0.18	1.78 ± 0.08	9.29 ± 1.78
Fermentative compounds				
Acetaldehyde[b]	53.5 ± 4.83	1.38 ± 1.95	nd	nd
Acetoin[b]	13.1 ± 2.44	nd	nd	nd
1-Butanol[b]	1.20 ± 0.01	nd	nd	nd
Isobutanol[b]	16.0 ± 2.06	nd	nd	nd
Isoamyl alcohol[b]	74.2 ± 0.94	3.52 ± 0.05	nd	nd
β-Phenylethanol[b]	14.3 ± 0.79	nd	nd	nd
Isobutyric acid[b]	0.43 ± 0.08	0.41 ± 0.05	nd	nd
Isovaleric acid	19.7 ± 2.28	253 ± 2.95	1.43 ± 0.02	0.59 ± 0.34
2-Methylbutyric acid	11.5 ± 1.53	140 ± 12.1	1.02 ± 0.09	0.43 ± 0.35
Isoamyl acetate[b]	0.07 ± 0.01	nd	nd	nd
Phenylethyl acetate	153 ± 9.79	nd	nd	nd
γ-Butyrolactone[b]	0.20 ± 0.01	0.25 ± 0.05		
Ethyl decanoate	8.57 ± 1.23	2.13 ± 0.45	0.48 ± 0.01	0.17 ± 0.01
Ethyl lactate[b]	1.39 ± 0.03	nd	nd	nd
Diethyl succinate[b]	0.23 ± 0.13	1.00 ± 0.12	nd	nd
Butyric acid[b]	0.41 ± 0.13	0.14 ± 0.20	nd	nd
Hexanoic acid[b]	2.25 ± 0.24	1.33 ± 0.52	nd	nd
Octanoic acid[b]	1.73 ± 0.20	2.06 ± 0.43	nd	nd
Decanoic acid[b]	0.26 ± 0.04	0.18 ± 0.04	nd	nd

The two first ones (L1450B and L372B) are SNM without precursors fermented by the corresponding yeasts. B1time is an unfermented control containing the precursors during all the experiment. B2HAH is the aroma composition of the fraction of precursors hydrolyzed by harsh acid hydrolysis. Data are average of two replicates. Except where indicated, all data are $\mu g\, l^{-1}$.

Zingerone: Vanillin acetone; Riesling acetal: 2,2,6,8-tetramethyl-7,11-dioxatricyclo[6.2.1.0(1,6)]undec-4-ene; TDN: 1.1.6-trimethyl-1.2-dihydronaphthalene.

[a] Data are area normalized to the internal standard.

[b] Data in $mg\, l^{-1}$.

The levels of nearly all the varietal aroma compounds were found to significantly differ between the different yeast strains used (data not shown). In order to better interpret the influence of the yeast strain on the varietal aroma profile of the obtained wines, the blank samples were removed from the dataset and the principal component. Analysis was run again. These results are shown in Fig. 3. As the figure clearly shows, there was a strong influence of the yeast genus: samples fermented by *Torulaspora* are found on the left-bottom part of the plane, those of *Saccharomyces* are found in the center-upper region, samples fermented with *Hanseniaspora* and *Kloeckera* are near the center of the plane, samples fermented with *Brettanomyces* lie in the right-down part and those fermented with *Debaryomyces* can be found in the center-down region of the plane. As in the previous case, the existence of these clusters was further demonstrated by different techniques of cluster analysis.

According to the PCA plots and to data in Table 3, the most different samples were the two wines made by *Torulaspora* which were the richest in some important aroma compounds such as Riesling acetal (only in these samples was this compound detected), ethylvanillate (6 times more concentrated than in the rest of samples), terpinyl acetate (3 times), γ-nonalactone and γ-decalactone (2 and 5 times, respectively), eugenol and 2-phenoxyethanol, ethyl dihydrocinnamate (3 times more in average), the actinidiols (2 times), farnesol (2–3 times), vanillin, isoeugenol, 3-oxo-β-ionone and α-terpineol.

Similarly, wines made with *Brettanomyces* were, as expected (Chatonnet, Dubourdieu, Boidron, & Lavigne, 1993) were the richest in 4-ethylphenol and 4-ethylguaiacol (more than 100 times more concentrated) and in isovaleric and 2-methylbutyric acids (20–30 times). Interestingly, these wines were also the richest in many vainillin-derivatives, such as methylvanillate, acetovanillone, acetosyringone, homovanillyl alcohol and homovanillic acid (between 2 and 3 times richer in all cases). They were also the richest in the pre-fermentative compound, *Z*-3-hexenol,

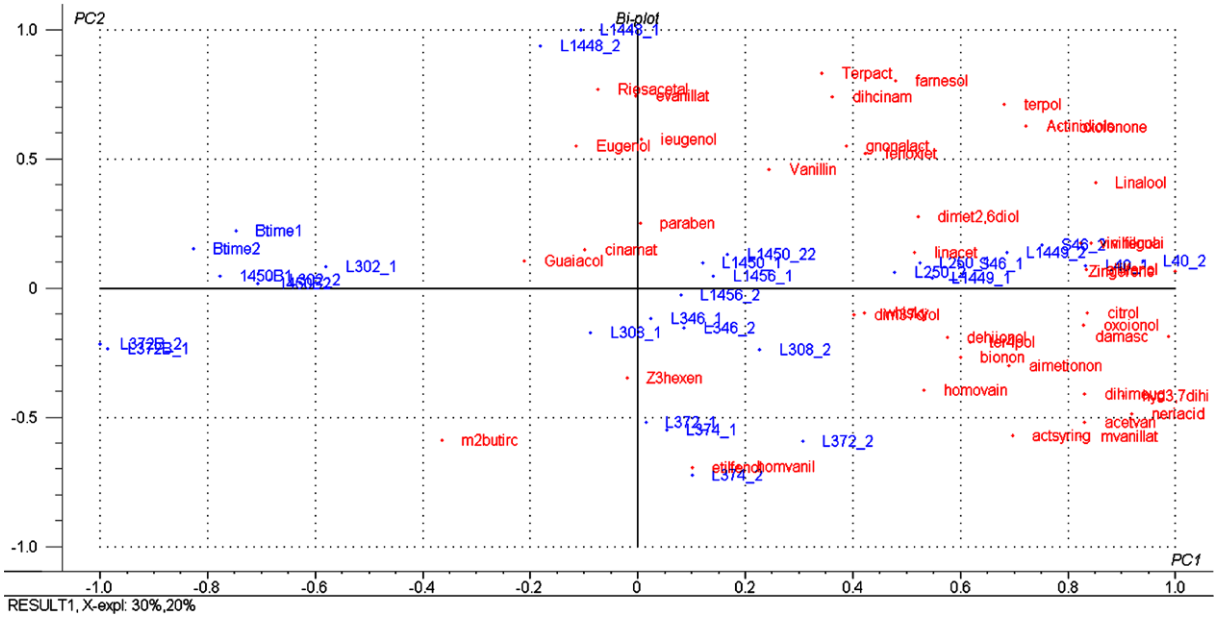

Fig. 2. Principal component plot with the sample loadings and variable weights. All samples are represented here. Samples denoted with B are samples fermented without precursors. Samples denoted B_{time} are the unfermented controls. For the codes of samples see Table 1.

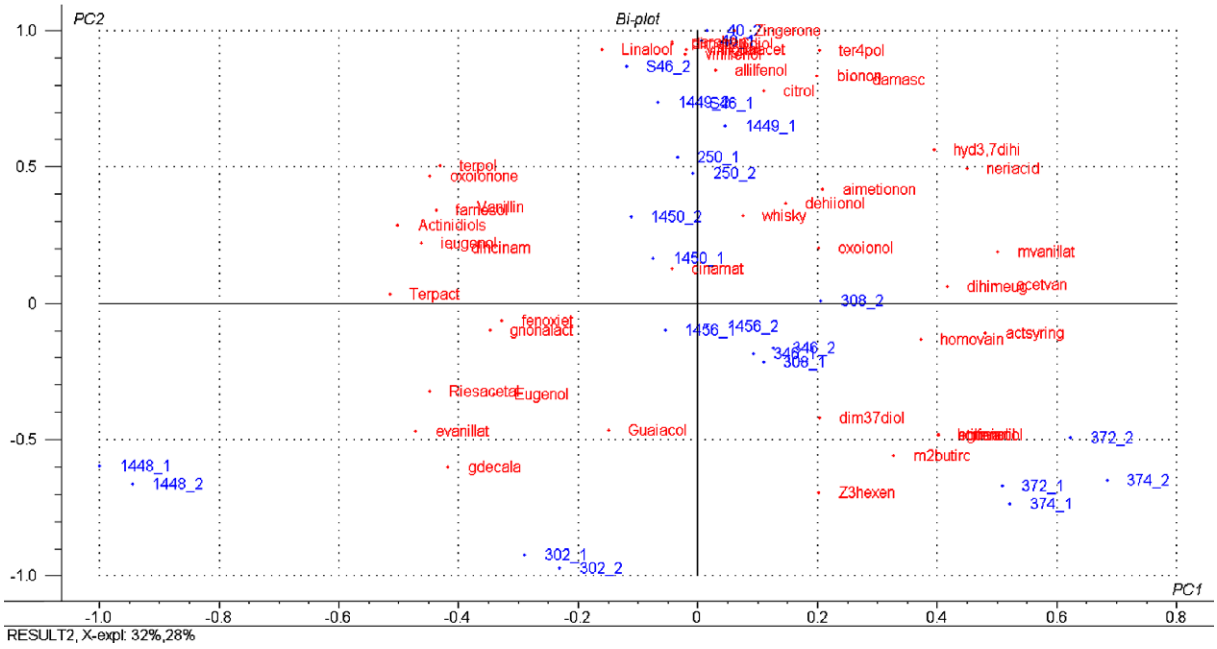

Fig. 3. Principal component plot with the sample loadings and variable weights. Only samples fermented with precursors are represented here. For the codes of samples see Table 1.

in the volatile phenol dihydromethyleugenol and in the terpenol 3,7-dimethyl-1,5-octadien-3,7-diol.

The group of samples made with *Saccharomyces* was less homogeneous, but these samples were richest in some of the most important wine aroma compounds, such as β-damascenone, β-ionone (1.5–3 times higher), linalool (3–5 times) and the vinylphenols (10 to 20 times higher). In addition, these samples were also the richest in some other wine terpenols such as linalyl acetate, terpinen-4-ol, citronellol and 2,6-dimethyl-1,7-octadien-3,6-diol, as well as in other compounds such as 4-allyl-2,6-dimethoxyphenol and zyngerone. Samples fermented with *Kloeckera* or *Hanseniaspora* did not present levels particularly high or low of any aroma compound. Finally, it should be noted that samples fermented with *Debaryomyces*, in spite of a nearly null fermentative activity, showed maxima or near to maxima levels of some aroma compounds, such as *Z*-3-hexenol, γ-decalactone, guaiacol, eugenol and nerol and minima concentrations of many other compounds.

There are in addition, some differences worth mentioning in the levels of some fermentative compounds which may have important sensory consequences: samples fermented by *Kloeckera* had extraordinarily high levels of some carbonyls, such as acetaldehyde, acetoin and phenylalcetaldehyde; samples fermented by *Hanseniaspora* had also amazing levels of phenylethyl acetate, while samples fermented with the L250 *Saccharomyces bayanus* had the highest levels of β-phenylethanol and quite high levels of its acetate; finally, samples fermented with *Brettanomyces* had, as commented earlier, highest levels of isovaleric and 2-methylbutyric acid.

3.3. Sensory differences

Although the different levels of residual sugar and of ethanol in the wine samples make it difficult to establish accurate sensory comparisons, there are some obvious correlations between some of the sensory attributes of the samples and the levels of some aroma-active compounds. For instance, the highest levels of ethylphenols and of isovaleric and 2-methylbutyric acids are obviously related to the ethylphenol and cheese odor notes of the samples fermented by *Brettanomyces*, respectively. Similarly, the highest levels of aliphatic lactones and of ethyl dihydrocinnamate of the wines made with *Torulaspora* may help explaining the highest scores of the descriptors dry fruit and coconut found in these wines. The rose descriptor can be also explained by the levels of linalool, β-phenylethanol and β-phenylethyl acetate, in accordance with previous results (Campo et al., 2005). In general, the higher scores in the floral notes of wines made with *Saccharomyces* yeasts may be related to their higher levels of linalool, β-ionone and β-damascenone. It should be also remarked, that leaving aside the particular cases of ethylphenols and vinylphenols, most aroma compounds are produced at relatively low concentrations, most often below the corresponding odor threshold of the compound. However the numbers of aroma compounds produced are very high and some of them bear similar odor properties that can exert a concerted action, as it has been recently shown (Loscos et al., 2007). This means that the sensory action derived from the fermentation of the aroma precursor-containing musts, must be understood as the result of the presence of a relatively large number of aroma compounds whose contribution is not specific (they do not transmit their specific sensory descriptors) but generic (they transmit some of the generic aroma attributes, such as floral or sweet).

4. Discussion

Results presented here confirm that large pools of aroma compounds, many of which have been previously identified as important wine odorants, are formed from non-floral grape precursors by the action of yeasts belonging to quite different genera. The pattern of aroma production from precursors is significantly linked to the genus of yeast, it being possible to state that a large diversity of enzymatic activities is displayed by the different genera and that such diversity is going to have a sensory consequence. While *Saccharomyces* yeasts produce maximal amounts of β-damascenone, β-ionone and linalool, *Brettanomyces* (apart from ethylphenols and isoacids) is able to form high amounts of most vanillin-derivatives, *Torulaspora* forms the highest amounts of lactones, Riesling acetal, ethyl vanillate and ethyl dihydrocinnamate, and even the inactive *Debaryomyces* forms relatively large amounts of some important aroma compounds, such as guaiacol and eugenol. Remarkably, the aroma production from precursors is not linked to the amount of sugar transformed by the yeast: some of the studied yeast transformed only tiny amounts of sugar, but the levels of some aroma compounds produced were the highest found in the experiment. This situation clearly contrasts with the relatively small diversity observed between different *Saccharomyces* strains, as is deduced from the present data and from a recent report (Loscos et al., 2007).

This diversity not only affects to compounds produced from glycosidic precursors, but also to compounds with fermentative or other origins. As commented earlier, there are large differences in the production of isovaleric acids, phenylethyl acetate and β-phenylethanol, and also in the levels of compounds coming directly from ferulic and coumaric acids. It is somewhat surprising that a link exists, particularly evident and sensory noticeable in the case of *Brettanomyces*, between the presence of precursors and the levels of isovaleric acids. Most likely, and as has been recently suggested (Ugliano et al., 2006), the fraction of precursors contains ferulic and coumaric acids that act as precursors for ethyl and vinylphenols. In any case, the existence of such link makes us think that the reduction of vinylphenols to ethylphenols is closely related to some of the oxidative processes involved in the amino acid metabolism.

All these observations could have a practical consequence on winemaking and could also give some clue about why some great wines are still today produced by spontaneous fermentation in which a large number of yeasts (and other microorganisms) may act concurrently or successively.

Results also confirm that, as expected, the enzyme driven hydrolytical activity of yeast is much more efficient than the natural acid hydrolysis, since in most cases, the amount of aroma formed is higher in the samples in which fermentation has taken place. However, in many cases there are some other processes, apart from the simple hydrolysis, taking place in aroma formation. In simple cases, such as β-damascenone, it is well known that the aroma molecule is not formed by hydrolysis, but by chemical rearrangement of different precursors, some of which require previous hydrolysis (Puglisi et al., 2005; Puglisi, Elsey, Prager, Skouroumounis, & Sefton, 2001; Skouroumounis & Sefton, 2000). In these cases, the higher

amount of aroma found in the fermented samples must be attributed to the higher instability of the hydrolyzed aglycone, as has been recently suggested (Skouroumounis & Sefton, 2000). In the case of other important aroma compounds which were only found in the harsh acid hydrolysates of the precursor fraction, such as TDN and vitispiranes (see Table 4), there is no apparent effect of the fermentation, which suggests that these compounds are formed mainly by slow chemical rearrangement from precursors which most likely are not glycosides. In some other cases, such as benzaldehyde, ethyl paraben, isoeugenol, vanillin or syringaldehyde, the opposite effect is observed, i.e., the levels in the fermented samples are lower than those found in the control. A possible explanation is that these compounds are formed also by rearrangement, but in this case such chemical rearrangement takes place faster in the original glycoside. A second possibility, however, is that the yeast could induce a different transformation of the precursor leading to a different molecule. Finally, there is a small group of compounds for which both phenomena are observed simultaneously: in some yeasts the levels are higher than in the controls, and in other yeasts the levels are equal to or smaller than those observed in the controls. Compounds following this complex trend are *Z*-3-hexenol (maxima in *Brettanomyces* and in *Debaryomyces*), eugenol, ethyl vanillate, 3-oxo-β-ionone and the actinidiols (maxima in *Torulaspora*), 4-vinylphenol and 4-vinylguaicol, linalyl acetate and 2,6-dimethyl-1,7-octadien-3,6-diol (maxima in *Saccharomyces*). In all these cases different alternative pathways to form the corresponding compounds must coexist and, leaving aside the vinylphenols (Chatonnet et al., 1993; Dugelay, Gunata, Sapis, Baumes, & Bayonove, 1993), the nature of such processes for the most, remain unknown.

In conclusion, this research has demonstrated that the different genera of yeasts have quite different abilities to release or form aroma compounds from odorless precursors. The diversity in the patterns of aroma formation is much wider than that observed within yeast of the *Saccharomyces* genus, which may have important practical consequences in winemaking. Leaving aside ethylphenols and vinylphenols, most aroma compounds are produced at relatively low concentrations, but in numbers enough to cause a sensory effect. The patterns of aroma production also suggest that in many cases the simple cleavage of the *O*-glycosydic bond is not enough to form the aroma compound and that additional research should be conducted to exactly understand the formation of the different aroma molecules.

Acknowledgements

This work has been funded by the Spanish Government (project AGL2004-06060). P.H.-O. has received a grant from the CAI-Europa XXI program. N.L. has received a grant from the Spanish FPU program.

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