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Changes in free and bound fractions of aroma compounds of four *Vitis vinifera* cultivars at the last ripening stages

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ABSTRACT

The volatile composition of white *Agudelo*, *Blanco lexitimo*, *Godello* and red *Serradelo* cultivars (NW Spain) harvested at two different stages of ripening have been evaluated. C_6-compounds, alcohols, volatile fatty acids, monoterpenes, C_{13}-norisoprenoids, volatile phenols and carbonyl compounds were identified and quantified in free and glycosidically bound forms by gas chromatography–mass spectrometry (GC–MS). The total volatile concentration showed a significant increase between the two ripening stages studied for all cultivars. The free volatile composition increased during maturity for *Godello* and *Serradelo* cultivars; however the glycosidically bound concentration increases for all cultivars with exception of *B. lexitimo*. Free C_6-compounds ((*E*)-2-hexanal, 1-hexanol and (*E*)-2-hexen-1-ol) and bound alcohols (benzyl alcohol and 2-phenylethanol) showed the highest concentrations of volatile compounds for all grape cultivars in the two dates studied. *Godello* cultivar showed the highest change of volatile concentration between two ripening dates because of the high value of free C_6-compounds. *B. lexitimo* was the most terpene-rich cultivar at the last ripening stage due to linalool; however C_{13}-norisoprenoids in free form were detected in low concentrations for all cultivars but not in *Godello* and *B. lexitimo* cultivars at the last ripening stage. Free hexanoic acid increased during ripening in all cultivars. The evolution of volatiles during ripening of grape juice from the cultivars studied was not proportional to the changes in sugar content, which shows that the technological and aromatic maturities did not occur at the same time in these cultivars. The results also showed the cultivar * ripening date interaction for all, free and bound, groups of compounds.

1. Introduction

Grape ripening is a physiological process that starts at the moment of *véraison* and lasts about 40 days, depending on the variety, environment and agricultural practices (Coello et al., 2007). This is a very important period that influences the composition of the grapes and, consequently, the wine, allowing grapes to develop their varietal characteristics (Gomez et al., 1995). The changes produced by the grapes during this period included physical changes (volume, weight, color and rigidity) and chemical changes (pH, sugars, acidity, phenolics and volatile composition).

The volatile composition is one of the most important parameters responsible for wine quality and, hence, for consumer acceptance. Given the importance of the aroma on the quality of the wine it has been the subject of numerous studies. The chemical basis and the diversity of grape and wine odoriferous compounds have been reviewed in detail (Bayonove et al., 1998; Oliveira et al., 2000; Ribéreau-Gayon et al., 2000; Mateo and Jiménez, 2000; Swiegers and Pretorius, 2005).

The aroma of a grape must derive from aromatic free volatiles and from non-volatile, odorless precursors, which may be revealed during the winemaking process (Arevalo et al., 2006). Several families of compounds are responsible for primary aroma of grapes such as monoterpenols, abundant in Muscat varieties, methoxypyrazines, which characterize the Cabernet family, C_{13}-norisoprenoids, abundant in Chardonnay, volatile thiols in Sauvignon, volatile phenols in *Traminer aromatico*, and dimethyl sulfide in Syrah. These compounds, however, could also contribute significantly to the aroma of several other varieties (Versini, 1985; Allen et al., 1991; Sefton et al., 1993; Tominaga et al., 2000; Segurel et al., 2004, 2005).

Most grape aroma compounds are present as free volatiles, which may contribute directly to odor, or as bound sugar conjugates, which are nonvolatile. Conjugates (including glycosides) can undergo acid or enzyme hydrolysis, releasing free volatiles and potentially enhancing aroma (Cordonnier and Bayonove, 1974; Zoecklein et al., 1997). Initial research on grape glycosides

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focused on monoterpenes and determined their role as aroma/flavor components of floral varieties. Subsequent research demonstrated the role of C_{13}-norisoprenoid compounds and shikimic acid-derived metabolites as precursors of nonfloral grape aroma/flavor (Williams et al., 1989).

Most of the volatile flavor components are produced after veraison until harvest. However notable aroma compounds that are produced during the first period of growth, decline during fruit ripening (Hardy, 1970; Hashizume and Samuta, 1999; Belancic and Agosin, 2007). C_6-aldehydes and alcohols are formed from linoleic acid and linolenic acid when grapes enter into contact with the air, and are formed by the actions of lipoxygenase, peroxidase and alcohol dehydrogenase enzymes (Garcia et al., 2003). C_{13}-norisoprenoid and terpene compounds are generated from carotenoids such as lutein and β-carotene (Winterhalter et al., 1990; Cox et al., 2005; Camara et al., 2004). Terpenes are important compounds as varietal aromas with floral and fruity notes and are present in green berries only in very small amounts, but their concentrations gradually rise during ripening until around maturity, after which concentrations fall (Wilson et al., 1984; Günata et al., 1985).

The concentration of varietal aroma compounds in grapes is influenced by several factors such as grape variety and degree of maturity, vintage, climate or vineyard management techniques (Bueno et al., 2003; Oliveira et al., 2006; Genisheva and Oliveira, 2009; Belancic and Agosin, 2007; Vilanova et al., 2007). It is generally recognized that grape maturity will affect the flavor profile parallel to the sugar content, however it is well accepted that aromatic maturity is achieved before technological maturity (Bayonove and Cordonnier, 1971; Marais, 1983; Marais and van Wyk, 1986). The knowledge of the grape varietal volatile composition offers a means of evaluating the aroma potential, and the period of time that the maximum potential is exhibited (Coello et al., 2007). Therefore it is important to determine the concentrations of varietal volatiles (terpenes, C_{13}-norisoprenoids and C_6-compounds) as a criterion to define the date of harvest (Salinas et al., 2004).

Betanzos is a geographical denomination of wines from NW Spain, where are grown several white cultivars such as *Godello*, *Agudelo*, *Blanco lexitimo* and red cultivars such as *Serradelo*. Previous work performed in our laboratory (Vilanova et al., 2009) reported a first study on the volatile composition of wines produced with these cultivars from Betanzos.

In this study, the determination of volatile compounds has been performed on cultivars from Betanzos (*B. lexitimo*, *Agudelo*, *Godello* and *Serradelo*) in the last ripening stages. The aims were to:

- Know the aromatic maturity of grapes and their relation with the technologic maturity (sugar content).
- Evaluate the significance of changes produced in the last stages of ripening to recognize the importance of volatile composition on the harvest date decision.
- Know the aroma potential of each cultivar and evaluate the period of time that the maximum potential is exhibited.

2. Results and discussion

2.1. Changes in classic parameters during berry maturity

Analytical results (Table 1) revealed differences in the dynamics of ripening in the four grape varieties considered in this study. White cultivars *B. lexitimo* and *Godello* displayed high sugar accumulation rates and attained maturity levels suitable for quality wines production in the last ripening stage (30 August). By contrast, the red *Serradelo* showed less maturity at the same date, followed by the white *Agudelo* cultivar. *B. lexitimo* showed the largest increase (2.2°Brix) in sugar concentration during the ripening stages studied. These results were in accord with those observed in previous studies on wines where the higher ethanol concentration was obtained for *B. lexitimo* wines in contrast to *Agudelo* and *Serradelo* wines (Vilanova et al., 2009).

2.2. Volatile composition of grapes

The aroma is one of the main factors related to the quality of white wines and compounds responsible for wine aroma should be taken in account in the evaluation of the optimal stage of grape ripening (Sánchez Palomo et al., 2007). Fig. 1 shows the total volatile composition of grape cultivars from Betanzos during the last ripening stages. *Godello* cultivar showed the highest increase of volatiles between the two ripening dates, however *B. lexitimo* showed an opposite behavior to *Godello*. Other authors have shown that the results for a given harvest may be strongly influenced by weather conditions during ripening and that the results for volatile composition varied independently of sugar content (Marais et al., 1992; Dieguez et al., 2003).

2.2.1. Free volatile compounds

Table 2 shows the evolution of groups of free volatile compounds (means and standard deviations) during ripening stages, together with the ANOVA results for the factors "cultivar (C)" and "ripening date (D)" and interaction C * D. The total concentration of each group of compounds in free fraction was obtained as the sum of individual concentrations of all compounds detected under the experimental conditions used. This study includes 36 free volatile compounds: 9 alcohols, 6 C_6-compounds, 6 volatile fatty acids, 8 monoterpenes, 2 C_{13}-norisoprenoids, 4 volatile phenols and 1 carbonyl compounds (Table 3). Analysis of variance (ANOVA) by groups shows statistically significant differences among cultivars for all groups of free volatile compounds studied. The differences between the two ripening dates also were significant for all groups of free volatile compounds except for monoterpenes, where only *B. lexitimo* shows significant differences in monoterpenes among ripening stages. The variation of the total free volatile concentration was only significant between the two ripening dates for *Godello* ($p < 0.001$) and *Serradelo* ($p < 0.05$) cultivars. *Godello* showed the highest aromatic ripening at harvest date (3.610 mg/L). The free volatile composition and the evolution of each group

Table 1
Characteristic of cultivars and grape juice during ripening.

Cultivars	Color	Training system	Ripening date					
			23.08.2007			30.08.2007		
			°Brix	Sugar (g/L)	Total acidity (g/L)	°Brix	Sugar (g/L)	Total acidity (g/L)
Agudelo	White	Single cordon	19.2	184	7.5	20.6	200	6.2
Godello	White	Single cordon	19.6	188	8.5	21.0	204	7.1
Blanco lexitimo	White	Single cordon	20.0	193	7.8	22.2	217	6.1
Serradelo	Red	Single cordon	18.0	171	6.6	19.6	188	5.3

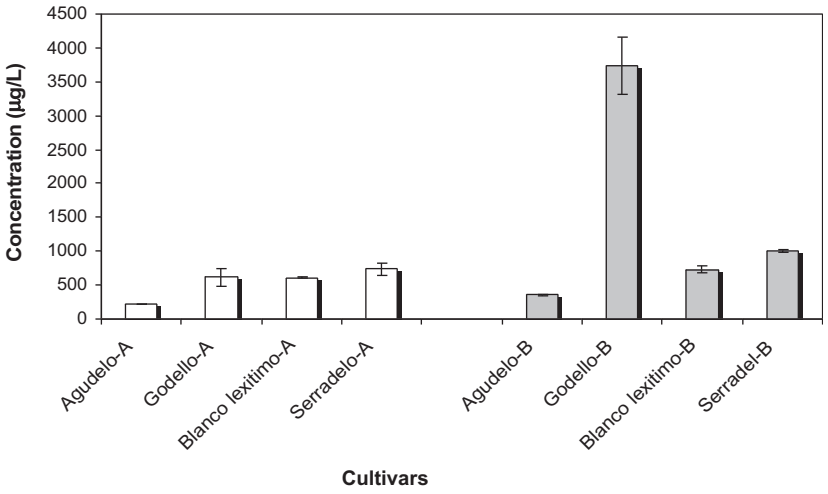

Fig. 1. Changes in volatile composition (mean and standard deviation) for Betanzos cultivars in two ripening dates: 23.08.2007 (A) and 30.08.2007 (B). All the compounds were quantified as 4-nonanol equivalents.

Table 2
Free volatile composition (μg/L) of Betanzos cultivars from two ripening dates. Mean values ($n = 3$) and standard deviation in parentheses, analysis of variance and contrast significance.

Cultivar	Alcohols	C_6-compounds	Monoterpenes	C_{13}-norisoprenoids	Volatile fatty acids	Volatile phenols	Carbonyl compounds	Total
Mean (SD) of free volatile compounds								
Ripening date 23.06.2007 (A)								
Agudelo	3.0 (0.0) a	146.5 (1.2) a	2.5 (0.1) b	0.3 (0.0) b	1.5 (0.3) a	0.9 (0.6) a	0.4 (0.0) a	155.1 (0.4) a
Godello	6.0 (0.1) b	572.1 (139.9) b	1.1 (0.1) a	0.2 (0.0) b	3.0 (0.6) a	2.1 (0.1) b	0.3 (0.0) b	584.8 (124.5) b
Blanco lexitimo	3.1 (0.3) a	522.3 (2.5) b	22.5 (0.9) c	nd a	1.4 (0.5) a	10.0 (0.9) c	nd c	559.2 (6.9) b
Serradelo	6.2 (1.3) b	619.8 (100.40) b	2.4 (0.0) b	0.7 (0.0) c	6.4 (1.8) b	7.6 (0.0) d	0.3 (0.1) b	643.3 (86.7) b
Ripening date 30.08.2007 (B)								
Agudelo	5.7 (0.2) a	200.2 (1.2) a	3.1 (0.1) a	0.4 (0.1) b	12.1 (0.1) b	2.4 (0.4) b	0.7 (0.0) b	224.5 (0.2) a
Godello	10.8 (3.6) b	3595.3 (1104.7) c	nd a	nd a	1.8 (0.7) a	1.5 (0.1) a	1.3 (0.1) c	3610.8 (407.2) c
Blanco lexitimo	9.9 (0.1) b	637.8 (80.6) b	28.0 (5.2) b	nd a	3.9 (3.2) a	3.1 (0.4) c	0.6 (0.1) b	683.3 (51.8) b
Serradelo	4.9 (0.4) a	917.8 (17.6) b	2.1 (0.1) a	0.2 (0.0) c	9.6 (3.9) b	3.1 (0.2) c	0.3 (0.1) a	938.0 (13.1) b
Analysis of variance p-values								
Cultivar	0.001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Repetition	0.226	0.655	0.519	0.979	0.049	0.442	0.959	0.627
Harvest date	<0.0001	<0.0001	0.162	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Cultivar * ripening date	0.001	<0.0001	0.041	<0.0001	0.000	<0.0001	<0.0001	<0.0001
Contrast p-values cultivar vs. ripening date								
Agudelo-A vs. *Agudelo*-B	0.026	0.686	0.738	0.012	<0.0001	0.001	<0.0001	0.604
Godello-A vs. *Godello*-B	<0.001	<0.0001	0.487	<0.0001	0.039	0.130	<0.0001	<0.0001
Blanco lexitimo-A vs. *Blanco lexitimo*-B	0.001	0.389	0.003	1.000	0.080	<0.0001	<0.0001	0.357
Serradelo-A vs. *Serradelo*-B	0.261	0.038	0.861	<0.0001	0.031	0.0001	0.143	0.041

nd – Not detected. Duncan test: different letters indicates significant differences among cultivars. All the compounds were quantified as 4-nonanol equivalents.

of volatiles was different for cultivars from Betanzos during grape ripening, showing interaction cultivar * ripening.

The C_6-compounds group, which showed the highest concentration of free volatile compounds in the two ripening dates for all cultivars, accounted for 90% (*Agudelo*) and 99% (*Godello*). Three compounds, (*E*)-2-hexenal, 1-hexanol and (*E*)-2-hexen-1-ol, had the highest values in all varieties studied. C_6-compounds are related to varietal origin because they can be formed, via C_6-aldehydes, through lipoxygenase activity, from linoleic and linolenic acids present in grapes (Oliveira et al., 2006; Cabaroglu et al., 1997; Moio et al., 2004; Kalua and Boss, 2009). C_6-compounds such as 1-hexanol and (*Z*) and (*E*)-3-hexen-1-ol are sometimes at a rather high level as in the *Müller-Thurgau* wines, wherein these compounds has been suggested as a parameter for discrimination of this cultivar (Nicolini et al., 1996). In contrast we can see (Table 2) that the influence of ripening date on the free C_6-compounds group was only significant for *Godello* ($p < 0.001$) and *Serradelo* ($p < 0.05$) cultivars. *Godello* cultivar showed a significant increase of 83.7% between the two ripening dates mainly due to two compounds (*E*)-2-hexanal and (*E*)-2-hexen-1-ol (Table 3). This behavior was not the same for all cultivars showing a cultivar * ripening date effect for these compounds. Only one compound of this group, (*Z*)-2-hexen-1-ol, did not show significant differences between the two ripening stages for the cultivars studied. During ripening period, the concentration of these C_6-alcohols tended to increase, stabilize, and then even decrease in the *Airén* variety from La Mancha region (Spain) at 9 °Baumé to 11 °Baumé and in *Macabeo* and *Chardonnay* varieties at 11 °Baumé to

Table 3
Changes in free volatile composition of Betanzos grape cultivars during ripening.

Free compounds (μg/L)	Ripening date								ANOVA		
	23.08.2007 (A)				30.08.2007 (B)						
	Agudelo	*Godello*	*B. lexitimo*	*Serradelo*	*Agudelo*	*Godello*	*B. lexitimo*	*Serradelo*	C	D	C * D
Monoterpenes											
trans-Pyran linalool oxide	0.56	0.29	11.34	0.89	0.35	nd	10.26	0.97	***	ns	ns
cis-Pyran linalool oxide	0.31	0.09	4.75	nd	0.31	nd	3.46	nd	**	ns	**
trans-Furan linalool oxide	nd	nd	nd	nd	nd	nd	0.37	nd	***	***	***
cis-Furan linalool oxide	nd	nd	nd	nd	nd	nd	0.43	nd	***	***	***
Geraniol	nd	nd	nd	nd	0.47	nd	nd	nd	***	***	***
α-Terpineol	0.96	0.56	1.77	0.72	0.18	nd	0.33	0.13	***	***	***
Linalool	nd	0.06	1.26	nd	0.12	nd	9.22	nd	***	***	***
3,7-Dimethylocta-1,5-dien-3,7-diol	0.72	0.11	3.43	0.76	1.64	nd	3.94	0.99	***	ns	ns
C_{13}-norisoprenoids											
β-Damascenone	0.27	nd	nd	0.30	0.10	nd	nd	nd	***	***	***
β-Ionone	nd	0.21	nd	0.36	0.26	nd	nd	0.19	***	**	***
C_6 compounds											
(*E*)-2-Hexenal	23.14	288.83	123.22	136.57	30.18	1685.95	102.40	114.21	***	**	***
1-Hexanol	84.52	121.47	137.50	338.41	67.85	409.47	145.30	537.78	***	***	***
(*E*)-3-Hexen-1-ol	1.20	4.86	4.05	1.63	0.88	64.42	3.23	4.10	***	***	***
(*Z*)-3-Hexen-1-ol	29.10	11.36	41.62	45.27	19.70	100.71	26.48	19.03	***	*	***
(*E*)-2-Hexen-1-ol	0.30	144.59	214.19	97.93	81.31	1324.94	358.65	241.78	***	***	***
(*Z*)-2-Hexen-1-ol	8.22	0.96	1.70	nd	0.27	9.82	1.70	0.86	***	ns	***
Alcohols											
2-Pentanol	0.65	0.58	nd	0.93	0.41	nd	0.34	1.52	***	ns	***
1-Butanol	nd	nd	nd	nd	0.45	nd	0.45	nd	***	***	***
1-Penten-3-ol	nd	2.12	nd	2.27	nd	nd	2.95	2.00	***	ns	***
E-2-Penten-1-ol	1.12	1.94	1.95	1.73	1.10	10.85	2.26	nd	**	**	***
1-Octen-3-ol	nd	0.32	nd	0.30	0.44	nd	0.24	0.39	***	***	***
1-Octanol	0.38	0.38	nd	0.32	0.12	nd	0.10	nd	***	***	***
Benzyl alcohol	0.33	0.23	nd	0.30	0.25	nd	0.76	0.52	***	***	***
2-Phenylethanol	0.55	0.28	1.12	0.32	2.17	nd	1.78	0.51	***	***	***
2-Phenoxyethanol	nd	0.19	nd	nd	0.74	nd	1.01	nd	***	***	***
Volatile fatty acids											
Hexanoic acid	1.03	1.35	1.39	1.55	3.86	1.83	2.57	5.50	ns	***	ns
(*E*)-2-Hexenoic acid	nd	nd	nd	nd	0.93	nd	nd	nd	***	***	***
Octanoic acid	nd	nd	nd	nd	3.74	nd	1.37	0.64	***	***	***
Nonanoic acid	0.52	0.16	nd	nd	1.20	nd	nd	nd	***	***	***
Decanoic acid	nd	nd	nd	nd	2.35	nd	nd	0.29	***	***	***
Hexadecanóic acid	nd	1.50	nd	4.82	nd	nd	nd	3.19	***	*	ns
Volatile phenols											
Methyl salicilate	nd	1.53	nd	0.26	0.15	1.53	1.21	0.63	***	***	***
4-Vinylguaiacol	0.86	0.13	3.43	0.36	0.23	nd	nd	nd	***	***	***
Vanillin	nd	0.43	6.53	6.94	1.67	nd	1.93	2.27	***	***	***
Methyl vanilate	nd	nd	nd	nd	0.36	nd	nd	0.24	***	***	***
Carbonyl compounds											
Benzaldehyde	0.42	0.29	nd	0.32	0.70	1.30	0.59	0.25	***	***	***

C: cultivar; D: harvest date; ns: not significance; nd: not detected. All the compounds were quantified as 4-nonanol equivalents.
* Significance at 95% level.
** Significance at 99% level.
*** Significance at 99.9% level.

13 °Baumé (Garcia et al., 2003). (*E*)-2-Hexenal was the most abundant volatile compound in Riesling grapes, and it showed a significant increase in concentration after *véraison* until harvest (Kalua and Boss, 2010).

On the other hand, monoterpenes and C_{13}-norisoprenoids are considered to be very important in determining the flavor and varietal character of *Vitis vinifera* cultivars and this concentration in wine may be increased by appropriate winemaking procedures (Rocha et al., 2007; Gomez et al., 1995; Garcia et al., 2003). With regard to the total concentration of free monoterpenes, *B. lexitimo* cultivar was the richest in the two ripening dates studied and the concentration increased during ripening due to linalool. Monoterpenes are the source of the characteristic floral and fruity characteristics of wines made from grape varieties such *Muscat* and *Gewürztraminer*, and to a lesser extent, of wines made from *Riesling*, *Albariño*/*Alvarinho* and *Loureiro* (Wilson et al., 1986; Versini et al., 1994; Muñoz-Organero et al., 1998; Bureau et al., 2000a,b; Oliveira et al., 2008). Muscat/floral grape varieties may generally synthesize more terpenes than the non-Muscat/nonfloral grape varieties during ripening (Kalua and Boss, 2009). The concentration of these compounds gradually rises during ripening until maturity (Wilson et al., 1984; Günata et al., 1985). However terpenes may decrease once optimal sugar levels are attained, although this may be influenced by temperature and water availability during ripening (Ribéreau-Gayon et al., 2000). In *Godello* cultivar free monoterpenes were not detected at last ripening date and geraniol was only found in *Agudelo* cultivar at the same date. Geraniol was the most abundant monoterpene in *Alvarinho* (45.7%) from the north of Portugal, followed by linalool (19.5%) and then by *trans*-pyran linalool oxide (10.9%) (Oliveira et al., 2004; Genisheva and Oliveira, 2009). However concentrations of the main free terpenols increased during grape development of *Muscat Hamburg*, except for geraniol (Fenoll et al., 2009). 3,7-Dimethyl-1,5-octadien-3,7-diol (diendiol I) and pyran oxides of linalool did not show significant

differences between the two ripening dates studied. In *Airén* grape variety normally the concentrations of the *trans*-isomer of pyran linalool oxide and diendiol I decreased continuously over the ripening period studied (Garcia et al., 2003). However results found in the *Muscat Hamburg* cultivar by Fenoll et al. (2009), showed that an increase of level of diendiol I was been related to an increase of linalool. Linalool has been proposed as the substrate for conversion to higher oxidation state compounds such as diendiol I (Wilson et al., 1984, 1986). In agreement with this proposal, in our study a significant correlation according Pearson's test ($p > 0.05$) was observed between linalool and diendiol I concentrations (data not shown). On the other hand, a decrease in the concentration of α-terpineol, a terpene alcohol with floral character in berries (Ribéreau Gayon et al., 1975), was observed between the two ripening dates for all cultivars studied. Similar results were found in *Muscat Hamburg* during ripening by Fenoll et al. (2009).

C_{13}-norisoprenoids have been identified as potential impact odorants in wines, particularly the compounds β-damascenone and β-ionone (Ferreira et al., 2000; Gomez-Miguez et al., 2007; Vilanova and Martinez, 2007). As is shown by Enzell (1985), carotenoid degradation can give rise to different highly flavorant C_{13}-norisoprenoid derivatives. Grapes and wines contain several C_{13}-norisoprenoids such as β-damascenone, α-ionone and others, so it would be expected that the carotenoid composition of grapes would have a noticeable effect on wine flavor (Razungles et al., 1988; Marais et al., 1992). β-Damascenone (fruity aroma) plays an important role in development of the characteristic *Riesling* aroma in wines (Chisholm et al., 1994). In our study, these compounds showed lower concentration in white cultivars than red cultivar *Serradelo* in the first date studied, and decreased at harvest date. In contrast it can be seen (Table 2) that C_{13}-norisoprenoids show significant differences for all cultivars between the two ripening dates studied except for *B. lexitimo*. Studies realized with *Fernão-Pires* cultivar from Portugal showed that the total amount of monoterpenoids and C_{13}-norisoprenoids increased from *véraison* to 20 days, where the maximum was reached, decreasing in the following weeks (Coello et al., 2007). In our study, C_{13}-norisoprenoids β-damascenone and β-ionone were not present in *Godello* and *B. lexitimo* cultivars at the last ripening stage, however β-damascenone was one of the most powerful odorants for *B. lexitimo* and *Agudelo* white wines from Betanzos (Vilanova et al., 2009). Due to the fact that between *véraison* and maturity the glycosylation of C_{13}-norisoprenoids may occur, a decrease of these compounds in free form is expected (Razungles et al., 1988; Baumes et al., 2002). In Shiraz grape berries, increased concentrations of glycosylated precursors to β-damascenone have been associated with increased sunlight penetration to the fruiting zone of the vine (Bureau et al., 2000a,b; Ristic et al., 2010).

Total alcohols in the free fraction show an increase for all cultivars between the two dates studied with the exception of red *Serradelo*. White *Godello* and *B. lexitimo* cultivars showed higher values of total alcohols in the last ripening stage than those obtained for the other cultivars studied. A general conclusion from an earlier study reported that C_6-compounds and alcohols reach their highest concentration during late ripening (Gomez et al., 1995; Kalua and Boss, 2009). The dominance of alcohols during late berry development, preceded by aldehydes, permits the use of the alcohols to aldehydes ratios in the prediction of harvest date for enhanced grape and wine aroma (Kalua and Boss, 2009). Among alcohols, 2-pentanol and 1-penten-3-ol were the only compounds stable during ripening (Table 3). *E*-2-Penten-1-ol and 1-penten-3-ol dominated the alcohol group in the two ripening stages for all cultivars with exception of the *Agudelo* cultivar. In general, benzyl alcohol and 2-phenylethanol presented the concentrations for all cultivars in the last ripening stage. Similarly, these compounds were detected in low concentrations in *Muscat Hamburg* cultivar and fluctuated during ripening (Fenoll et al., 2009). The levels of benzyl alcohol and 2-phenylethanol are quite high in non-Muscat grape cultivars in which terpenols are less abundant (Selli et al., 2003; Voirin et al., 1992; Dieguez et al., 2003; Rocha et al., 2000). At the last sampling time, it was possible to detect very low concentrations of 2-phenylethanol, which has a pleasant "rose" aroma, whereas the other alcohols are more herbaceous/purgent. The Literature indicates that 2-phenylethanol is very abundant in wines; however, it arises mainly from fermentation, with 2-phenylalanine being its precursor (Laminkanra et al., 1996). 2-Phenylethanol has a dual sensory effect, positive sensory attributes at low concentration and negative sensory attributes at elevated levels (Schwab et al., 2008).

Other compounds detected and quantified in the free fraction of the cultivars studied were volatile fatty acids, volatile phenols and carbonyl compounds (Tables 2 and 3). These three groups of volatile compounds showed significant differences among cultivars at the two ripening dates studied. Total free volatile fatty acids present a significant increase between the two dates in *Agudelo* and *Serradelo* cultivars, dominated by hexanoic acid. Hexanoic acid characterized to all cultivars showed an increase at the last ripening stage, with the highest concentration for red *Serradelo* cultivar. A decreased content of volatile fatty acids between ripening dates was shown for *Godello* white cultivar, in which hexanoic acid was detected only at last ripening stage. The interaction cultivar * ripening date was significant for all compounds except for hexanoic acid and hexadecanoic acid. Previous studies shown that among volatile fatty acids, the concentration of octanoic acid was the highest for *B. lexitimo*, *Agudelo* and *Serradelo* wines (Vilanova et al., 2009). In this study also *Agudelo* presented the highest value of octanoic acid at last ripening stage.

No significant changes were detected in concentrations of total free volatile phenols and carbonyl compounds among the dates studied for *Godello* and *Serradelo* cultivars. Volatile phenols presented a decrease of total concentration for *B. lexitimo* and *Serradelo* during ripening while *Agudelo* increased its concentration in the last ripening stage. The evolution of these compounds has a tendency to a slight decrease during the ripening period in *Macabeo*, *Airén* and *Chardonnay* musts (Garcia et al., 2003). These authors suggested that this could be attributed to the dilution effect produced by water accumulation in the grape during this period. 4-vinylguaiacol and vanillin concentrations showed low values and in most cases decreased during ripening of berries. Vanillin was the most abundant compound for all cultivars at last ripening stage with exception for *Godello*. Finally the level of carbonyl compounds also showed an increase during ripening for all cultivars except for *Serradelo* cultivar.

Results of ANOVA (Table 3) showed that the effect of "cultivar", "ripening date" and interaction "cultivar * ripening date" on volatiles is important because 97.2%, 83.3% and 88.8%, respectively of the volatiles quantified showed significant differences among cultivars.

2.2.2. Glycosidically bound compounds

Tables 4 and 5 show the evolution of groups of bound volatile compounds during ripening stages studied (mean and standard deviation), together with the ANOVA results for the factors "cultivar (C)" and "ripening date (D)" and interaction C * D. This study included 45 bound volatile compounds: 8 alcohols, 6 C_6-compounds, 6 volatile fatty acids, 12 monoterpenes, 5 C_{13}-norisoprenoids, 7 volatile phenols and 1 carbonyl compound. Differences among cultivars, ripening date and the effect of the ripening date on glycosidically bound volatiles were also analyzed.

ANOVA shows statistically significant differences among cultivars for all groups of volatile compounds studied with the exception of volatile phenols (Table 4). Differences between the two

Table 4
Glycosidically bound composition (μg/L) of Betanzos cultivars from two ripening dates. Mean values ($n = 3$) and standard deviation in parentheses, analysis of variance and contrasts significance.

Cultivar	Alcohols	C_6-compounds	Monoterpenes	C_{13}-norisoprenoids	Volatile fatty acids	Volatile phenols	Carbonyl compounds	Total
Mean (SD) of bound volatile compounds								
Ripening date 23.08.2007 (A)								
Agudelo	40.7 (2.1) a	7.0 (0.6) b	2.5 (0.1) a	3.4 (0.1) a	5.8 (0.4) a	5.5 (0.4) a	0.2 (0.0) b	65.1 (2.6) b
Godello	11.5 (4.4) b	1.7 (0.5) a	2.5 (0.8) a	5.3 (2.0) b	1.3 (0.1) a	5.0 (3.3) a	1.0 (0.1) c	28.4 (11.3) a
Blanco lexitimo	20.0 (2.5) c	5.2 (0.6) b	12.1 (3.1) b	2.3 (0.1) a	1.6 (0.1) b	9.9 (1.6) b	nd a	51.2 (7.8) b
Serradelo	47.7 (2.7) d	13.7 (1.8) c	9.7 (0.8) b	3.5 (0.1) ab	7.3 (1.3) c	12.5 (1.0) b	0.2 (0.0) b	94.6 (5.0) c
Ripening date 30.08.2007 (B)								
Agudelo	56.5 (0.9) a	9.1 (0.1) b	39.6 (0.4) a	5.0 (1.4) b	6.7 (0.6) a	11.5 (0.4) b	nd a	128.4 (1.7) c
Godello	77.7 (10.3) b	8.6 (1.4) b	9.1 (1.0) b	8.6 (0.5) c	2.3 (0.0) b	14.4 (0.6) c	3.2 (0.2) c	123.9 (11.7) c
Blanco lexitimo	14.8 (1.6) c	5.2 (1.3) a	13.2 (2.8) c	1.6 (0.3) a	4.9 (0.0) c	9.7 (1.6) ab	0.1 (0.0) ab	49.6 (7.6) a
Serradelo	36.2 (2.9) d	10.4 (1.5) b	5.3 (0.8) d	1.1 (0.1) a	7.9 (1.1) d	6.7 (0.8) b	0.2 (0.0) b	68.0 (5.1) b
Analysis of variance p-values								
Cultivar	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.419	<0.0001	<0.0001
Repetition	0.228	0.344	0.034	0.959	0.732	0.244	0.211	0.071
Harvest date	<0.0001	0.007	<0.0001	0.242	0.000	0.002	<0.0001	<0.0001
Cultivar * ripening date	<0.0001	<0.0001	<0.0001	0.001	0.014	<0.0001	<0.0001	<0.0001
Contrast p-values cultivar vs. ripening date								
Agudelo-A vs. *Agudelo*-B	0.000	0.035	<0.0001	0.052	0.152	0.000	0.007	<0.0001
Godello-A vs. *Godello*-B	0.0001	0.0001	0.0001	0.001	0110	<0.0001	<0.0001	<0.0001
Blanco lexitimo-A vs. *Blanco lexitimo*-B	0.153	0.996	0.328	0.400	<0.0001	0.878	0.086	0.768
Serradelo-A vs. *Serradelo*-B	0.005	0.003	0.001	0.007	0.272	0.000	0.742	0.000

nd: not detected. Duncan test: different letters indicates significant differences among cultivars. All the compounds were quantified as 4-nonanol equivalents.

ripening dates were significant for all groups of bound volatile compounds with exception for bound C_{13}-norisoprenoids. The interaction cultivar * ripening date was significant for all bound volatile groups. Significant changes of total bound concentration were found for all cultivars ($p < 0.0001$) except for *B. lexitimo*. *Agudelo* and *Godello* cultivars show a significant increase between the two ripening dates studied, showing the highest concentration of total volatile composition at the last date (0.13 and 0.12 mg/L, respectively).

The bound alcohols showed the highest concentration for all cultivars at both dates studied, accounting for between 30% for *B. lexitimo* and 62% for *Godello* of the total bound fraction. Significant differences for alcohols were found among cultivars for the two ripening dates with exception of *B. lexitimo*. *Godello* cultivar showed the highest concentration of bound alcohols at the last date. Benzyl alcohol and 2-phenylethanol, with floral aromas, were the most abundant glycosidically bound compounds for all cultivars at the two ripening stages (Table 5). Both compounds were the two main aromatic compounds present in *Muscat Hamburg* grapes (Fenoll et al., 2009). Voirin et al. (1992) indicate that the presence of aromatic alcohols is associated with neutral cultivars.

C_6-compounds and monoterpenes showed similar behavior, increasing their concentration for *Agudelo* and *Godello* cultivars and decreasing for *Serradelo*. This result shows clearly the interaction cultivar * ripening date for these groups of compounds. Bound C_6-compounds showed the highest value for *Serradelo* at first ripening and then, at the last ripening date this concentration was similar to those of *Agudelo* and *Godello*. (*E*)-2-Hexenal and 1-hexanol account for the highest concentrations, at the last date, for *Agudelo* and *Godello* cultivars, respectively. (*Z*)-2-Hexen-1-ol was not detected at harvest for any cultivar.

The concentration of monoterpenes was higher in bound form than free form, for all cultivars except for *B. lexitimo*. The highest level of total bound monoterpenes was detected for white *Agudelo* cultivar at the last ripening date. Red cultivar *Serradelo* had much more discrete levels of monoterpenic compounds than the other cultivars and this concentration was minor at the last ripening date. Similar results were observed in *Vinhão* red cultivar from Portugal, where only traces of monoterpenes were found (Oliveira et al., 2004). Bound 3,7-dimethyl-1,5-octadien-3,7-diol (diendiol I) was the most abundant compound for *Agudelo*, *B. lexitimo* and *Serradelo* in the first date and decreased in the last date, except for *Agudelo* cultivar. A significant positive correlation according to Pearson's test ($p > 0.05$) was observed between bound linalool and diendiol I concentrations (data not shown). Nerol, (*E*)-8-hydroxy-linalool, (*Z*)-8-hydroxy-linalool and HO-trienol were only detected in the bound form for the Betanzos cultivars. Levels of geraniol in the bound fraction were much higher than those found in the free fraction and this compound showed an increase between the two dates studied for *Godello* and *Agudelo* cultivars. Similar results were found by Fenoll et al. (2009) for *Muscat Hamburg* during ripening.

Among the cultivars studied, *Godello* showed the highest concentration of bound C_{13}-norisoprenoids at the last date followed by *Agudelo* cultivar. With regard to bound C_{13}-norisoprenoids, the concentrations of 3-hydroxy-β-damascone and 3-oxo-7,8-dehydro-α-ionol did not show changes between the two ripening dates studied. 3-Oxo-α-ionol presented the highest concentration for *Agudelo* cultivar in the last date, followed by 3-oxo-α-ionol for *Godello*. Qualitatively, the C_{13}-norisoprenoid compositions of the berries for the most varieties are quite similar at the last date. The major aglycons are 3-hydroxy-β-damascone, 3-oxo-α-ionol, and 3-oxo-7,8-dehydro-α-ionol in accordance with data reported for other cultivars (Razungles et al., 1988).

Other compounds detected and quantified in the bound fraction were volatile fatty acids, volatile phenols and 1 carbonyl compound. The level of volatile fatty acids showed an increase between the two ripening stages only for all white *B. lexitimo* cultivar. *Serradelo* red cultivar presented major level of bound volatile acids in the two dates studied. Hexanoic acid was the most abundant compound for *Agudelo* and *Godello* cultivars at ripe stage, however hexadecanioc acid showed major values for *B. lexitimo* and *Serradelo*.

Volatile phenols in bound form increased their concentration for *Agudelo* and *Godello* during ripening showing significant

Table 5
Changes in glicosidically bound composition of Betanzos grape cultivars during ripening.

Bound compounds (μg/L)	Ripening date								ANOVA		
	23.08.2007 (A)				30.08.2007 (B)						
	Agudelo	*Godello*	*B. lexitimo*	*Serradelo*	*Agudelo*	*Godello*	*B. lexitimo*	*Serradelo*	C	D	C * D
Monoterpenes											
trans-Furan linalool oxide	0.17	0.33	0.80	0.63	3.26	0.93	1.13	0.42	***	***	***
cis-Furan linalool oxide	nd	nd	0.70	1.33	2.80	2.60	0.78	0.67	***	***	***
3,7-Dimethylocta-1,5-dien-3,7-diol	1.60	nd	3.59	2.53	8.31	nd	3.36	1.45	***	***	***
trans-Pyran linalool oxide	nd	0.20	0.25	0.31	0.76	0.39	0.22	0.24	**	***	***
cis-Pyran linalool oxide	0.12	nd	0.14	0.21	nd	nd	0.14	0.19	***	**	**
(*E*)-8-Hydroxy-linalool	nd	0.81	0.38	1.02	1.42	1.45	0.34	0.37	***	***	***
(*Z*)-8-Hydroxy-linalool	nd	0.29	1.09	0.84	6.42	0.75	1.13	0.59	***	***	***
HO-trienol	0.29	nd	0.37	0.21	1.06	nd	0.42	0.11	***	***	***
Geraniol	0.33	0.71	2.26	1.13	3.24	2.15	2.00	0.62	***	***	***
Nerol	nd	0.13	0.76	0.21	0.60	0.36	0.66	0.21	***	***	***
Linalool	nd	nd	0.59	0.48	9.77	0.45	1.98	0.29	***	***	***
α-Terpineol	nd	nd	1.21	0.76	1.96	nd	1.10	0.18	***	***	***
C_{13}-norisoprenoids											
3-Hydroxy-β-damascone	1.56	1.01	0.77	nd	nd	2.50	nd	0.67	***	ns	***
3-Hydroxy-7,8-dehydro-β-ionol	nd	1.32	nd	1.63	nd	3.84	nd	nd	***	*	***
3-Oxo-7,8-dihydro-α-ionol	nd	0.93	nd	nd	nd	nd	nd	nd	*	ns	*
3-Oxo-α-ionol	0.99	1.01	1.50	0.95	5.00	1.15	1.61	nd	***	***	***
4-Oxo-β-ionol	0.83	1.08	nd	0.95	nd	1.15	nd	0.47	***	***	***
C_6-compounds											
(*E*)-2-Hexenal	3.85	1.10	2.65	6.56	1.54	4.77	1.35	1.62	***	***	***
1-Hexanol	1.46	0.58	1.38	4.04	6.43	2.20	1.81	3.49	***	***	***
(*E*)-3-Hexen-1-ol	nd	nd	nd	3.08	nd	nd	nd	1.47	**	***	**
(*Z*)-3-Hexen-1-ol	0.77	nd	0.88	nd	1.14	0.76	1.43	1.88	***	***	***
(*E*)-2-Hexen-1-ol	nd	nd	0.31	nd	nd	0.83	0.60	1.94	***	***	***
(*Z*)-2-Hexen-1-ol	0.90	nd	nd	nd	nd	nd	nd	nd	***	***	***
Alcohols											
2-Pentanol	0.85	nd	1.87	1.85	nd	1.41	1.53	1.91	***	ns	**
1-Butanol	1.55	nd	nd	nd	nd	nd	nd	nd	***	***	***
3-Methyl-1-butanol	0.98	0.41	0.28	1.38	12.51	1.91	0.82	3.59	***	***	***
3-Methyl-2 butanol	0.59	nd	0.36	0.40	nd	1.25	0.17	0.27	***	ns	***
1-Octanol	0.09	nd	nd	0.20	0.52	nd	0.13	nd	***	***	***
Benzyl alcohol	33.16	nd	12.96	31.78	22.56	43.17	8.62	24.20	***	***	***
2-Phenylethanol	3.42	10.92	4.58	11.56	20.94	29.41	3.53	6.25	***	***	***
2-Phenoxyethanol	nd	0.21	nd	0.54	nd	0.56	nd	nd	***	***	***
Volatile fatty acids											
Hexanoic acid	1.36	0.29	nd	nd	3.52	0.81	0.45	nd	***	***	***
(*E*)-2-Hexenoic acid	1.29	nd	nd	nd	2.53	nd	nd	nd	***	**	***
Octanoic acid	0.94	0.27	0.63	0.53	0.62	nd	0.38	0.36	***	***	ns
Nonanoic acid	0.95	0.09	0.16	0.27	nd	nd	0.24	0.37	***	***	***
Decanoic acid	0.66	nd	0.42	nd	nd	nd	0.31	nd	***	***	***
Hexadecanoic acid	nd	nd	nd	5.71	nd	nd	2.89	7.03	***	***	***
Volatile phenols											
Methyl salicilate	0.28	0.70	0.54	2.37	0.63	1.52	1.02	1.57	***	*	***
Eugenol	0.75	0.46	3.10	0.58	1.85	1.07	2.94	0.26	***	***	***
4-Vinylguaiacol	0.88	3.08	2.01	2.64	3.25	11.83	2.58	2.08	***	***	***
4-Vinylphenol	0.46	nd	0.43	nd	nd	nd	nd	nd	***	***	***
Vanillin	0.89	0.28	1.21	3.70	nd	nd	0.91	1.42	***	***	***
Methyl vanilate	1.55	0.27	1.54	2.24	4.27	nd	1.50	0.96	***	***	***
Acetovanillone	0.73	0.23	1.09	0.95	1.51	nd	0.80	0.47	***	ns	***
Carbonyl compounds											
Benzaldehyde	0.22	0.99	nd	0,22	nd	3.22	0.13	0.25	***	***	***

C: cultivar; D: harvest date; ns: not significance; nd: not detected. All the compounds were quantified as 4-nonanol equivalents.
* Significance at 95% level.
** Significance at 99% level.
*** Significance at 99.9% level.

differences among cultivars at both dates. Eugenol, 4-vinylphenol and acetovanillone only were detected in the bound fraction. These compounds showed the highest values for most cultivars with fluctuations between the two ripening stages. Eugenol glycosides were detected at low concentration in *Muscat Hamburg* grapes (Fenoll et al., 2009). Acetovainillone was the only stable phenol volatile between both data analyses.

Finally, the carbonyl compounds group showed the lowest concentration for all cultivars at the two ripening dates. Benzaldehyde was found in both fractions in ripe cultivars, except for *Agudelo* in the bound fraction.

Results of the ANOVA (Table 5) showed that the effect of "cultivar" on volatiles is important because 100% of the volatiles quantified showed significant differences among cultivars. Data in Table 5 also presents ANOVA results for the factor ripening date, where the concentration of five compounds did not vary significantly among dates (two C_{13}-norisoprenoids, two alcohols and one phenol volatile). Finally, a total of 44 volatile compounds (97.7% of total

volatiles quantified) present a significant interaction between the two factors, cultivar and ripening date.

3. Concluding remarks

During ripening, substantial changes take place in the composition of grapes. Therefore, it is important to choose the correct moment for their harvest because grape composition will determine the quality of the resulting wine. In this work the volatile composition of cultivars from Betanzos (NW Spain) was strongly influenced by the cultivar and the ripening stage. The results also showed significant interaction "cultivar * ripening date" for 93.8% of volatile compounds quantified. The increase of free total volatile concentration was only significant for *Godello* and *Serradelo* cultivars between the two ripening dates studied, because of the high levels of C_6-compounds, (*E*)-2-hexenal, (*E*)-2-hexen-1-ol and 1-hexanol. *Godello* cultivar showed the maximum value of volatile composition coincident with the last ripening data studied (August 30). White *B. lexitimo* was characterized by a higher concentration of monoterpenes (linalool) at last ripening date. It will permit to produce more terpene-rich wines when the harvest is delayed. *Agudelo* cultivar showed a high level of bound monoterpenes on the first harvest date. The red cultivar *Serradelo* also showed a high level of bound compounds at first harvest date analyzed, however technologic maturation is incomplete at this time. The results of this study showed that the evolution of the volatile composition during ripening was not proportional to the changes in the sugar content and ratio sugar/acid content of grapes, which shows that the technological and aroma ripening did not occur at the same time. For this reason, it is of great importance to know the evolution of volatiles during ripening and the corresponding sugar/acidity ratio. This type of information about the raw material of wine represents a helpful tool to support decisions of the winemaker based on the wine type.

4. Experimental

4.1. Sampling and processing

Grapes from *V. vinifera B. lexitimo*, *Agudelo*, *Godello* and *Serradelo* cultivars were collected in Betanzos Geographical Denomination (NW Spain) from Lorenzo Bescansa vineyard (latitude 43°17′N and longitude 0°13′W). The samples were collected in triplicate, at two ripening dates. For each sample, 300 grape berries were picked randomly throughout the vine, taking into consideration the number of berries per bunch, and the balance between shadow and sun exposure in the vineyard. Samples were transported immediately to the laboratory. Grape berry samples were manually crushed for analysis of the °Brix and titratable acidity (TA). The other samples were stored and frozen at −20 °C until volatile compounds analysis. °Brix was determined using a hand-held refractometer while TA, being expressed as percentage of tartaric acid, was determined using 100 mmol/L NaOH (OIV Official Methods). The characteristics of grape cultivars from Betanzos for the two ripening data are shown in Table 1.

4.2. Solvents

All the solvents used were of at least gas chromatographic grade: ethyl acetate (Merck, SupraSolv), dichloromethane (Merck, SupraSolv), methanol (Merck, SupraSolv) and pentane (Merck, UniSolv). Azeotrope pentane-dichloromethane (2:1 v/v) was distilled after combination of pentane and dichloromethane and redistilled whenever necessary.

4.3. Extraction of free and bound fractions

About 550 g of frozen berries were thawed at 4 °C overnight, and then manually crushed, centrifuged (*RCF* = 9660, 20 min, 4 °C) and filtered through a glass wool bed. To 200 mL of juice, 2.35 μg of 4-nonanol (Merck, Ref. 818773) were added and passed through a LiChrolut EN cartridge (Merck, 500 mg, 40–120 μm) according to Ibarz et al. (2006) and Oliveira et al. (2008). The resin was previously pre-conditioned with 5 mL of methanol and 10 mL of aqueous alcoholic solution (10%, v/v). Free and bound fractions were eluted successively with 5 mL of pentane–dichloromethane azeotrope and 7 mL of ethyl acetate. The pentane–dichloromethane elute was dried over anhydrous sodium sulfate and concentrated to 200 μL by solvent evaporation at 34 °C through a Vigreux and then a Dufton column, prior to analysis. The ethyl acetate eluate was concentrated to dryness in vacuum (40 °C) and re-dissolved in 200 μL of 100 mmol/L citrate–phosphate buffer (pH = 5.0). Residual free compounds were extracted five times with azeotropic mixture and discarded. Fourteen milligrams of enzyme AR2000 (Gist-Brocades) were added to the glycoside extract and the mixture was incubated at 40 °C, for 12 h. Released aglycons were extracted with pentane–dichloromethane azeotrope, after addition of 2.35 μg of 4-nonanol as internal standard. The organic phase was then concentrated to 200 μL through a Dufton column. The extractions were made in triplicate.

4.4. Chromatographic analysis

Gas chromatographic analysis of volatile compounds was performed using a GC–MS system constituted by a Varian 3400 chromatograph and an ion-trap mass spectrometer Varian Saturn II. A 1 μL injection was made into a capillary column, coated with CP-Wax 52 CB (50 m × 0.25 mm i.d., 0.2 μm film thickness, Chrompack). The temperature of the injector (SPI-septum-equipped programmable temperature) was programmed from 20 to 250 °C, at 180 °C/min. The oven temperature was held at 40 °C, for 5 min, then programmed to rise from 40 to 250 °C, at 3 °C/min, then held 20 min at 250 °C and finally programmed to go from 250 to 255 °C at 1 °C/min. The carrier gas was helium N60 (Air Liquide) at 103 kPa, which corresponds to a linear speed of 180 cm/s at 150 °C. The detector was set to electronic impact mode (70 eV), with an acquisition range from 29 to 360 *m*/*z*, and an acquisition rate of 610 ms.

4.5. Identification and quantification of volatile compounds

Identification was performed using the software Saturn version 5.2 (Varian), by comparing mass spectra and retention indices with those of pure standard compounds. All of the compounds were quantified as 4-nonanol equivalents.

4.6. Statistical analyses

The data were analyzed using XLstat-Pro (Addinsoft 2009). A mixed model ANOVA was performed on the volatile compounds analysis data. Cultivar, repetition and ripening date, as well as the two-way interaction treatment cultivar * ripening date were evaluated for significance. The differences among cultivars and cultivar composition between two ripening stages were evaluated using a priori contrast ($p < 0.05$). The mean differences between cultivars were calculated using the least significant difference Fishers' test.

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