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Effect of Macerating Enzymes on the Oligosaccharide Profiles of Merlot Red Wines

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ABSTRACT: Commercial pectinase preparations are applied in winemaking to improve wine processing and final quality. These preparations contain pectolytic enzyme activities such as polygalacturonases, pectin esterases, pectin lyases, and rhamnogalacturonases. These enzymes modify the polysaccharide and oligosaccharide composition of wines. The influence of various commercial enzyme preparations on wine oligosaccharide composition was studied, on Merlot wines from the Bordeaux area. Wine oligosaccharides were isolated by high-resolution size-exclusion chromatography on a Superdex-30 HR column. The glycosyl residue and glycosyl linkage compositions of the oligosaccharide fractions obtained were determined. The MS spectra of the Merlot oligosaccharide fractions from control and enzyme-treated wines were recorded on an AccuTOF mass spectrometer equipped with an electrospray ionization (ESI) source and a time-of-flight (TOF) mass analyzer. Oligosaccharides in the control wines were partly methylated homogalacturonans, corresponding to smooth regions of pectins, whereas those of the enzyme-treated wines were mostly rhamnogalacturonan-like structures linked with neutral lateral chains, arising from the hairy regions. The enzyme preparations used thus cleaved the rhamnogalacturonan backbone of the hairy zones and demethylated and hydrolyzed the smooth regions. Besides, different structures were detected, depending on the enzyme preparation used, indicating that they contained rhamnogalacturonase activities with different specificities. The oligosaccharide profiles can serve as a marker of enzymatic treatments.

KEYWORDS: wines, pectinases, oligosaccharides, Merlot, ESI-TOF, oligogalacturonic acids, oligorhamnogalacturonic acids

INTRODUCTION

Commercial pectinases that act on grape cell wall polysaccharides are often used in the food industry and particularly in winemaking[1,2] not only to solve clarification and filtration problems,[3−5] but also to increase the pressing efficiency and juice extraction,[6] to release aroma compounds,[7] or to increase and stabilize wine color.[8−11] Pectinases naturally occur in grapes berries, but grape enzymes are poorly active under the pH conditions and SO_2 levels associated with winemaking practice. Fungal enzymes, including pectinases, hemicellulases, glucanases, and glycosidases, are widely used in the food industry.[12] With the exception of glucanases, all of the commercial enzymes are produced by *Aspergillus niger*. The use of commercial enzyme preparations for winemaking arose as a result of increased knowledge on enzymatic activities involved in musts and wines and the nature and structure of the macromolecules found in musts and wines.[13] Wine polysaccharides arising from the pectocellulosic cell walls of grape berries include polysaccharides rich in arabinose and galactose (PRAGs), which comprise arabinogalactan−proteins (AGPs), arabinans, and rhamnogalacturonans (RG-I and RG-II).[14−17] Besides, a recent study has shown that wines contain rather large concentrations (approximately 300 mg L^{-1}) of oligosaccharides structurally related to plant cell wall polysaccharides.[18] Studies on the effect of enzymes on wine polysaccharide composition have shown an increase of RG-II and a decrease of PRAGs, along with a particular modification of AGPs, with loss of their terminal arabinose residues.[10,19] In contrast, the impact of enzymes on oligosaccharide composition is still unknown. Oligosaccharides are involved in plant physiology and in particular in plant defense responses,[20] and they can also play an important role in medicinal, food, and agricultural applications.[21] It was shown that some oligosaccharides have physicochemical properties such as chelation of cations,[22] which can be significant for wine quality.

The present work focuses on the analytical and structural composition of oligosaccharides of Merlot wines released by enzymatic treatments during winemaking. The effect of different commercial enzyme preparations on wine oligosaccharide composition and structure has been studied.

EXPERIMENTAL PROCEDURES

Winemaking. Red wines were made from *Vitis vinifera* var. Merlot grapes grown in a parcel located near Bordeaux in southern France (Château Goudichaud, Saint Germain du Puch) and harvested at maturity in 2004 and 2006. Wines were made in the experimental cellar of Novozymes and Laffort Œnologie at Château Goudichaud. The

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Merlot grapes were randomly distributed in 170 kg lots that were destemmed, crushed, and put into 200 L stainless steel tanks equipped with temperature control, enabling fermentation kinetics to be regulated. Bisulfite (6 g/hL) was first added, then, after homogenization, different enzymatic preparations from Novozymes were added in each tank as listed in Table 1.

After 12 h of maceration at 12 °C, all fermentations were started by implanting Excellence SP (commercial yeast, Lamothe-Abiet, Bordeaux, France) at 20 g/hL; alcoholic fermentation started at 15 °C; the temperature was allowed to rise during fermentation and was then maintained at around 24−25 °C. When alcoholic fermentation was finished (11 days), the free-run juice of each trial was transferred to another 200 L tank. After devatting, the young wines were inoculated with freeze-dried bacteria culture (1 g/hL) to induce malolactic fermentation and maintained at 20−21 °C. Wines were filtered after 6 months of aging on clarifying 20 × 20 cm cellulose-Kieselguhr Seitz K200 plates prior to bottling. Wine samples were stored in the wine experimental cellar at the same temperature and analyzed after the same period of aging, that is, 20 months after the end of alcoholic fermentation.

Table 1. Enzymes and Doses Used in This Study

vintage	expt code	enzyme used	dose
2004	control04	without enzyme	
	A04	Vinozym Vintage FCE[a]	3.5 g/100 kg
	AB04	Vinozym Vintage FCE/Vinoflow FCE	(3.5 g/100 kg)/(5 g/100 kg)
2006	control06	without enzyme	
	A06	Vinozym Vintage FCE	3.5 g/100 kg
	AB06	Vinozym Vintage FCE/Vinoflow FCE	(3.5 g/100 kg)/(5 g/100 kg)
	B06	Vinoflow FCE	5 g/100 kg
	D06	Lafase Fruit	4 g/100 kg
	E06	enzyme E FCE	2 g/100 kg
	Acx06	Vinozym Vintage FCE/Cx	(3.5 g/100 kg)/(10 mL/hL)
	F06	PG/PE and PL/Cx[b]	(10 mL/hL)/(10 mL/hL)/(10 mL/hL)
	G06	PG/PE/PL/Cx	(10 mL/hL)/(10 mL/hL)/(10 mL/hL)/(10 mL/hL)
	I06	enzyme I	10 mL/100 kg
	J06	enzyme J FCE	10 mL/100 kg

[a] FCE, purified from cinnamoyl esterase. [b] PG, polygalacturonase; PE, pectin esterase; PL, pectin lyase; Cx, cellulase.

Isolation of Oligosaccharide Fractions. The oligosaccharide fractions were isolated as previously described.[18] Merlot wines (5 mL) were partially decolorized by adsorption of the pigments on a column of MN Polyamide SC6 (5 × 1 cm) previously equilibrated with 1 M NaCl. Wine oligosaccharides were not retained on the polyamide column and were eluted by 2 bed volumes of 1 M NaCl.[23] High-resolution size-exclusion chromatography was performed by loading 2 mL of the previously concentrated fraction on a Superdex 30-HR column (60 × 1.6 cm, Pharmacia, Sweden) with a precolumn (0.6 × 4 cm), equilibrated at 1 mL/min in 30 mM ammonium formiate, pH 5.6. The elution of polysaccharides was followed with an Erma-ERC 7512 (Erma, Japan) refractive index detector combined with Waters software. One fraction was collected according to elution time between 60 and 93 min. The isolated fraction was freeze-dried, redissolved in water, and freeze-dried again four times to remove completely the ammonium salt. This fraction corresponds to the Merlot wine oligosaccharide fraction.

Glycosyl Residue Composition. Neutral and acidic glycosyl residues of Merlot oligosaccharides from vintage 2006 were determined after acidic methanolysis with anhydrous MeOH containing 0.5 M HCl (80 °C, 6 h), by GC of their per-O-trimethylsilylated methyl glycoside derivatives as described previously.[24]

Glycosyl-Linkage Determination. The glycosyl-linkage composition of wine oligosaccharides from vintage 2006 was determined by GC-MS of the partially methylated alditol acetates. One milligram of oligosaccharides in 0.5 mL of dimethyl sulfoxide was methylated using methyl sulfinyl carbanion and methyl iodide.[25] The methylated materials were then treated with 2 M TFA (1.15 h at 120 °C). The released methylated monosaccharides were converted to their corresponding alditols by treatment with $NaDH_4$ and then acetylated.[26] Partially methylated alditol acetates were analyzed by GC-EI-MS using a DB-1 capillary column (30 m × 0.25 mm i.d., 0.25 μm film); temperature programming was at 135 °C for 10 min, raised at 1.2 °C/min to 180 °C, coupled to a HP5973 MSD.[27] The validity of the methods used and their repeatabilities were checked according to Vidal et al.[17]

ESI Mass Spectrometry. Oligosaccharide samples from Merlot wines from vintages 2004 and 2006 (50 μg) in 1:1 MeOH/water (5 μL) were injected directly into an AccuTOF (AccuTOF JMS-T100LC,

Table 2. Glycosyl Composition (Milligrams per Liter of Wine) of Merlot Oligosaccharides Isolated from Wine[a]

	control06	A06	B06	E06	D06	AB06	F06	G06	Acx06	I06	J06
Rha	22	22	44	42	26	11	37	33	37	34	31
Fuc	1	1	1	1	1	1	2	1	1	1	1
Ara	47	11	22	67	13	6	33	24	19	24	13
Gal	19	28	31	23	20	23	21	23	22	21	18
Glc	33	36	37	33	29	29	19	27	27	21	24
Man	24	23	27	22	19	20	22	21	22	23	18
Xyl	21	13	18	20	4	9	14	16	17	13	15
Gal A	62	62	56	54	46	45	52	53	49	56	40
Glc A	4	7	6	8	5	5	4	5	6	6	4
4-O-MeGlc A	9	7	7	9	7	7	8	6	7	8	7
xylitol	3	2	3	2	2	2	1	1	2	1	1
total	245	212	253	279	181	157	214	210	210	209	173

[a] Rha, rhamnose; Fuc, fucose; Ara, arabinose; Gal, galactose; Glc, glucose; Man, mannose; Xyl, xylose; Gal A, galacturonic acid; Glc A, glucuronic acid; 4-O-MeGlc A, 4-*O*-methylglucuronic acid.

Table 3. Glycosyl Ratio Ara/Gal, Rha/Gal A, and (Ara+Gal)/Rha of Oligosaccharides Isolated from Merlot Wine[a]

	control06	A06	B06	AB06	E06	D06	F06	G06	Acx06	I06	J06
Ara/Gal	2.42	0.41	0.73	0.27	2.87	0.67	1.54	1.06	0.87	1.15	0.73
Rha/Gal A	0.35	0.35	0.78	0.23	0.78	0.55	0.71	0.62	0.75	0.61	0.79
(Ara+Gal)/Rha	1.06	0.63	0.94	0.63	1.67	0.72	1.04	0.90	0.85	0.79	0.77

[a] Rha, rhamnose; Ara, arabinose; Gal, galactose; Gal A, galacturonic acid.

Table 4. Glycosyl Linkage Composition (Mole Percentage) of Merlot Oligosaccharides Isolated from Control and Enzyme-Treated Wines

glycosyl residue	linkage	control06	A06	B06	E06	D06	AB06	F06	G06	Acx06	I06	J06
2,3,4 Rha[a]	T-Rha*p*	3.2	6.2	9.6	5.1	5.8	6.9	7.4	4.9	9.7	8.2	6.0
3,4 Rha	1→2	14.5	23.3	20.4	14.7	23.3	21.6	17.5	19.5	17.7	20.9	24.0
3 Rha	1→2,4	2.5	3.7	2.0	2.3	3.0	1.9	2.4	3.3	1.8	2.4	3.2
2,3,4 Fuc	T-Fuc	0.6	0.9	0.8	tr	0.8	3.0	0.9	1.1	1.7	0.8	0.8
2,3,5 Ara	T-Ara*f*	6.3	4.9	8.2	8.7	4.0	5.5	11.2	6.4	9.6	11.0	4.1
2,3,4 Ara	T-Ara*p*	2.2	2.4	2.7	2.3	2.7	4.3	3.3	2.7	3.9	3.0	2.7
2,5 Ara	1→3	0.7	2.0	2.2	0.9	1.8	2.4	2.3	1.9	2.2	1.7	1.0
3,5 Ara	1→2	tr[b]	tr	0.9	tr	tr	tr	1.0	0.6	1.0	1.2	tr
2,3 Ara	1→5	17.4	1.5	1.4	23.6	1.4	1.5	2.0	1.8	1.3	1.6	0.6
2 Ara	1→3,5	2.4	tr	0.8	1.1			1.9	1.2	0.8	0.7	
2,3/3,4 Xyl	1→2/4	10.8	8.2	7.1	8.9	9.4	11.8	8.2	8.2	10.8	8.5	10.2
2,3,4,6 Gal	T-Gal*p*	0.7	1.8	3.4	0.6	1.1	1.8	2.8	1.2	4.0	2.8	1.0
2,3,4 Gal	1→6	1.1	2.1	2.5	1.1	2.1	2.2	1.9	2.1	2.5	2.1	2.1
2,4,6 Gal	1→3	3.1	2.8	2.0	3.3	4.5	2.5	3.1	5.0	2.3	5.1	5.2
2,3,6 Gal	1→4	2.3	5.3	5.3	2.9	4.5	4.6	4.7	4.9	5.8	4.2	4.8
2,4 Gal	1→3,6	1.3	1.7	1.1	tr	1.1		0.8	1.2	3.2	0.6	1.4
2 Gal	1→3,4,6	1.3	0.8	1.6	1.0	0.9	tr	1.4	1.5	1.0	1.2	0.9
2,3,4,6 Glc	T-Glc*p*	1.5	1.9	1.8	1.2	2.0	2.4	1.8	1.7	1.9	1.7	1.5
2,3,4 Glc	1→6	5.0	5.1	3.8	2.0	5.3	2.9	3.9	3.1	2.5	3.2	4.5
2,3,6 Glc	1→4	2.4	2.8	2.4	2.2	2.9	3.1	2.5	2.9	2.2	2.6	2.6
2,4 Glc	1→3,6	1.7	2.4	2.3	1.4	1.8	3.1	1.0	2.5	1.5	2.8	3.6
2,6 Glc	1→3,4	1.5	2.1		3.5	1.6	0.8		4.2			2.2
2,3 Glc	1→4,6		1.0	0.9	0.9	1.3	0.6	1.4	1.6	1.5	0.8	1.0
2,3,4,6 Man	T-Man*p*	5.7	5.5	6.2	4.0	6.6	7.8	4.4	6.0	5.2	5.1	6.0
3,4,6 Man	1→2	6.8	5.3	4.3	3.7	6.7	6.0	3.7	5.8	2.8	3.6	5.8
2,4,6 Man	1→3	2.4	2.7	2.4	2.2	2.9	3.1	4.1	2.9	2.2	2.6	2.6
2,4 Man	1→3,6	2.1	1.7	1.6	1.2	2.3	1.6	1.0	2.0	0.9	0.9	2.6

[a] 2,3,4-Rha is 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylrhamnitol, etc. [b] tr, trace (<0.5 mol %).

JEOL, Japan) mass spectrometer equipped with an ESI source and a time-of-flight (TOF) mass analyzer, in the negative ion mode. The source voltage was set at −2000 V (negative ESI), the orifice voltage at −45 V (negative ESI), the desolvating chamber temperature at 250 °C, and the orifice temperature at 80 °C, with the mass range being from 200 to 4000 Da. Mass center software was used for analysis.

To confirm the structures of Merlot oligosaccharides, further MS experiments and MS^n fragmentation analysis were performed on a ThermoFinnigan LCQ Advantage (San Jose, CA) mass spectrometer equipped with an ESI source and an ion trap mass analyzer, which were controlled by the LCA navigator software. The mass spectrometer was operated in the negative ion mode in the range m/z 150 to 1200 and under the following conditions: source voltage, 4.5 kV; capillary voltage, 40.26 V; capillary temperature, 200 °C; and collision energy for fragmentation, 25% for MS^2 and 30% for MS^3. The first 30 s of the total ionic current (TIC) was used to have an average spectrum. The ESI-TOF spectrum was then extracted to an ASCII file and exported to Matlab software (version 7.3, The Mathworks Inc.) for statistical analysis.

Statistical Analysis. Principal component analysis (PCA) was used to explore the variability of the whole set of mass spectra collected on the wine oligosaccharide fractions. Before PCA, spectra underwent a pretreatment to obtain a homogeneous data matrix; each spectrum was then normalized by dividing each intensity recorded by the sum of all the intensities (TIC).

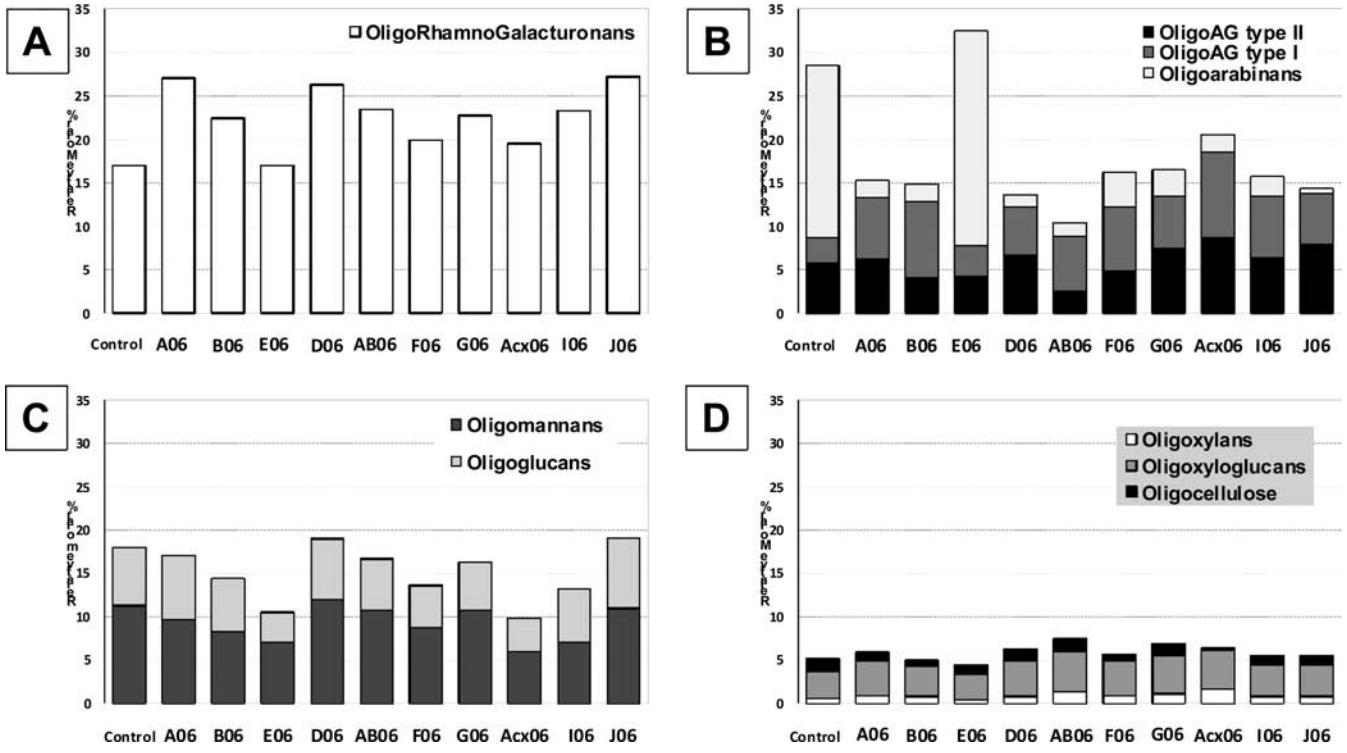

Figure 1. Evolution (mol %) of major families of Merlot oligosaccharides isolated from control and enzyme-treated wines. Xyloglucan is calculated from the sum of T-Xyl, T-Fuc, 4,6 Glc, and one-third of its proportion in 4 Glc. The rest of 4-Glc is assumed to be cellulose. The sum of 4,6 Man and T-Man is considered as mannan, and the sum of 5 Ara and 3,5 Ara is considered as arabinan. Arabinogalactan type I was estimated as the sum of 4 Gal and 3,4 Gal plus T-Gal and arabinogalactan type II as the sum of 3-Gal and 3,6 Gal and equivalent in T-Ara to the value for 3,6 Gal. 2 Rha and 2,4 Rha were attributed to the rhamnogalacturonan backbone.

RESULTS AND DISCUSSION

Wine oligosaccharides of each Merlot wine were recovered in the fraction eluted from the size-exclusion column in the range from 60 to 93 min. These corresponded to oligosaccharides of degrees of polymerization up to 2 as described previously.[18] Differences in the total oligosaccharide concentration (ranging from 157 to 279 mg L^{-1}) were observed between the Merlot wines (Table 2). Enzyme-treated wines, except those treated with preparations B and E, contained lower amounts of oligosaccharides than the control. The observed concentration differences can be related to differences in enzyme activities, resulting in increased release and/or degradation of oligosaccharides. As enzyme-treated wines have different concentrations in polysaccharides, more RG-II and less PRAGs,[10] lower concentrations of oligosaccharides reflect the fact that homogalacturonans and arabinans are highly degraded by pectinases and not recovered in the oligosaccharide fraction.

In agreement with this hypothesis, the most significant decrease of the oligosaccharide fraction (DP > 2) was obtained for wine AB06, which corresponds to the use of two industrial enzymatic preparations (Vinozym Vintage FCE and Vinoflow FCE).

The glycosyl residue composition of Merlot oligosaccharides was determined by GC after methanolysis and trimethysylilation [24] (Table 2). It contains most of the sugars identified in the composition of wine polysaccharides and oligosaccharides.[17,18] We can note that none of the characteristic sugars of RG-II was identified in the glycosyl residue composition of Merlot oligosaccharides, confirming that RG-II is resistant to pectolytic enzyme hydrolysis.[28] The glycosyl composition includes sugars such as rhamnose, arabinose, galactose, xylose, and galacturonic and glucuronic acids from the pectocellulosic cell walls of grape berries[14−17,29−33] and also mannose and glucose released from yeast polysaccharides or from lactic bacteria during malolactic fermentation.[30,34,35] Identification of xylose, glucuronic acid, and 4-*O*-methylglucuronic acid residues indicated that traces of hemicelluloses might be solubilized from grape berry cell walls[36,37] and recovered as oligosaccharide structures in wines. These oligosaccharides of glucuronoxylan type were previously described in oligosaccharide fractions isolated from Carignan and Merlot wines.[18] The oligosaccharide fractions of all enzyme-treated wines contained lower amounts of arabinose residues than that of the control, except the oligosaccharide fraction of the wine treated with enzyme preparation E, which was much enriched in arabinose, originating probably from the pectin side chains. We also observed an increase of rhamnose except for wine AB06 treated by the combination of two enzymes. The association of preparation A (Vinozym Vintage FCE) with preparation B (Wine AB06) (Vinoflow FCE) led to a loss of arabinose residues compared to the control, whereas preparation B alone resulted in an increased release of oligosaccharides containing arabinose and preparation A alone had no effect. This suggests that preparation B releases particular structures which can then be degraded by preparation A. This suggests a synergistic effect between these enzymatic preparations, preparation B releasing particular structures that can then be degraded by preparation A.

To try to have a better understanding of the degradation of grape cell wall induced by enzymes, several ratios were calculated (Table 3). The Ara/Gal ratio is characteristic of the wine poly-

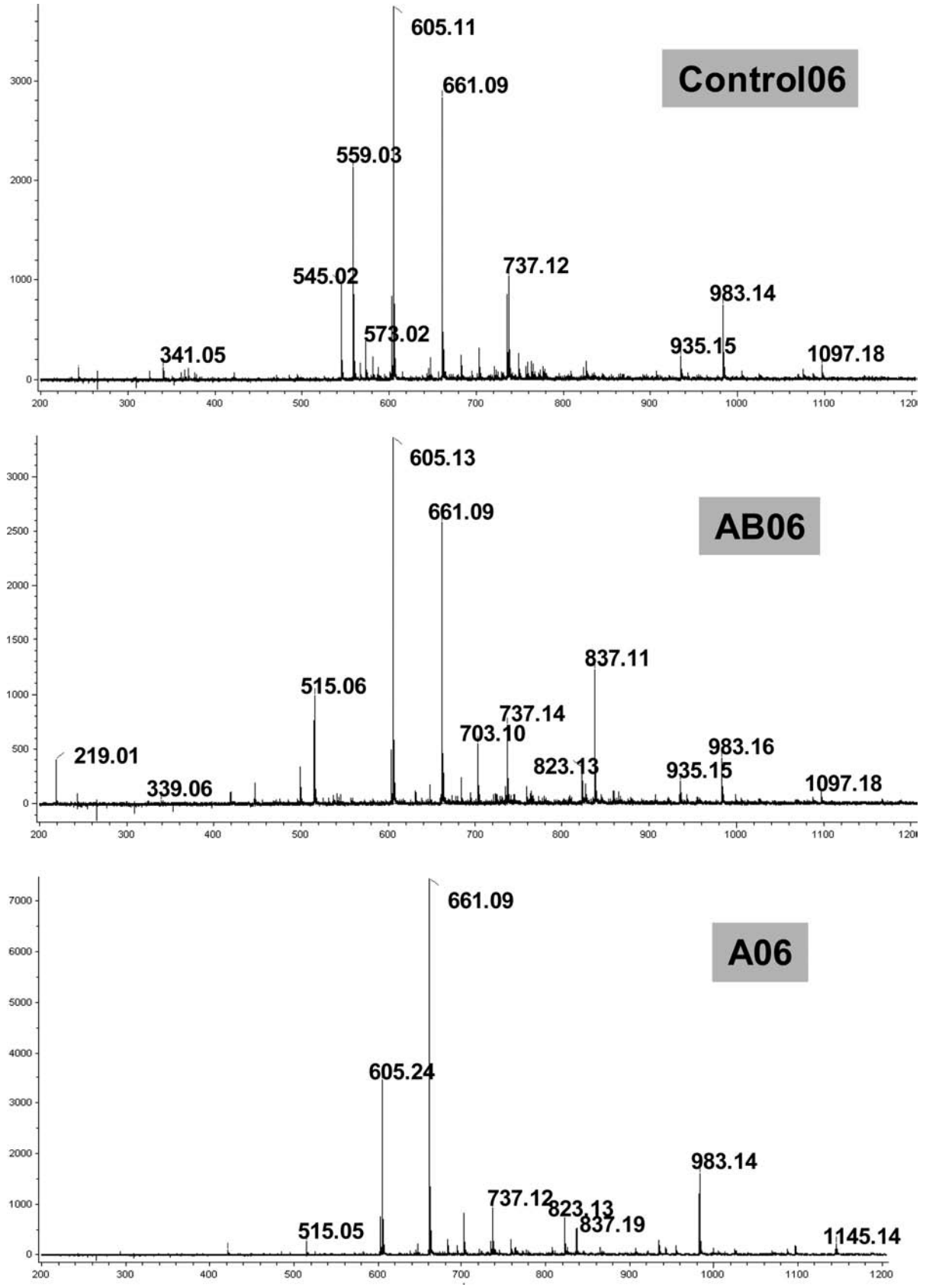

Figure 2. ESI-TOF spectra in the negative ion mode of the Merlot oligosaccharides from three wines (control06, AB06, and A06).

saccharides rich in arabinose and galactose (PRAG).[10,17,19,38] The rhamnose/galacturonic acid ratio reflects the relative richness of wine oligosaccharides in homogalacturonans versus rhamnogalacturonans.[39] Finally, as most of the Ara and Gal are

associated with pectin hairy regions, the ratio arabinose plus galactose to rhamnose ((Ara+Gal)/Rha) enables estimation of the relative importance of the neutral side chains to the rhamnogalacturonan backbone and, thus, the degradation of the side chains by enzymes.

The Ara/Gal ratio obtained for the oligosaccharide fraction of the control wine was very close to the ratios obtained previously for Carignan and Merlot wines, 2.2 and 2.8, respectively.[18] The Ara/Gal ratio obtained for Merlot oligosaccharide fractions was strongly modified by the use of pectic enzymes, decreasing from 2.4 for the control to <1 for the treated wines except that treated by enzyme E, which showed a ratio of 2.87. The low value determined for the rhamnose/galacturonic acid ratio in Merlot oligosaccharides (0.35) indicates that homogalacturonans predominate, whereas the higher ratio in most treated wines (0.5−0.8) indicates a majority of rhamnogalacturonan structures organized with a repeat unit of [→2)-α-L-Rha*p*-(1→4)-α-D-Gal*p*A-(1→]. Both types of structures have been described in wine oligosaccharides.[18]

This suggests that the enzyme preparations release rhamnogalacturonan-based oligosaccharides, as confirmed by the increased level of rhamnose and galacturonic acid residues in the oligosaccharide fractions of enzyme-treated wines. Moreover, some degradation of homogalacturonans by the polygalacturonase activities present in the enzyme preparations used may also take place and would also result in increased rhamnose to galacturonic acid ratios. This ratio was lower in the fraction of oligosaccharides treated with both Vinozym Vintage FCE and Vinoflow FCE, again suggesting synergistic effects between the two preparations, that is, release of rhamnogalacturonan oligosacharides by preparation B followed by their degradation by preparation A, which was inactive when used alone. The ratio (Ara+Gal)/Rha indicates that the arabinan and arabinogalactan side chains carried by the rhamnose residues of the pectin hairy zone were degraded or modified: 1.06 for control wine compared to 0.75 on average for enzyme-treated wines, except for wine treated by enzyme E. The modification of the ratio was mainly due to loss of arabinose. Branched arabinose residues arising from arabinans substituting rhamnogalacturonan chains of pectins and terminal arabinose that may also arise from free arabinans have been detected in wine oligosaccharides.[18] The increase in the ratio (Ara+Gal)/Rha for the enzymatic preparation E indicates a greater release of oligosaccharides of rhamnogalacturan type carrying side chains of arabinans and arabinogalactans from the hairy zones or by the presence of arabino-oligosaccharides released by an endoarabinanase present in enzymatic preparation E.

Analysis of the glycosidic linkages after permethylation and acidic hydrolysis enabled us to determine the structures of oligosaccharides that are released during the winemaking in control and enzyme-treated wines (Table 4).

Mannose was linked in positions →2, → 3, and →3,6 and in nonreducing terminal position, as typically found in yeast mannoproteins.[30,40] The enzymatic treatment during winemaking does not seem to modify the oligomannosides, and the molar percentage of each methyl ether was identical to that previously found in Carignan and Merlot wines.[18] The oligosaccharides present in control and enzyme-treated wines (Table 4) contain arabinose linked in →5 and →3,5, characteristic of arabinans present as side chains of rhamnogalacturonans (RG) of pectins. The presence of rhamnogalacturonan backbone was confirmed by identification of 2 Rha and 2,4 Rha.[17] Arabinose in nonreducing terminal position, which substitutes side chains of β-(1→ 6)-galactans of AGPs, was also present in oligosaccharides from control and enzyme-treated wines. Merlot oligosaccharides contain all methyl ethers corresponding to the galactose chains of AGPs: galactose linked in −3; −6; −3,6; −4,6; and −3,4,6.[15,27] The presence of 2,3-di-*O*-methyl- and 3,4-di-*O*-methyl-D-xylopyranose in the methylation analysis showed that the wine oligosaccharides contain xylan structures, arising from the (1→4)- and (1→2)-linked xylose chain units. For oligosaccharides of control wine and wine E06, the main monosaccharide residue was arabinose (29.5 and 37.1%, respectively), whereas for oligosaccharides isolated from the other enzyme-treated wines, A06, AB06, B06, Acx06, F06, G06, I06, and J06, rhamnose was the major sugar. Analysis of methyl ethers also shows that the proportions of arabinose linked in 1→5 and in 1→3,5 decreased in the enzyme-treated wines, except in wine E06. Indeed, arabinose linked in 1→5 decreased from 17.4% in wine control to <2% in wines treated by all enzymatic preparations except enzyme E. In this case, arabinose linked in 1→5 increased, indicating that arabino-oligosaccharide types were not degraded by preparation E. The presence in all of the samples of xylose linked in 1→4 and identification of 4-*O*-methylglucuronic acid on the glycosyl composition indicated that wine oligosaccharides contain 4-*O*-methylglucuronoxylan oligosaccharides. These oligosaccharides were already identified in Carignan and Merlot wines.[18] Proportions of various families of cell wall polysaccharides from grapes (celluloses, xylans, rhamnogalacturonans, arabinans, and arabinogalactans) or yeasts (mannans and glucans) are given in Figure 1. These calculations are based on the contribution of various residues characteristic of the different families of polysaccharides and are used as indicators of the respective contents of each polysaccharide family.[41−44]

These results indicate a release of rhamnogalacturonans of hairy region (Figure 1A) and a degradation of arabinose of PRAG or arabinans (Figure 1B) of the rhamnogalacturonan side chain by the enzymes used during winemaking. Other oligosaccharide

Table 5. Principal Structures present in Merlot wine oligosaccharides identified in ESI-TOF Spectra in the Negative Ion Mode according to Ducasse et al.[18]

M	$[M-H]^-$	structure[a]
516	515	[GalA-Rha]-GalA
546	545	$[GalA]_3$
560	559	$[GalA]_3Me_1$
574	573	$[GalA]_3Me_2$
604	603	GalU-[Rha-Ara-Ara],
606	605	[4-OMe-GlcA-$[Xyl]_2$-xylitol]
648	647	Rha-GalU-[Rha-Gal]
661	661	$[Rha-GalA]_2$
722	721	$[GalA]_4$
736	735	$[GalA]_4Me_1$
738	737	[4-OMe-GlcA-$[Xyl]_3$-xylitol]
750	749	$[GalA]_4Me_2$
764	763	$[GalA]_4Me_3$
808	807	$[Rha-GalA]_2$-Rha
838	837	$[GalA-Rha]_2$-GalA
984	983	$[Rha-GalA]_3$

[a] GalA, galacturonic acid; Rha, rhamnose; Me, CH_3; 4-Ome-GlcA acid; 4-O-CH_3-glucuronic acid; Xyl, xylose.

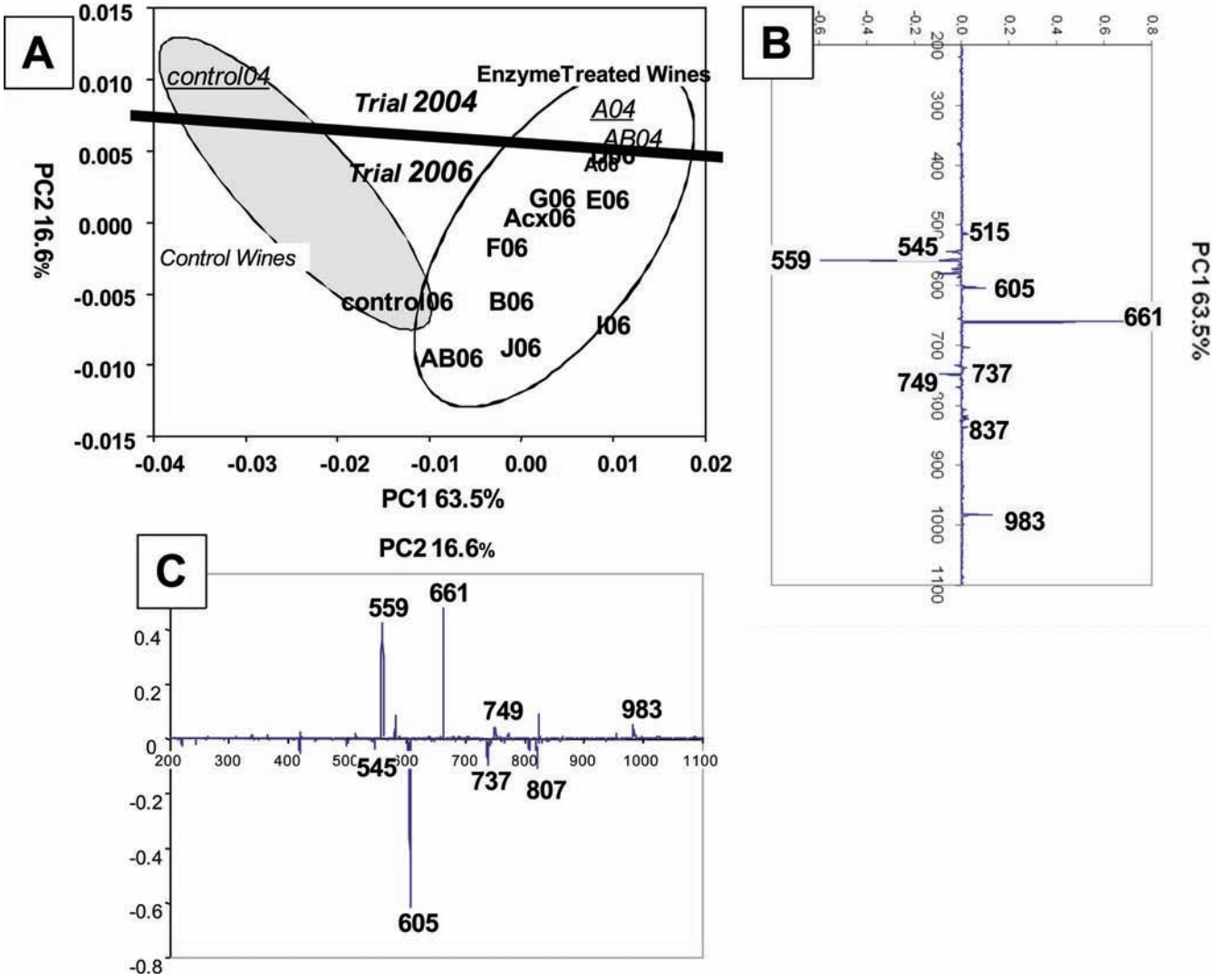

Figure 3. Analysis in principal components of oligosaccharide mass spectra of Merlot wines 2004 and 2006: (A) wine projections on axis formed by the first two principal components; (B) contribution of the variables to principal component 1 (PC1 63.5%) and (C) principal component 2 (PC2 16.6%).

structures (Figure 1C,D) seem little affected by enzyme activities added to wines in 2006, except the glucans in wine treated with enzyme E. The use of enzymatic preparations with slightly different spectra of activities yielded oligosaccharides of different structures, which may have different impacts on wine quality.

The ESI-TOF spectra in the negative ion mode of the Merlot oligosaccharides from three wines (control06, AB06, and A06) are given in Figure 2 as examples. The MS spectra show all oligosaccharide molecules as the deprotonated $[M - H]^-$ ions. The ions observed in the mass spectra were ions at m/z 545, 559, 605, 661, 737, 749, 837, and 983. Structures of these main ions (Table 5) could be determined by fragmentation using a mass spectrometer equipped with an ESI source and an ion trap mass analyzer and corresponded to those described previously for oligosaccharides from Carignan and Merlot wines.[18] The spectra showed oligosaccharidic structures corresponding to oligogalacturonic acids, partially esterified by methyl groups (trigalacturonic acid detected at m/z 545 (no methyl), m/z 559 (one methyl), and m/z 573 (two methyl groups) or to the repetition of the basic unit [→4)-α-D-GalA*p*-(1→2)-α-L-Rha*p*-(1→] two (m/z 661) or three (m/z 983) times. These repetition units can be substituted by a galactose, by an arabinose, or by both (m/z 603, m/z 647). The [4-OMe-GlcA-[Xyl]2-xylitol] (m/z 605) and [4-OMe-GlcA-[Xyl]3-xylitol] (m/z 737) were identified in all spectra from Merlot wine oligosaccharides. The structures identified correspond to oligosaccharides arising from pectins as a result of degradation of grape cell wall berries by pectinases during grape maturation and/or winemaking when winemakers added exogenous enzyme activities.

The data set corresponding to whole ESI-TOF mass spectra of oligosaccharides isolated from wines 2004 (control04 and enzyme-treated wines A04 and AB04) and 2006 (control06 and enzyme-treated wines A06, AB06, B06, Acx06, F06, G06, I06, and J06), after normalization, was treated by principal component analysis (PCA) to a best visualization of the impact of enzymatic treatment on oligosaccharides released in wines during winemaking. Mass spectrometry cannot be considered a quantitative method because oligosaccharide species may exhibit different desorption capacities according to their structure and in source competition may alter the response of individual

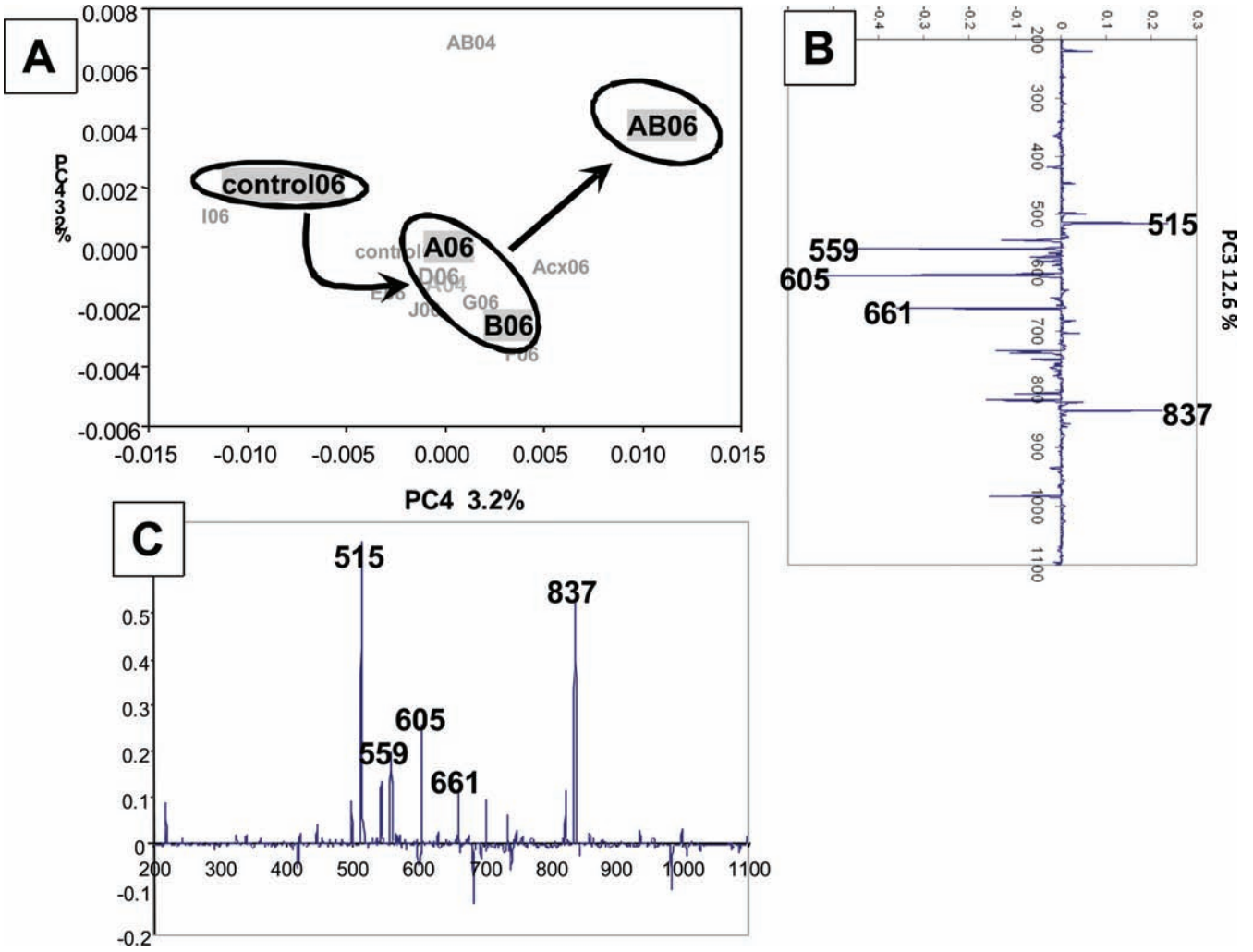

Figure 4. PCA of oligosaccharide mass spectra of Merlot wines 2004 and 2006: (A) wine projections on the level formed by principal components 3 and 4; contributions of (B) principal component 3 (PC3 12.6%) and (C) principal component 4 (PC4 3.2%).

compounds in different backgrounds. However, the MS profiles (i.e., relative intensities of mass signals) obtained were highly reproducible for each sample and very different between samples (as shown in Figure 2), thus allowing comparison between samples. The first two principal components (PC1 and PC2) explain together 80% of the variability of the data. Axis 1 (PC1), accounting for 63.5% of the variability, separates control (04 and 06) wines from enzyme-treated wines (Figure 3A). This first axis (Figure 3B) is defined positively by ions corresponding to the repetition of the basic unit [→4)-α-D-GalA*p*-(1→2)-α-L-Rha*p*-(1→] (*m*/*z* 661, 837, and 983), representing rhamnogalacturonic zones (hairy regions), associated with oligosaccharides of enzyme-treated wines, and negatively by ions corresponding to methylated galacturonic acids (*m*/*z* at 545, 559, and 749), representing the structures of homogalacturonans (smooth regions), which are more abundant in control wines. This confirms that enzymatic preparations induce a greater release of pectin hairy regions (rhamnogalacturonan-like structures carrying neutral lateral chains) and that they degrade the smooth zones of pectins (homogalacturonans). Moreover, oligosaccharides responsible for the separation along axis 1 indicate that pectin methylesterase activities and polygalacturonase present in enzymatic preparations demethylate and degrade oligogalacturonic acids ($[\rightarrow 4)$-α-D-GalA*p*-$(1\rightarrow]_n$ with $n = 3-5$) released from the smooth regions of grape berry cell walls during stages of winemaking. Axis 2 (PC2), accounting for 16.6% of the variability, separates years 2004 and 2006 (Figure 3A). Axis 2 is defined negatively by oligosaccharides containing 4-*O*-methylglucuronoxylans (Figure 3C, $[M - H]^-$ with *m*/*z* 605 and 737), more abundant in 2006 wines. This type of structure corresponding to glucuronoxylans is released in all wines and does not seem to be specific of an enzymatic treatment. Principal components 3 and 4 (PC3 and PC4) explain 15.8% of the variability of the whole spectra. They separate trials 2004 and 2006 treated with two enzyme preparations (wines AB04 and AB06, treated with Vinozym Vintage FCE and Vinoflow FCE) from other wines (Figure 4A). PC3 and PC4 (Figure 4B,C) were defined positively by rhamnogalacturonan structures having a galacturonic acid in terminal nonreducing position ($[M - H]^-$ with *m*/*z* 515, α-D-GalA*p*-(1→2)-α-L-Rha*p*-(1→4)-α-D-GalA, and *m*/*z* 837, α-D-GalA*p*-(1→2)-α-L-Rha*p*-(1→4)-α-D-GalA*p*-(1→2)-α-L-Rha*p*-(1→4)-α-D-GalA. This result indicates a specificity of enzymatic activities of preparations A and B (Vinozym Vintage FCE and Vinoflow FCE) used together. Oligosaccharides of wines treated during winemaking by preparations F, G, I, and J also contain structures of rhamnogalacturonans but having a residue of

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rhamnose in terminal position ($[M - H]^-$ with m/z 807, α-L-Rha*p*-(1→4)-α-D-GalA*p*-(1→2)-α-L-Rha*p*-(1→4)-α-D-GalA*p*-(1→2)-α-L-Rha*p*. These results indicate a cleavage mechanism of pectin hairy regions different from that of enzymatic preparations A and B. Oligosaccharides of wine E06 have no α-D-GalA*p*-(1→2)-α-L-Rha*p*-(1→4)-α-D-GalA, α-D-GalA*p*-(1→2)-α-L-Rha*p*-(1→4)-α-D-GalA*p*-(1→2)-α-L-Rha*p*-(1→4)-α-D-GalA, or α-L-Rha*p*-(1→4)-α-D-GalA*p*-(1→2)-α-L-Rha*p*-(1→4)-α-D-GalA*p*-(1→2)-α-L-Rha*p*; they contain only structures of repeating unit [→4)-α-D-GalA*p*-(1→2)-α-L-Rha*p*-(1→]$_{2n}$ twice, four times, etc.

Our analytical results suggest that oligosaccharides are markers of enzyme activities and of the mechanisms of pectin degradation. Thus, we can deduce that all of the enzyme preparations (A, AB, B, D, Acx, F, G, I, and J, see Table 1) tested in this trial have a rhamnogalacturonase activity, but with cleavage sites which release different products. Thus, enzyme preparations A (Vinozym Vintage FCE), AB (Vinozym Vintage FCE/Vinoflow FCE), and D (Lafase Fruit) probably have a rhamnogalacturonase that hydrolyzes between a residue of galacturonic acid and a residue of rhamnose and released an oligosaccharide with a galacturonic acid in terminal position, whereas preparations F, G, I, and J have the same site of recognition but release an oligosaccharide with a rhamnose in terminal position. Enzyme preparation E has a rhamnogalacturonase activity that cuts the repeating unit of rhamnogalacturonans and liberates the following structures: [→2)-α-L-Rha*p*-(1→4)-α-D-GalA*p*-(1→] with galacturonic acid in terminal position and with an even degree of polymerization (2, 4, etc.).

In conclusion, oligosaccharide fractions of Merlot enzyme-treated and control wines could be isolated and characterized by three complementary methods: analysis by TMS derivatives gave access to monosaccharide compositions, analysis by permethylation determined the glycosidic linkages of oligosaccharides, and analysis by ESI-TOF mass spectrometry made it possible to deduce the main structures of oligosaccharides present in wine. The analysis of the oligosaccharides from trials in 2004 and 2006 highlighted that some of them are markers of enzyme activities degrading the grape berry cell wall polysaccharides. The enzymes used demethylate and hydrolyze smooth regions (HG zones) of pectins. Except preparation E, the other enzymes release fragments of arabinogalactan type I, degrade the side chains of arabinans, and release hairy regions of pectins (rhamnogalacturonan-like structures carrying neutral lateral chains). The identification by mass spectrometry of RG structures ending by rhamnose or galacturonic acid showed that they are characteristic of mechanisms implied in the degradation of pectins. This study led to a better understanding of the effect of commercial enzyme preparations on the oligosaccharides of Merlot wines. The understanding of the release of polysaccharides and oligosaccharides by commercial enzymes into the must is the first step to a comprehensive study of the effect of these fractions on the organoleptic and technological properties of wines.

Future investigations will aim at elucidating the impact of the different oligosaccharide structures and of the various enzymatic activities on wine quality.

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