Use of Electrospray Mass Spectrometry for Mass Determination of Grape (*Vitis vinifera*) Juice Pathogenesis-Related Proteins: A Potential Tool for Varietal Differentiation

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Methods based on liquid chromatography−mass spectrometry (LC-MS) and protein trap mass spectrometry (trap-MS) were developed to determine the complement of pathogenesis-related (PR) proteins in grape juice. Trap-MS was superior to LC-MS in terms of simplicity, rapidity, and sensitivity. Proteins with a wide range of masses (13−33 kDa) were found in the juices of 19 different varieties of grape (*Vitis vinifera*) and were identified as mostly PR-5 type (thaumatin-like) and PR-3 type (chitinases) proteins. Although the PR proteins in juices of grapes are highly conserved, small consistent differences in molecular masses were noted when otherwise identical proteins were compared from different varieties. These differences persisted through different harvest years and in fruits grown in different Australian locations. With the definition of four different masses for PR-5 proteins (range = 21239−21272 Da) and nine different masses of PR-3 proteins (range = 25330−25631 Da) and using statistical analysis, the methods developed could be used for varietal differentiation of grapes grown in several South Australian locations on the basis of the PR protein composition of the juice. It remains to be seen whether this technology can be extended to grapes grown worldwide and to wine and other fruit-derived products to assist with label integrity to the benefit of consumers.

Keywords: *Grape proteins; pathogenesis-related proteins; thaumatin-like proteins; chitinases; varietal differentiation; LC-MS; electrospray; mass spectrometry; Vitis vinifera*

INTRODUCTION

Grapes constitute one of the most important horticultural crops in the world, and ∼5000 varieties or cultivars of grapes may exist (*1*). Traditionally, grapevine identification has relied on the skills of ampelographers, who use up to 150 traits to identify varieties by their appearance (*2*). Despite such detail, it is still possible for even experienced ampelographers to confuse varieties, and in some cases, unknown vines need to be grown for several years and under the same conditions as known vines before identification can be achieved with a high degree of certainty. Although ampelography has a rightful place in viticultural operations, it clearly is not applicable to juice or wine.

Alternative methods to overcome or complement the limitations of ampelography have been developed over the past two decades. Most notably the use of DNA fingerprinting techniques has greatly enhanced the scope of ampelography and allowed genetic relationships between existing varities to be defined (*3*−*6*). This DNA-based microsatellite method is successful when DNA is extracted from various vine tissues and berry juice, but this technology cannot be extended to wine due to the release of nucleases by yeast during fermentation. Although wine aroma compounds, particularly monoterpenes, pigments, or other trace organic compounds, can be used in some cases to indicate the variety of grape used to produce a given wine (*7*−*9*), there is at present limited scope for the application of molecular techniques to determine the grape variety(ies) from which a given wine has been produced. This would particularly be so in cases of varietal blending.

In recent years, it has become clear that all grape cultivars synthesize a set of pathogenesis-related (PR) proteins following veraison (onset of ripening) and that these proteins furthermore are identical to those forming a haze in wine (*10*−*14*). Specifically, it has become clear that a number of isoforms exist within individual varieties (*15*−*20*) and that the molecular weights of these might differ slightly across varietal boundaries. For example, compare thaumatin-like proteins in Muscat of Alexandria (*11*) and Sultana (*21*) and chitinases in Muscat of Alexandria (*16*) and Shiraz (*17*). Because small but detectable amounts of these stable PR proteins, particularly the PR-5 (thaumatin-like) and PR-3 (chitinases) representatives, may persist in wine even following "protein stabilization" by treatment with bentonite (unpublished observations), grape proteins might therefore constitute a molecular tag for identification of the grape varieties that have been employed to produce a given wine. Previous attempts to differentiate grapes and their products by electrophoretic analyses

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Table 1. Level of Soluble Solids (°Brix) in Grape Juices Used in This Study

harvest year:		1997	1998	1999						
vineyard:		W[a]	W	W	P	S	A	L	C	B
				White Varieties						
Chardonnay	CHA[b]		23.7[c]	26.1	21.7	22.0	**20.3**			
Sauvignon Blanc	SAB	**25.6**	**20.1**	**24.2**	**23.1**		**18.6**			
Riesling	RIE		19.2	20.3				21.6		
Muscat of Alexandria	MUA		**24.2**	**22.4**				**14.7**		
Sultana	SUL	19.5	19.2	25.8						
Doradillo	DOR	19.5	17.5	20.1						
Crouchen	CRO	**19.0**	**19.8**	**22.7**						
Semillon	SEM		**24.1**	**17.4**						
Muscadelle	MUS	**23.9**	**22.4**	**23.9**						
				Red Varieties						
Pinot Noir	PIN		**24.6**	23.9	**20.5**		**23.3**			
Gamay	GAM		**21.2**	**26.2**						
Cabernet Franc	CAF	24.1	**23.5**	**24.5**				21.5		
Petit Verdot	PEV	*20.0*	20.5	20.1				16.8		
Malbec	MAL		17.7	**23.7**				23.4		
Touriga	TOU	24.3	19.7	21.2						
Tarrango	TAR	*19.7*	*21.4*	*22.0*						
Merlot	MER	**22.7**	23.8	*22.8*				**23.1**		
Barbera	BAR	24.8	27.2	25.2						
Cabernet Sauvignon	CAS	*24.0*	23.2	*25.6*	22.8		*21.8*	24.5		
Shiraz	SHI			*25.6*	*23.4*			25.3	*23.1*	22.6

[a] Abbreviation of the vineyard detailed under Materials and Methods. [b] Abbreviation of the variety. [c] °Brix values are presented in bold, plain, and underscored italic characters indicating high (>0.5 μg/μL), medium (<0.5 μg/μL), and low (<0.1 μg/μL) levels of the total proteins, respectively. The level of total proteins in the concentrated juice was estimated by SDS-PAGE and Coomassie blue staining.

of their protein complement have been reported (*22*−*28*). Electrospray ionization mass spectrometry (ESI/MS) is likely to exhibit far greater resolving power than electrophoretic techniques, so the present study was set up to explore its feasibility as a complement to DNA-based technologies for varietal differentiation of grapes and wines.

The present study had three aims: first, to establish a facile analytical method for the qualitative determination of PR proteins in juice using ESI/MS; second, to determine the extent to which molecular masses of PR proteins present in juices of different varieties differ; and, third, to investigate whether these differences were sufficient to develop varietal differentiation of grapes.

MATERIALS AND METHODS

Source of Grape Berries. The berries of 20 different varieties (*Vitis vinifera* cv.) were harvested from seven different vineyards including those located at the Waite Campus of the University of Adelaide (abbreviated as W in Table 1), Padthaway (P), Summertown (S), Adelaide Hills (A), Langhorne Creek (L), Coonawarra (C), and Barossa Valley (B). These regions are all located in South Australia but span a considerable range of climatic and physical conditions. The details of the berry collection schedule are summarized in Table 1. The harvest year, variety, and vineyard source of the berry or its juice sample are presented hereafter in the tables using the abbreviations shown in Table 1.

Berry Sampling and Juice Preparation. Clusters of berries were randomly taken from the same grapevine. Berries were transported to the laboratory without strict temperature control except in the case of extended transportation time, when berries were stored in an insulated box with ice. The berry samples were stored at −20 °C prior to processing.

Fifty berries were thawed, and juice was obtained by crushing the berries using a stainless steel juicer and clarified by centrifugation (10000*g* for 30 min at 4 °C). The supernatant was collected through coarse cheesecloth to remove any debris, and a 1 mL aliquot was used for measurement of °Brix (total soluble solids) using a digital refractometer (Erma Inc., Tokyo, Japan). Two milliliter aliquots of the supernatant were loaded onto a Centricon YM10 centrifugal filter device (Millipore) with a nominal molecular weight cutoff of 10 kDa. The sample was centrifuged (4000*g* at 4 °C) until the juice was concentrated to ~100 μL.

Sodium Dodecyl Sulfate—Polyacrylamide Gel Electrophoresis (SDS-PAGE). The composition and semiquantitative estimation of proteins in the concentrated juice were assessed by SDS-PAGE analysis as described by Tattersall et al. (*11*). Proteins were stained with 0.1% (w/v) Coomassie brilliant blue R-250.

Liquid Chromatography Electrospray Mass Spectrometry (LC-MS). The concentrated juice was loaded using a flow injector (Rheodyne model 8125, Cotati, CA) fitted with a 5 μL loop onto a C8 reverse-phase HPLC column (1 × 250 mm, Vydac, Hesperia, CA) fitted with a guard column (1 × 20 mm, Upchurch, Oak Habor, WA) packed with Perisorb 8 (Upchurch) and equilibrated in a mixture of 75% solvent A [0.05% (v/v) trifluoroacetic acid (TFA) in water] and 25% solvent B [0.05% (v/v) TFA in 90% (v/v) aqueous acetonitrile]. The mobile phases were delivered at a flow rate of 15 μL/min by a dual syringe pump (140B solvent delivery system, Applied Biosystems, Perkin-Elmer). Proteins were eluted by applying a linear gradient of 25−90% of solvent B over 60 min, and then the final concentration of solvent B was held for 30 min. After the reduction of solvent B to 30% in 10 min at a flow rate of 30 μL/min, the column was washed with a gradient of 30−90% of solvent B over 20 min.

The column was directly connected to an electrospray ion source of a mass spectrometer (API-300, PE Sciex, Thornhill, ON, Canada) or was connected with a UV−vis detector (HP1100, Hewlett-Packard) monitored at 220 nm, followed by the mass spectrometer. The mass spectrometer was operated in positive ion mode and was scanned from m/z 1200 to 2900 with a step size of 0.1 Da and a dwell time of 0.3 ms. The electrospray and orifice potentials were set at 5.5 kV and 30 V, respectively. The curtain (nitrogen) and nebulizer (air) gases were set at 8 and 10 units, respectively. The mass spectrum consisting of the multiple charge ions was taken from the protein peak and was processed to determine the most probable molecular weights of the proteins using Bio-Multiview software 1.2β3 (PE Sciex).

Protein Trap Electrospray Mass Spectrometry (Trap-MS). The concentrated juice was loaded using the flow injector

fitted with a 5 μL loop onto a protein trap cartridge (3 × 8 mm, Michrom BioResources, Auburn, CA) equilibrated in a mixture of 70% solvent C [2% (v/v) formic acid in water] and 30% solvent D [2% (v/v) formic acid in 80% (v/v) aqueous acetonitrile]. The trap cartridge was washed with 30% of solvent D at a rate of 100 μL/min for 5 min. After the reduction of the flow rate to 10 μL/min in 5 min, the trap cartridge was directly connected with the mass spectrometer, and proteins were eluted with a gradient of 30−60% of solvent D in 10 min, held for 10 min at 60% of solvent D, and then increased to 80% of solvent D in 10 min and held there for a further 5 min. After the completion of the analysis, the cartridge was replaced with a prewashed cartridge for the next analysis. The used cartridge was washed sequentially with 3 mL of 50% and 3 mL of 80% aqueous acetonitrile containing 2% formic acid at a flow rate of 200 μL/min by a syringe pump (Cole-Parmer, Vernon, IL) before being reused. The mass range from *m*/*z* 1500 to 2800 was scanned with a step size of 0.1 Da and a dwell time of 0.5 ms. The orifice potential was set at 70 V. Other electrospray mass spectrometric conditions were the same as those of the LC-MS method described above.

Total Protein Quantification by Amino Acid Analysis. Proteins in the concentrated juices were hydrolyzed in 6 M HCl containing 0.5% (w/w) phenol and 0.4% (w/v) dithiopropionic acid under argon gas at 116 °C for 16 h (*29*). Amino acid analysis of the hydrolyzed and unhydrolyzed samples was performed on an AminoQuant series II amino acid analyzer (Hewlett-Packard) according to the manufacturer's instructions. The amount of protein was estimated by subtracting the sum of the masses of free amino acids from that of anhydrous amino acids found after hydrolysis.

Cluster Analysis. Cluster analysis was carried out using NTSYS-pc version 1.80 software (F. James Rohlf, Exeter Software, Setauket, NY). The qualitative presence or absence (1 or 0) of protein was determined by mass spectrometric data. Pairwise similarities were calculated using the Dice coefficient for two-state data (1 or 0). The unweight pair-group method (UPGMA) was used for cluster analysis.

Varietal identification by DNA Fingerprinting. The DNA fingerprinting technique was carried out by the Analytical Service of the Australian Wine Research Institute on leaves from some of the grapevines used as sources of berry samples, according to the method developed by Thomas et al. (*5*).

RESULTS AND DISCUSSION

Protein Composition of Grape Juices Determined by LC-MS. The LC-MS elution profiles of the proteins from juices of grapes harvested in 1998 and 1999, monitored by UV absorbance and ESI/MS, varied considerably among varieties but not within most of the same varieties (data not shown). This indicated that the composition of the proteins in the juice might be specific to each variety. Varietal differences in the protein composition of juice, must, and wine have been observed by others using electrophoresis (*23*, *26*, *27*) and, to a much lesser extent, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) as applied to wines made from three varieties of white grapes (*28*).

The relative molecular masses (M_r values) obtained for the proteins present in juice samples from 19 varieties harvested in 1998 and 1999 are shown in Table 2 and listed in order of increasing retention time. The identity of individual proteins was determined by its elution order and M_r by LC-MS. The M_r values in Table 2 represent mean values for proteins, detected in the different juice samples. The mean M_r values of all the proteins had very small variations with a standard deviation of $<\pm 3$, except for the $M_r = 33212$ (± 4.2), 33230 (± 4.3), and 33284 (± 4.7) species, thus indicating that the M_r values of the proteins could be reliably identified. As shown in Table 2, 24 and 21 different proteins in the juices were defined from white and red varieties, respectively. Additional proteins were detected, but due to their low abundance and/or poor ionization characteristics, it was not possible to reliably determine their M_r values.

The grouping of the proteins into four categories based on their retention times and M_r values was made as follows. First, the group categorized as VVPR-4 proteins eluted at retention times between 25 and 30 min (data not shown) and consisted of proteins with $M_r = 12905$, 12995, and 13020. The protein of $M_r = 13020$ from Muscat of Alexandria was recently characterized as a PR-4 type protein (VVPR-4a) by cDNA sequence analysis (*30*). The proteins of $M_r = 12905$ and 12995 have not yet been identified. However, they were assumed to be PR-4 type proteins because their M_r values and retention times were very close to those of VVPR-4a. In addition, it is known that the VVPR-4a gene is part of a small, highly related multigene family, consisting of at least two, and possibly five, genes (*30*).

Second, the VVTL group eluted at retention times between 30 and 40 min (data not shown) and consisted of proteins with $M_r = 21272$, 21248, 21239, and 21260. These proteins have been previously characterized as PR-5 type (thaumatin-like) proteins (*10*, *11*, *14*, *21*), and a group of related proteins is consistent with the presence of a small multigene family encoding PR-5 proteins in grapes (*11*).

Third, the VVCHI group eluted at retention times between 40 and 55 min (data not shown) and had a wide range of M_r values ranging from 25329 to 25958. The chromatographic behaviors and M_r values of these proteins were very similar to those of chitinases found in the grape juices, reported by Waters et al. (*16*) and Pocock et al. (*14*). In addition, Derckel et al. (*18*) observed as many as 13 chitinase isoforms in grapevine tissues based on electrophoretic analyses. The diversity of M_r values and the chromatographic behaviors indicated that the proteins in this group could be characterized as PR-3 proteins (chitinases).

The last elution group (designated 33 kDa) consisted of proteins with a range of M_r values from 33212 to 33383. The nature of these proteins has not been identified, and further investigation by sequence analyses is required to ascertain their true identity.

Multiple charged ion spectra derived by ESI were very similar among the proteins in the same group but were different between the groups (data not shown). This observation indicated that all proteins in each group could share a similar structural nature because the distribution and intensities of the multiply charged molecular ions produced by ESI for proteins are directly related to the number of basic amino acid residues present and the structural conformation of the protein (*31*−*33*). This is further support for the structural similarity of the proteins in the same group.

Characterization of Varietal Differences in Grape Protein Composition by LC-MS. *Seasonal Variation in Protein Composition.* The protein compositions, as evidenced by LC-MS of juice from the same variety of grape harvested in both the 1998 and 1999 harvest years, were similar, with the exception of the 33 kDa proteins. This group of proteins was present in most of the juices from the 1998 harvest but to a lesser extent in those from the 1999 harvest (see Table 2). The

Table 2. Protein Composition of the Juice Samples from the 19 Grape Varieties from the Waite Campus Vineyard Obtained in the 1998 and 1999 Harvest Years[a]

A. White Varieties

protein	CHA		SAB		RIE		MUA		SUL		DOR		CRO		SEM		MUS	
(M_r)	1998	1999	1998	1999	1998	1999	1998	1999	1998	1999	1998	1999	1998	1999	1998	1999	1998	1999
12905	−	−	−	−	+	−	−	−	−	−	−	−	+	+	+	−	+	+
13020	+	+	−	−	+	−	+	+	+	+	+	+	−	−	+	+	−	−
21272	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−
21248	+	+	−	−	+	+	+	+	+	+	−	−	+	+	+	+	+	+
21239	+	+	+	+	+	+	−	+	−	−	−	−	−	−	−	−	+	+
21260	−	−	−	−	−	−	−	+	+	+	−	−	−	−	+	+	−	−
25927	−	−	−	+	−	−	−	−	+	+	+	+	+	+	+	+	−	−
25943	−	−	+	+	−	−	+	+	−	−	+	−	−	−	+	+	+	+
25329	+	+	−	−	+	−	−	−	+	+	−	−	−	−	−	−	−	−
25341	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+
25557	+	+	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−
25574	+	+	+	+	+	+	−	−	+	+	+	+	−	−	+	+	+	+
25590	−	−	−	−	+	+	+	+	−	−	−	−	+	+	−	−	−	−
25633	−	−	+	+	−	+	+	−	−	−	+	+	+	+	+	+	−	−
25411	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−
25426	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−
25457	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	+	+
33336	+	−	−	−	+	−	−	−	−	−	−	−	−	−	+	+	−	−
33367	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
33383	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−
33212	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−
33230	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
33284	−	−	+	+	−	−	−	−	−	−	−	−	+	−	−	−	+	+
33296	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−

B. Red Varieties

protein	PIN		GAM		CAF		PEV		MAL		TOU		TAR		MER[b]		BAR		CAS	
(M_r)	1998	1999	1998	1999	1998	1999	1998	1999	1998	1999	1998	1999	1998	1999	1998	1999	1998	1999	1998	1999
12905	−	−	−	−	+	+	−	−	+	+	+	−	−	−	+	−	−	−	−	−
12995	−	−	−	−	+	+	+	+	−	−	+	−	−	−	+	−	+	+	−	−
13020	+	+	+	+	−	−	−	−	+	+	−	−	+	+	−	−	+	+	−	−
21272	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−
21248	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	−	+	+	+	−
21239	+	+	+	+	−	−	+	+	+	+	+	+	−	−	−	−	−	−	−	−
21260	+	+	+	+	−	−	−	−	+	−	+	+	−	−	+	−	+	+	−	−
25927	−	−	−	−	−	−	+	−	−	−	+	+	−	+	−	−	−	−	−	−
25943	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	+	−	−	+	+
25958	−	−	−	−	+	+	−	−	+	+	−	−	−	−	+	+	+	+	+	−
25329	+	+	+	+	−	−	+	+	+	+	−	−	+	+	−	−	+	+	−	−
25574	−	−	+	+	−	−	+	+	−	−	−	−	−	−	−	+	−	−	+	+
25590	+	+	−	−	+	+	+	−	−	−	+	+	+	+	−	−	−	−	−	−
25633	−	+	−	−	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+
33336	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
33367	+	+	−	−	−	−	−	−	+	−	−	−	−	−	+	−	+	−	+	+
33383	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
33212	−	−	−	−	+	+	−	−	+	−	+	−	−	−	+	−	−	−	+	−
33230	+	+	+	−	−	−	−	−	+	−	+	−	−	−	−	−	−	−	−	−
33284	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	+	−
33296	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−

[a] The M_r values of the proteins were determined by LC-MS, and the presence and absence of the protein in the juice are shown as + and −, respectively. [b] The anomalous results for Merlot juice are explained in the text.

significance of this finding remains unclear, but it can be ruled out that a ripening maturity phenomenon was a determinant because all grapes were harvested at roughly the same °Brix.

Apart from the 33 kDa group of proteins, juice from the 1998 Chardonnay, Sultana, Crouchen, Muscadelle, Gamay, Cabernet Franc, and Barbera grapes exhibited protein compositions identical to that from the same variety in the 1999 harvest year. The protein composition of juice from Sauvignon Blanc, Doradillo, Semillon, Pinot Noir, Malbec, and Tarrango grapes was also identical between the two harvest years for the same variety apart from one protein (Table 2). On the other hand, the juice from grapes thought to be of the cultivar Merlot contained eight different proteins, but only two of the eight proteins appeared in both harvest years (Table 2); therefore, the percent of agreement was 25% (2/8), which was significantly lower than those of other varieties (>55%). This apparent anomaly is discussed and explained further below.

A member of the VVPR-4 protein group appeared on 41 occasions in the different juice samples for the two harvest years and on 34 of 41 occasions (34/41, 83% agreement) appeared in the same variety for both years. In regard to members of the VVTL and VVCHI groups, the agreements were 93% (78/84) and 89% (114/128), respectively. As mentioned above, the 33 kDa group was obviously the most variable with 55% (24/43) agreement. These data suggest that the VVTL and VVCHI groups probably were best suited for varietal identification. To

Table 3. Similarity of the Grape Varieties Based on the Composition of VVTL and VVCHI Proteins Determined by LC-MS[a]

A. White Varieties

	CHR	SAB	RIE	MUA	SUL	DOR	CRO	SEM	MUS
CHR	**1.00**								
SAB	0.52	**0.91**							
RIE	0.75	0.61	**0.83**						
MUA	0.37	0.46	0.56	**0.80**					
SUL	0.77	0.40	0.54	0.34	**1.00**				
DOR	0.21	0.66	0.32	0.18	0.38	**0.86**			
CRO	0.18	0.28	0.46	0.40	0.33	0.47	**1.00**		
SEM	0.33	0.61	0.42	0.45	0.62	0.73	0.55	**1.00**	
MUS	0.50	0.52	0.50	0.52	0.31	0.31	0.18	0.50	**1.00**

B. Red Varieties

	PIN	GAM	CAF	PEV	MAL	TOU	TAR	MER[b]	BAR	CAS
PIN	**0.92**									
GA	0.80	**1.00**								
CAF	0.61	0.36	**1.00**							
PEV	0.70	0.79	0.49	**0.71**						
MAL	0.77	0.72	0.70	0.67	**0.92**					
TOU	0.72	0.50	0.55	0.52	0.56	**1.00**				
TAR	0.50	0.21	0.47	0.40	0.40	0.52	**0.86**			
MER	0.29	0.20	0.58	0.22	0.54	0.41	0.29	**0.57**		
BAR	0.61	0.55	0.60	0.42	0.78	0.55	0.47	0.70	**1.00**	
CAS	0.18	0.29	0.43	0.43	0.37	0.29	0.27	0.57	0.43	**0.75**

[a] Degree of similarity was a mean value of four data pairs (n = 4) presented in plain typeface except for the pairs (n = l) of the same variety presented in boldface. [b] The anomalous results for Merlot juice are explained in the text.

test this hypothesis, the protein composition and M_r data obtained for the latter two groups of proteins are subjected to similarity analysis.

Similarity of Grape Varieties Based on the Composition of VVTL and VVCHI Proteins. The similarities of the grape variety samples were calculated on the basis of the data for proteins in the VVTL and VVCHI groups in the juice from all varieties and are shown in Table 3. The majority of the harvest year pairs of the same variety had a high degree of similarity (>0.80). In particular, Chardonnay, Sultana, Crouchen, Semillon, Muscadelle, Gamay, Cabernet Franc, Touriga, and Barbera exhibited a similarity of 1.00, showing perfect agreement in the VVTL and VVCHI protein composition between the two harvest years. The similarities of the harvest year pairs of Petit Verdot, Cabernet Sauvignon, and Merlot were calculated to be 0.71, 0.75, and 0.57, respectively, indicating that the occasions of disagreement in the protein composition of those varieties were relatively frequent. In addition, the similarities of the intervariety samples of Petit Verdot and Gamay (0.79), Merlot and Barbera (0.70), Merlot and Cabernet Franc (0.58), and Merlot and Cabernet Sauvignon (0.57) were higher than or equal to those of the harvest year pairs of Petit Verdot (0.71) and Merlot (0.57), respectively.

Nevertheless, the harvest year pair of every variety analyzed exhibited a higher similarity as compared with all intervariety pairs, with the exception of Merlot and Petit Verdot. This demonstrated that the variation of the VVTL and VVCHI protein compositions in the juices had potential to be a tool for the varietal differentiation.

The LC-MS method was accurately able to identify PR proteins in the juices according to their LC elution orders and M_r values (within ±3 Da), which revealed the high possibility of the varietal differentiation based on the variation of masses of PR proteins. However, the LC-MS method appeared to have two major drawbacks: (*1*) poor sensitivity for the VVTL proteins and (*2*) lengthy analysis time. The detection of the proteins by LC-MS is dependent upon the concentration of the protein, its intrinsic ease of ionization, and the absence of interfering substances. For proteins that, for one reason or other, ionize with low efficiency, occurrence at a low concentration in the juice may lead to a nonscore. Indeed, the berry proteins do differ significantly in their ionization efficiencies (data not shown). The ion intensity of the VVTL proteins was much lower than that of VVCHI proteins. We attributed the poor ionization of the VVTL proteins to ion signal suppressing effects of TFA used as a modifier in the mobile phases (*34*−*36*).

Characterization of Varietal Differences in Grape Protein Composition by Trap-MS. *Protein Trap-MS Method.* The trap-MS method was developed to improve the detectability of VVTL proteins by ESI/MS, as well as to shorten the analysis time. The method employs an 8-mm cartridge ("protein trap") instead of a 250-mm column, and proteins are eluted in one or two broad nonresolved fractions. This greatly shortens the analysis time from 120 to 45 min and subsequent data processing time, as discussed below. With this method, TFA can be replaced by formic acid in the mobile phase as resolution among the proteins is not required, and thus potential ion suppression and lower subsequent sensitivity due to TFA are avoided.

The protein elution profile of juice from the 1998 harvest of Sauvignon Blanc grapes grown at the Waite vineyards analyzed by the trap-MS method is shown in Figure 1a. The protein elution profile with this method was largely dependent upon the variety but usually had two major peaks as shown in Figure 1a. The early peak contained predominantly VVTL proteins, and the later one consisted predominantly of VVCHI proteins (data not shown). Although the elution order of proteins from the trap cartridge was not clearly determined due to the overlapping peaks, it seemed to show the same trends as that of LC-MS.

The sensitivity of the trap-MS method for detection of VVTL proteins was enhanced ~10-fold (data not shown) relative to that of the LC-MS method employing TFA as an ion-pairing agent. This estimation was made by comparing the ion intensities derived from the M_r = 21272 VVTL protein analyzed by both the methods using the identical juice samples. We believe this is due to the distribution of multiple charge ions being shifted to a higher charge state by the use of formic acid instead of TFA as a modifier of the mobile phase, most likely due to the strong ion-pairing abilities of TFA anions (*35*). In fact, the ESI mass spectra of the protein with M_r = 21272 obtained by trap-MS and LC-MS had the most intense multiply charged ions at m/z 2128.1 (10^+) and 2660.0 (8^+), respectively. The sensitivity of detection of VVCHI proteins by trap-MS appeared not to be enhanced relative to the more time-consuming LC-MS method. The VVCHI proteins already exhibited high charge states with TFA; therefore, the use of formic acid did not significantly increase the sensitivity of detection of these proteins. Although trap-MS appeared to have disadvantages over LC-MS in terms of peak broadening and complication of mass spectra due to the coelution of multiple proteins, the considerable improvement in the sensitivity to the VVTL proteins compensated for this inconvenience.

A consolidated mass spectrum (Figure 1b) was recorded during the elution of the entire "peak" (Figure

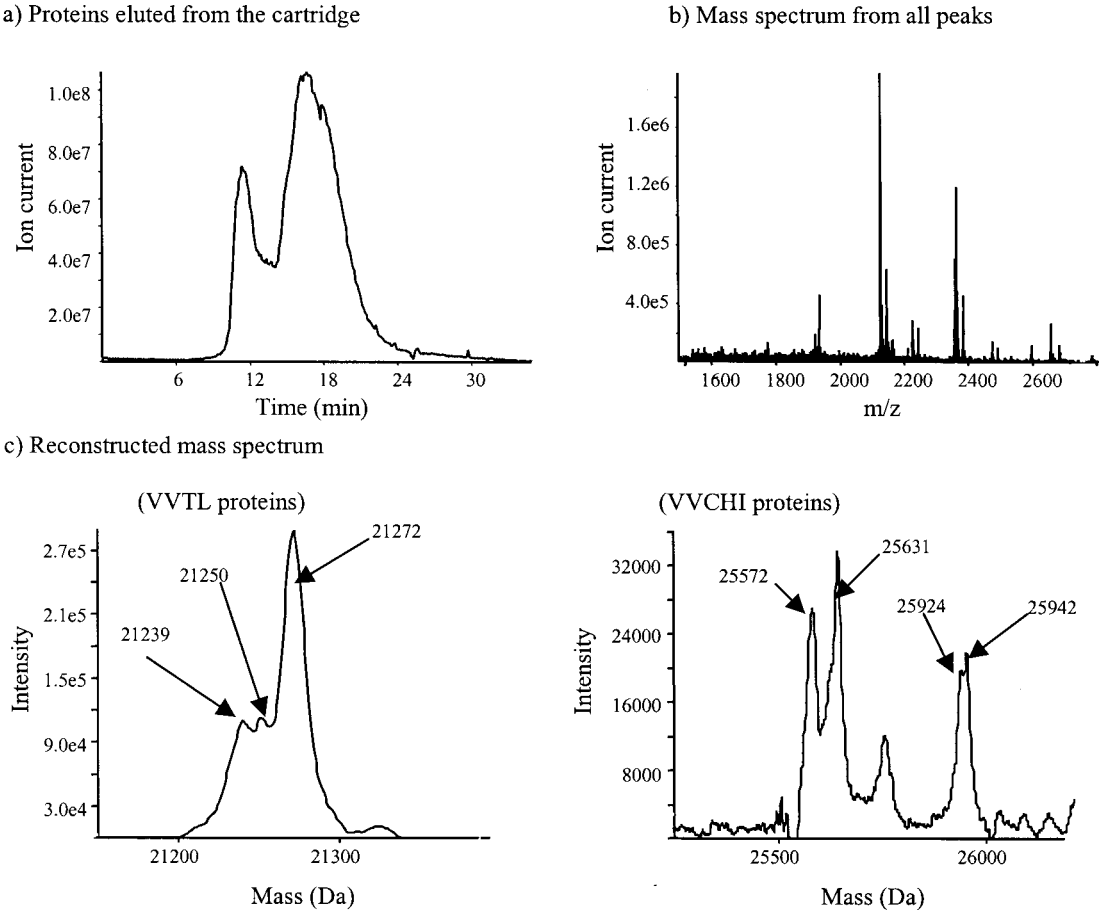

Figure 1. Determination of M_r values of proteins in juice from Sauvignon Blanc harvested from the Waite Campus vineyard in 1998 and separated by trap-MS. The (b) multiply charged ions detected when (a) the proteins eluted as a broad fraction from the trap cartridge (detected by total ion current) were processed to generate the (c) reconstructed mass spectrum, which yields the M_r values of the protein(s).

1a) obtained when Sauvignon Blanc juice was subjected to the trap procedure and processed to generate the reconstructed mass spectrum (Figure 1c) in the range of M_r values from 21000 to 26500 Da. In essence, the M_r values of all proteins were determined by the single mass spectrum from the entire protein fraction rather than by analyzing the spectrum from several individual protein peaks, as is the case for the LC-MS method. As a result, the data processing time was also shortened significantly for the trap-MS method.

Detection Limit. Concentrated juices from the 1999 harvest of Muscat of Alexandria and Cabernet Franc grapes with total protein concentrations of 5.9 and 5.7 μg/μL, respectively, were prepared. Figure 2 shows the reconstructed mass spectrum of the Muscat of Alexandria juice sample diluted 200-fold (a) and 500-fold (b) before trap-MS analysis. Proteins with $M_r = 21272$ and 21250 in the VVTL group and M_r = 25410, 25456, 25588, and 25942 in the VVCHI group were expected to be in the sample and were observed in the 200-fold diluted sample but not unambiguously so in the 500-fold diluted sample. Similar results were observed with the Cabernet Franc sample (data not shown), suggesting that the minimum amount of total proteins required for trap-MS was ~150 ng. However, these data might underestimate the detection limit of trap-MS because components that might interfere with the analysis would have been diluted along with the proteins.

Seasonal Variation in VVTL and VVCHI Protein Composition Determined by Trap-MS. The 19 varieties harvested from the Waite Campus vineyard in 1998 and 1999 harvest years as well as in 1997 for some varieties (Table 1) were analyzed by the trap-MS method to investigate the variation in the protein composition with the harvest years. The M_r values of the VVTL and VVCHI proteins in all of the juice samples analyzed are shown in Table 4. Fifteen and 11 different proteins were detected in the white and red varieties, respectively. The VVTL protein with M_r = 21250 was found in all varieties, and the VVCHI protein with M_r = 25631 was also commonly present in 14 of 19 varieties. A perfect agreement in the protein composition between the different harvest years was observed in 12 of the 19 varieties: Riesling, Muscat of Alexandria, Sultana, Doradillo, Crouchen, Semillon, Muscadelle, Pinot Noir, Gamay, Malbec, Touriga, and Barbera. The rest of the varieties also exhibited a high degree of repeatability with regard to their protein composition between the harvest years. Apart from Merlot and Tarrango, the disagreement between the harvest years for these other varieties pertained to proteins with M_r of 25924, 25942, or 25957. This group of proteins was also inconsistently observed in the same variety with LC-MS (Table 2). Furthermore, the LC-MS data were inconsistent with the trap-MS data for these proteins (compare Tables 2 and 4). In addition, the mass differences among the three proteins were very small, and it might be a result of the adduct formation on the same protein rather than the heterogeneity of these proteins.

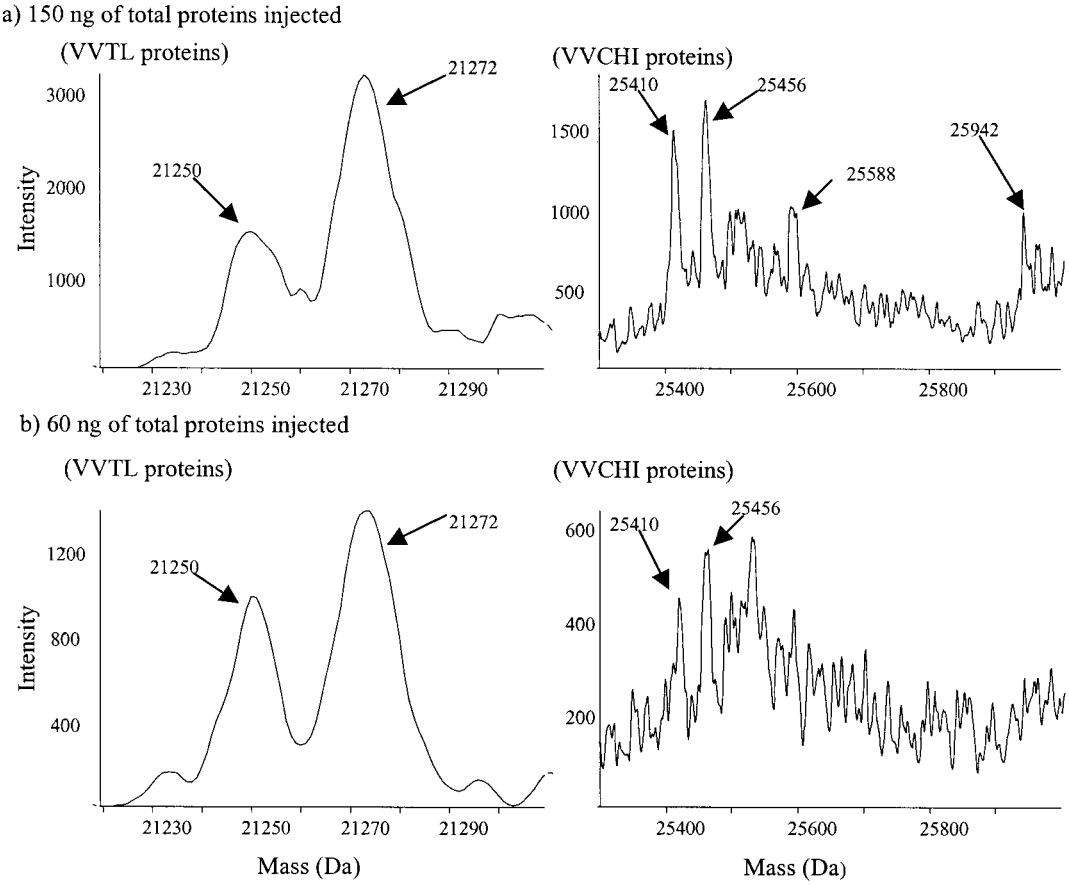

Figure 2. Reconstructed mass spectrum, from trap-MS analysis, representing M_r values of VVTL and VVCHI proteins found in a juice from Muscat of Alexandria fruit containing (a) 150 ng or (b) 60 ng of protein.

The trap-MS pattern was specific to each variety and demonstrated a greater consistency throughout the harvest years than was observed for the LC-MS method. In general, the trap-MS method improved the detection of VVTL proteins as expected, although not always. Some VVTL proteins seen by LC-MS, such as proteins with M_r = 21260 in juice from Pinot Noir, Gamay, Malbec, Muscat of Alexandria, and Sultana grapes and with M_r = 21239 in Muscat of Alexandria, were not observed in the same sample analyzed by trap-MS. This is most likely due to the poorer trap separation observed and subsequent masking of these relatively minor components by coeluting and more abundant homologues. Similarly, the VVCHI proteins with M_r = 25556 in Chardonnay and with M_r = 25572 in Riesling and Sultana were not defined by trap-MS.

Use of Trap-MS To Gauge Variation in the VVTL and VVCHI Protein Complement of Juices Obtained from Different Vineyards. Juice from Chardonnay, Sauvignon Blanc, Riesling, Muscat of Alexandria, Pinot Noir, Cabernet Franc, Petit Verdot, Malbec, Merlot, Cabernet Sauvignon, and Shiraz grapes harvested during the 1999 vintage from different vineyards (Table 1) was prepared and analyzed by trap-MS (Table 5). In 7 of the 11 varieties, namely, Chardonnay, Riesling, Muscat of Alexandria, Pinot Noir, Petit Verdot, Malbec, and Shiraz, the complements of proteins in juices from two different vineyards were identical. Sauvignon Blanc, Cabernet Sauvignon, and Cabernet Franc differed only in the VVCHI protein with M_r = 25942 or 25957. As discussed above, the appearance of these proteins was relatively inconsistent with the same variety. The juice from Merlot grapes harvested from one particular vine in the Waite vineyards differed both from that obtained with grapes from Langhorne Creek in the 1999 harvest (Table 5) and from those harvested from the Waite vineyard in previous years (Table 4) due to the appearance of an additional VVCHI protein with M_r = 25572. This will be discussed further below, as the appearance of this protein cannot be explained by adduct formation or sensitivity problems.

Similarity of Grape Varieties Based on the Composition of VVTL and VVCHI Data from Trap-MS. The proteins of M_r = 25924, 25942, and 25957 were excluded for the similarity assessment due to their inconsistent appearance. Perfect agreement (similarity of 1.0) between the protein composition of samples of the same variety from different harvest years and/or vineyards was observed for all varieties analyzed with the exception of Tarrango and Merlot (mean similarities of 0.93, respectively). UPGMA cluster analysis was performed with the similarity data. The resulting dendograms are shown in Figure 3. The cluster analysis showed that the individual grape varieties were clearly differentiated from each other as a result of the protein composition being highly specific to the variety.

As seen previously by LC-MS, the trap-MS data showed that the Merlot grapes obtained from one vine at the Waite Campus vineyard in 1999 were more similar to Cabernet Sauvignon samples than other Merlot samples (Figure 3b).

To obtain a definitive answer for the dissimilarity of the 1999 Merlot from the Waite vineyard, DNA fingerprinting analysis was carried out on the two grapevines

Table 4. Protein Composition of the Juice Samples from the 19 Grape Varieties from the Waite Campus Vineyard Harvested in Different Years[a]

A. White Varieties

protein M_r	CHA		SAB			RIE		MUA		SUL			DOR			CRO			SEM		MUS		
	1998	1999	1997	1998	1999	1998	1999	1998	1999	1997	1998	1999	1997	1998	1999	1997	1998	1999	1998	1999	1997	1998	1999
21272	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−
21250	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
21239	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+
21260	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−
25924	+	−	+	+	+	−	−	−	−	+	+	+	−	−	−	+	+	+	+	+	−	−	−
25942	−	−	−	+	+	−	−	+	+	−	−	−	−	−	−	−	−	−	+	+	+	+	+
25330	+	+	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−
25341	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+
25556	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−
25572	+	+	+	+	+	−	−	−	−	−	−	−	+	+	+	−	−	−	+	+	+	+	+
25588	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−	+	+	+	−	−	−	−	−
25631	−	−	+	+	+	+	+	−	−	−	−	−	+	+	+	+	+	+	+	+	−	−	−
25410	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
25423	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−
25456	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	+	+	+

B. Red Varieties

protein M_r	PIN		GAM		CAF			PEV			MAL		TOU			TAR			MER			BAR			CAS		
	1998	1999	1998	1999	1997	1998	1999	1997	1998	1999	1998	1999	1997	1998	1999	1997	1998	1999	1997	1998	1999	1997	1998	1999	1997	1998	1999
21272	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
21250	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
21239	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−
21260	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	+	+	+	+	+	+	−	−	−
25924	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
25942	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
25957	−	−	−	−	−	+	+	−	+	−	+	+	−	−	−	−	−	−	+	+	+	+	+	+	−	−	−
25330	+	+	+	+	−	−	−	+	+	+	+	+	−	−	−	+	+	+	−	−	−	+	+	+	−	−	−
25572	−	−	+	+	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+	+	+
25588	+	+	−	−	+	+	+	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−
25631	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

[a] The M_r values of the proteins were determined by trap-MS, and the presence and absence of the protein in the juice are shown as + and −, respectively.

Table 5. Protein Composition of the Juice Samples from the 11 Grape Varieties Harvested in 1999 from Different Vineyards[a]

A. White Varieties

protein M_r	CHA				SAB			RIE		MUA	
	W	P	S	A	W	P	A	W	L	W	L
21272	+	+	+	+	+	+	+	+	+	+	+
21250	+	+	+	+	+	+	+	+	+	+	+
21239	+	+	+	+	+	+	+	+	+	−	−
25924	−	−	−	−	+	+	+	−	−	−	−
25942	−	−	−	−	+	−	+	−	−	+	+
25330	+	+	+	+	−	−	−	−	−	−	−
25572	+	+	+	+	+	+	+	−	−	−	−
25588	−	−	−	−	−	−	−	+	+	+	+
25631	−	−	−	−	+	+	+	+	+	−	−
25410	−	−	−	−	−	−	−	−	−	+	+
25456	−	−	−	−	−	−	−	−	−	+	+

B. Red Varieties

protein M_r	PIN			CAF		PEV		MAL		MER		CAS					SHI				
	W	P	A	W	L	W	L	W	L	W	L	W	P	A	L	W	B	C	P	L
21272	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
21250	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
21239	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
21260	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−
25957	−	−	−	+	−	−	−	+	+	+	+	−	+	+	−	−	−	−	−	−	−
25330	+	+	+	−	−	+	+	+	+	−	−	−	−	−	−	+	+	+	+	+	+
25573	−	−	−	−	−	+	+	−	−	+	−	+	+	+	+	+	+	+	+	+	+
25588	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
25631	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−

[a] The M_r values of the proteins were determined by a trap-MS, and the presence and absence of the protein in the juice are shown as + and −, respectively.

from which the 1999 and 1998 berries were picked. The grapevines in the same vineyard from which other fruit in 1999 was sourced, Pinot Noir, Cabernet Sauvignon, Cabernet Franc, Shiraz, Sauvignon Blanc, Riesling, Chardonnay, Sultana and Semillon, were also examined. All vines were authenticated except the single Merlot vine from which the 1999 fruit was picked. This vine was identified as Cabernet Sauvignon.

The collective data gathered so far clearly demonstrate the potential for mass spectrometry of proteins to differentiate varieties of classical varieties of *V. vinifera* with a good degree of robustness. The varieties surveyed here originated from other parts of the world through recent importation. Although they would account for the majority of Australia's planted vineyard area, a more extensive analysis of varieties harvested from a broader range of locations not included in this study is needed to see how universally applicable the discriminatory power of this methodology would be in a global viticultural context.

We believe this approach to varietal discrimination to be unique and potentially expandable to other fruits and commodities. The extent to which the discriminating power can be expanded across a larger section of grapes remains to be seen. Similarly, a priority is to investigate whether the approach can be expanded to wine. The low concentration of protein in wine following fining can probably be overcome by concentration of the samples, but potential modifications of the PR proteins during vinification could represent a stumbling block. However, as DNA typing appears not to be possible for wine samples, the extension of our findings might represent one of very few avenues by which varietal authentication of wine can be executed with some degree of certainty, if necessary through a combination with analyses of secondary metabolites (*7*).

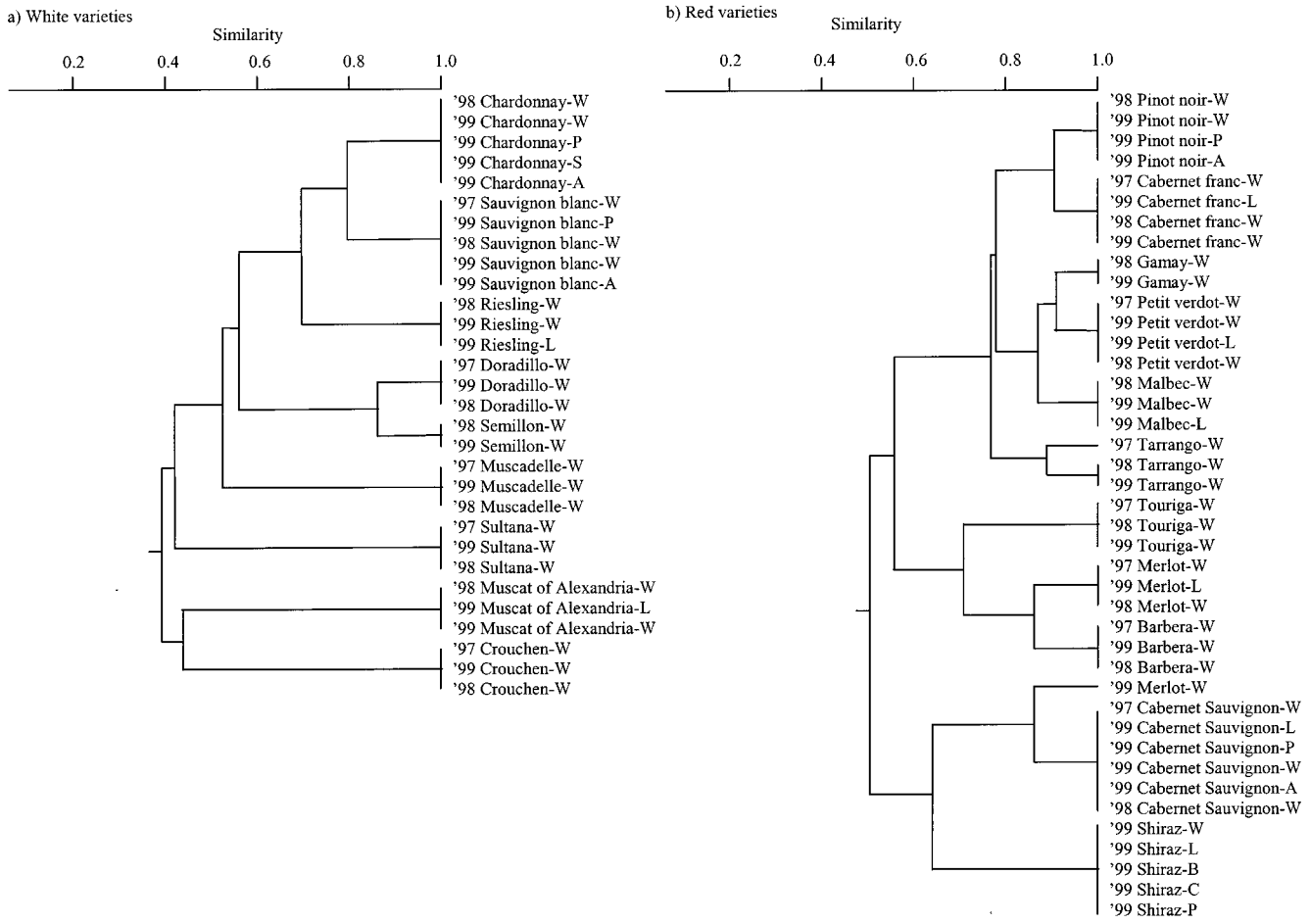

Figure 3. Dendogram depicting cluster analysis of the trap-MS data of proteins in juice from (a) white and (b) red grape varieties.

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