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Characterization of seed proanthocyanidins of thirty-two red and white hybrid grape varieties

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Summary

Grape seed extracts are known for their beneficial effects on health and on cardiovascular diseases due to antioxidant activity and the free radical-scavenging properties of proanthocyanidins (PAs). Moreover, grape seed tannins are used in oenology as additives to improve the organoleptic characteristics of wines, and for the clarification of must and wines. PAs in seed extract of 32 hybrid and three *V. vinifera* grape varieties were characterized by MALDI-TOF mass spectrometry. Signals of 148 compounds were identified as $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ adducts of B-type and A-type PAs formed from catechin/epicatechin subunits up to undecamers and with galloylation degree 0-7. The number-average molecular weight (M_n) of the samples, a parameter correlated with the molecular weight of polymers, and the polyphenolic content of extract, were also determined. Profiles of the hybrid grape varieties were compared with those of three *V. vinifera* samples studied as references. 'Terzi 108-6' showed high content of antioxidant polyphenols and 'Seyve Villard 12-390' higher content of higher oligomers. These two grape varieties are therefore potentially very interesting as sources of antioxidants and tannins for nutraceutical and oenological uses.

K e y w o r d s : proanthocyanidins; hybrid grapes; seeds, MALDI-TOF; polyphenols; mass spectrometry.

Introduction

The study of grape extracts from several varieties of vines is becoming increasingly important, due to their use in the food, nutraceutical, pharmaceutical and colorant industries. In grapes, condensed tannins - also called proanthocyanidins (PAs) - are present at high levels both in the skins and seeds (Downey *al*. 2003, Manè *et al.* 2007). These compounds play an important oenological role as they are responsible for some organoleptic properties of wine, particularly bitterness and astringency. Grape seed tannins are therefore sometimes used as additives to improve the organoleptic characteristics of products (Versari *et al.* 2012). Moreover, high-molecular weight grape seed tannins are commonly used in oenology for the clarification of must and wines: they should not change the olfactory properties or color of products and should adhere to certain chemical specifications, such as minimum level of total flavanols (> 10 mg catechin equivalent·g^{-1} of oenological tannin, OIV 2009).

Grape seed extracts are also known to have beneficial effects on health and on cardiovascular diseases, being mainly ascribed to the *in vivo* and *in vitro* antioxidant activity and free radical-scavenging properties of PAs (Shrikhande 2000, Yilmaz and Toledo 2004). These biological properties of PAs, and of polyphenols in general, have led to increased interest in the recovery of by-products in the enological industry, as seed and skin extracts are used as dietary supplements and food preservatives and in the nutraceutical industry.

Procyanidins are flavan-3-ol oligomers and polymers with structure containing (+)-catechin and (-)-epicatechin units linked by interflavanic bonds at C4-C8 and/or C4-C6 positions (B-type compounds). In A-type compounds one or more C2-C7 and C2-C5 ether bonds, are present (Fig. 1) (Macheix *et al.* 1990, Ricardo da Silva *et al.* 1991, Santos-Buelga and Scalbert 2000). PAs of grape seeds are complex mixtures of oligomers and polymers composed by (+)-catechin, (-)-epicatechin and (-)-epicatechin-3-O-gallate, with one galloyl group esterified to the heterocyclic C-ring and the presence of B-type and A-type bonds (Ricardo da Silva *et al.* 1991, Prieur *et al.* 1994, Passos *et al.* 2007). Prodelphinidins, containg (+)-gallocatechin and (-)-epigallocatechin units, have been found in grape skins, pulp and stems (Souquet *et al.* 1996, 2000, Manè *et al.* 2007).

The degree of polymerization (DP) of PAs in grape seeds varies widely and is generally lower than in grape skin (Souquet *et al.* 1996). Several authors reported that grape seed PAs correspond to oligomers: PAs with DP up to 11 were identified in seed extract profile determined by "Matrix Assisted Laser Desorption Ionization Time of Flight" mass spectrometry (MALDI-TOF) (Krueger *et al.* 2000), and up to 7 by MALDI and FTICR mass spectrometry (Yang and Chien 2000, Rockenbach *et al.* 2012). PAs with DP up to 18 were detected by performing thiolysis and gel permeation chromatography (Prieur *et al.* 1994,

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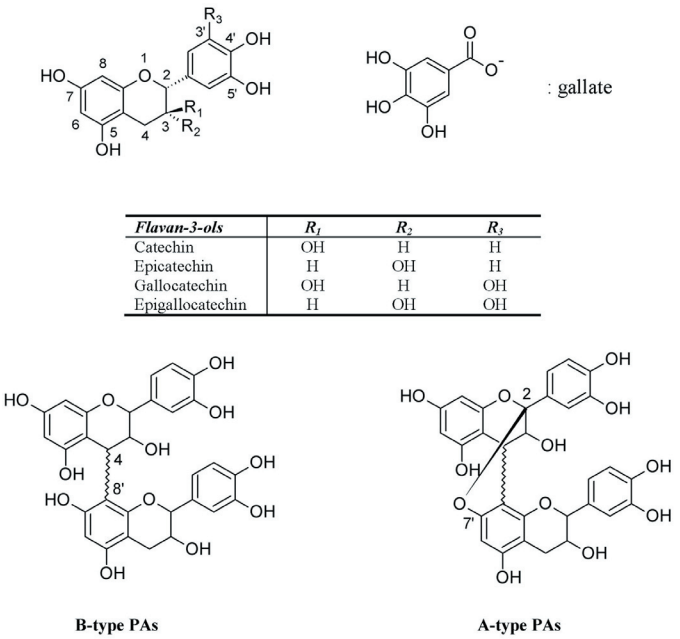

Flavan-3-ols	R_1	R_2	R_3
Catechin	OH	H	H
Epicatechin	H	OH	H
Gallocatechin	OH	H	OH
Epigallocatechin	H	OH	OH

Fig. 1: Structures of flavanols and procyanidins (PAs) in grape seeds.

Labarbe *et al.* 1999). Moreover, in the electrospray mass spectra obtained by direct-infusion the signals of $[M-3H]^{3-}$ PAs ions corresponding to up to 28 units, were observed (Hayasaka *et al.* 2003). In general, by mass spectrometric techniques a scarce detection of higher molecular weight PAs is often observed principally due to ion suppression phenomena related to the high abundance of lower molecular species (Taylor *et al.* 2003, Fulcrand *et al.* 2008, Monagas *et al.* 2010, Mouls and Fulcrand 2012).

The degree of polymerization and galloylation affects the "mouth-feel" properties caused by the interaction of PAs with salivary glycoproteins: a heightened perception of astringency has been observed with a concomitant increase in the degree of polymerization; similarly, an increased degree of galloylation favours heightened perception of coarseness (Vidal *et al.* 2003).

In the last century, a number of French-American hybrid grape varieties were produced, with the aim of having plants resistant to diseases. Currently, these cultivars have been little studied because in several countries (e.g., in the European Community) they are not included in the list of varieties used for producing wine (CE Regulation no. 479/2008), and in many cases are only conserved in germplasm collections. These hybrids are often characterised by a high grape production per hectare and, due to their resistance to diseases, without using pesticides. Moreover, they are often characterized by peculiar richness of chemical secondary metabolites with respect to the *V. vinifera* varieties. For example, a study of the anthocyanin profiles of twenty-one of these red grapes showed that most of them are qualitatively and quantitatively very rich in monoglucoside and diglucoside compounds (De Rosso *et al.* 2012); a study of grape seed triacylglycerols showed that some of these varieties (e.g. 'Bacò 1') have peculiar contents of trilinolein, a high-unsaturated triglyceride compound (De Marchi *et al.* 2012). In the present work, PAs profile of thirty-two hybrid grapes including both red and white varieties was studied. MALDI-TOF and MALDI-TOF/TOF analysis were performed, which have been proved to be effective in tannin studies (Reed *et al.* 2005, Monagas *et al.* 2010). PAs profile of seeds of these varieties was never studied before and for the high polyphenolic content some of them could be interesting for production of seed extracts for the food, oenological, and nutraceutical industry. Three *V. vinifera* samples, 'Aramon', 'Chardonnay' and 'Cabernet Sauvignon', were studied as references, to compare their polyphenol potential with the hybrid samples.

Material and Methods

Samples and reagents: Thirty-two hybrid grape samples (21 red and 11 white) and three *V. vinifera* grape samples ('Aramon', 'Chardonnay' and 'Cabernet Sauvignon') were collected in 2010 at full technological ripeness from the five plants of each variety present in the CRA-VIT (Viticulture Research Centre) Grapevine Germplasm Collection (Susegana, Treviso, Italy), and immediately frozen. The list of samples studied is reported in Tab. 1. Methanol, hexane and Folin-Ciocalteu reagent were purchased from Fluka Sigma-Aldrich (Milan, Italy).

Proanthocyanidins extraction: Seeds of 20 berries were manually separated from skins and pulps, dried on a paper sheet, weighted and ground with liquid nitrogen by using a mortar. The sample was defatted with hexane (ratio 1:10 w/v) by keeping the sample under stirring at room temperature overnight in the dark. The powder was separated from the solvent by a paper filter and extracted with a methanol/water solution (70:30 v/v) in ratio 1:10 (w/v) (XU *et al.* 2010). The solution was kept under stirring overnight, centrifuged at 2683 g for 10 min at 15 °C and filtered on a paper filter.

Table 1

Hybrid grape varieties studied; M_n: number-average molecular weight; R: red grape variety; W: white grape variety

		Grape variety	*V*IVC number*)	Total polyphenol index mg (+)-catechin · kg grape^{-1}	M_n
1	R	Bacó 1	870	871	1430
2	R	Bacó 30-12	3980	1999	1436
3	R	Bertille Seyve 1808	1243	566	1370
4	R	Bertille Seyve 4825	1271	326	1443
5	R	Burdin 4077	1823	857	1365
6	R	Clinton (?)	271	207	1042
7	R	Couderc 25	2926	500	1386
8	R	Galibert 238-35	4352	450	1367
9	R	Seibel 8357	2768	571	1236
10	R	Seibel 8745	11466	1642	1350
11	R	Seibel 10878	2519	997	1372
12	R	Seyve Villard 12-347	11587	563	1367
13	R	Seyve Villard 12-390	11592	1101	1518
14	R	Seyve Villard 23-369	11643	1522	1392
15	R	Seyve Villard 23-399	11663	1104	1331
16	R	Seyve Villard 29-522	14324	1066	1323
17	R	Terzi 100-31	12400	1182	1277
18	R	Terzi 108-6	12401	2597	1138
19	R	Terzi 9746	12398	738	1329
20	R	Unknown red 1	na	849	1341
21	R	Unknown red 2	na	566	1482
22	W	Bacó 2-16	862	881	1405
23	W	Burdin 7469	1887	1386	1388
24	W	GF.GA-48-12	4551	1149	1373
25	W	Galibert 15-2	4342	1277	1441
26	W	Galibert 255-43	8209	704	1571
27	W	Galibert Treblanc	4350	644	1444
28	W	GF.84-21-9	20347	1569	1441
29	W	Inc Bruni 624	1758	1099	1369
30	W	Seibel 9110	12952	366	1542
31	W	Seibel 10173	416	1411	1357
32	W	Seyve Villard 12-375	13081	614	1377
33	R	Aramon	544	1055	1356
34	W	Chardonnay	2455	1311	1400
35	R	Cabernet Sauvignon	1929	1667	1372

*) *Vitis* International Variety Catalogue http://www.vivc.de/
(?) Accession of CRA-VIT collection in course of confirmation.

Total Polyphenol index: Total polyphenolic index of extracts was determined by Folin-Ciocalteu method (DI STEFANO and CRAVERO 1991). A volume of 0.1 mL of methanolic extract was 4-fold diluted with water and transferred in a 20 mL flask. Five millilitres of distilled water, 1 mL of Folin-Ciocalteau reagent and 4 mL of a sodium carbonate solution (10 % w/v) were added to the sample, and the reaction was carried on for 1 ½ h. At the end of reaction the absorbance of solution at 700 nm was measured using a Cary 50 Scan UV-Vis Spectrophotometer (Varian, Milan, Italy). Total phenolic index was calculated by the equation (1): (+)-catechin ($mg \cdot kg^{-1}$) = $186.5 \cdot A_{700} \cdot (n \cdot V^{-1}) \times (V_{ex} \cdot g^{-1})$ (1) with n, number of dilution of the sample; V, volume of extract used (0.1 mL); V_{ex}, total volume of grape seed extract; g, weight of grapes.

MALDI-TOF mass spectrometry: Analyses were performed using a MALDI-TOF-TOF UltrafleXtreme (Bruker Daltonics, Bremen, Germany) instrument, equipped with 1 kHz smartbeam II laser (λ = 355 nm) and operating in reflectron positive ion mode. The instrumental conditions employed to analyze molecular species in the *m/z* range 450-3500 were: Ion Source 1: 25.00 kV; Ion Source 2: 22.30 kV, Lens: 7.70 kV, Pulsed ion extraction: 80 ns, Reflector: 26.45 kV, Reflector 2: 13.45 kV. The matrix solution was prepared by dissolving 10 mg of crystalline 2,5-dihydroxy benzoic acid (DHB) in 1 mL of methanol/water solution (70:30 v/v). The extract solutions were diluted ten times with a methanol/water solution (70:30 v/v). Five µL of this solution were mixed with the same volume of matrix solution and 1 µL of the resulting mixture was deposited directly on the stainless steel sample holder and allowed to dry before introduction into the mass spectrometer. External mass calibration (Peptide Calibration Standard) was based on monoisotopic values

of $[M+H]^+$ of Bradykinin, Angiotensin II, Angiotensin I, Substance P, Bombesin, ACTH clip (1-17), ACTH clip (18-39), Somatostatin 28 at *m/z* 757.39916, 1046.5420, 1296.6853, 1347.7361, 1619.8230, 2093.0868, 2465.1990 and 3147.4714, respectively. TOF/TOF experiments were performed using the LIFT device with the following experimental conditions: IS1: 7.5 kV; IS2: 6.75 kV; Lift1: 19 kV; Lift2: 3.7 kV; Reflector1: 29.5 kV; Delay time: 70 ns.

Statistical analysis: Relative intensities of the signals were referred to the highest signal and subjected to angular transformation in order to normalize data distribution. It was performed by using the equation (2):

$$y = arcsin(sqrt(p)) \quad (2)$$

where *y* is the value of the transformed variable, *arcsin* is the arcsine function, *sqrt(p)* is the squared root of the proportion (Gomez and Gomez 1984). To select the variables statistically significant for differentiation between *V. vinifera* and hybrid samples One-way Analysis of Variance (ANOVA) with the Least Significant Difference (Fisher test) and 95 % significant level, was applied (Cabredo-Pinillos *et al.* 2008). Statistical analyses were performed using MINITAB Statistical Software Inc. (2003), Release 14 for Windows (State College, Pennsylvania).

Results and Discussion

Characterization of proanthocyanidins: The first step of this study involved the identification of the best MALDI conditions in order to obtain the best reproducibility and the best signal-to-noise ratio. Various matrices were tested, such as sinapinic acid, α-cyano-4-hydroxycinnamic acid, 2,5-dihydroxybenzoic acid (DHB), dithranol, and the best spectra were obtained using DHB, with a reproducibility of ion abundance of about 95-97 % (measured on the main peaks of the spectra) and with a signal-to-noise value in the range of 7-35. Under such conditions the spectra are characterised by the presence of abundant cationised species (Na^+, K^+) and, in some cases, of protonated molecular species. Measurements were also performed in both linear and reflectron modes: our instrument provided results which differed from those described by Monagas *et al.* (2010), showing that the reflectron mode yields more significant and reproducible data.

Positive MALDI-TOF spectra of grape seed extracts show decreasing peak intensities with increasing *m/z* values (Fig. 1 of supplemental material). According to data in the literature, peaks corresponding to $[M+Na]^+$ and $[M+K]^+$ adducts of PAs are found, while $[M+H]^+$ were observed only in some cases (Tab. 2) (Krueger *et al.* 2000**,** Weber *et al.* 2007). MALDI data analysis revealed several series of molecular species originating from A-type and B-type tannins. Despite positive MALDI-TOF analysis did not produce the signals of oligomers higher than *m/z* 4000, the spectra provided much information which allowed identification of the signals of 148 putative PAs. The presence of 58 putative isobaric pairs (Tab. 2) - for example, the ions at *m/z* 1481 - may be due to a pentamer-K adduct or a digalloylated (2G) tetramer-Na adduct. It should be noted that the difference of 288 Da observed for many signals is due to addition of further catechin/epicatechin monomers, while that of 152 Da may be due to the presence of gallic acid moieties in the molecular structure (Fig. 2 of supplemental material). Moreover, it is worth a note that substitution with two gallic acid molecules gives equivalent mass of the addition of a gallocatechin/epigallocatechin unit in the molecule (+ 304 Da).

Other oligomeric series have been detected and their *m/z* values are lower than that of B-type series: in particular peaks at -2, -4 and -6 Da have been observed. These series can be ascribed to the presence of PAs with one or more A-type bonds in the sample. For example, in the case of $[M+H]^+$ trimers, a cluster with peaks differing 2 and 4 Da has been observed and this can be explained by the presence of two B-type bonds (ion at *m/z* 867) or of one B-type bond and one A-type bond (ion at *m/z* 865) or of two A-type bonds (ion at *m/z* 863). The signal at *m/z* 1149, identified as $[M+H]^+$ tetramer A-type, indicates the presence of three A-type bonds, although it is theoretically improbable because of steric hindrance. Looking at the obtained results shown in Tab. 2, this analytical approach leads to the identification of both A- and B-type tannins up to undecamers with degrees of galloylation (DG) ranging from 0 to 7. In general, DP and DG of the PAs determined are similar to those previously reported in *V. vinifera* grape seed extracts (Krueger *et al.* 2000, Hayasaka *et al.* 2003).

Two other signals at *m/z* 561 and *m/z* 559 were detected, possibly due to $[M+H]^+$ species of dehydrated B-type and A-type dimers, respectively, as described by Mouls and Fulcrand (2012). TOF/TOF experiments were carried out on more abundant A- and B-type oligomers to characterize their molecular structures. As an example, the TOF/TOF mass spectra of ions at m/z 1481 (B-type oligomer) and m/z 865 (trimer with an A-type and a B-type bond) are reported in Fig. 3 of supplemental material. As it can be seen, in the case of the ion at *m/z* 1481 fragment ions due to the losses of catechin/epicathechin moieties (288 Da) are well detectable together with losses of 170 Da (gallic acid) and 152 Da, originating from rearrangement of the dihydropyran heterocycle. When one A-type bond is present in the molecule, the TOF/TOF mass spectrum becomes more complicated. In the case of ion at *m/z* 865, the already described losses of 288 Da and 152 Da are well detectable, but new fragments, such as that at *m/z* 684 is present, originating from the loss of a radical species, whose molecular formula is $C_9H_9O_4$ (181 Da). This could be explained by rearrangements of protonated species induced by the A-type bond, which enhance steric hindrance of the molecule. Increasing the polymerization degree, the TOF/TOF mass spectra of the A-type PAs become very complicated: the losses of 152 Da and 288 Da are still detectable, but fragment ions originating from rearrangements are very abundant and it is very difficult to assign structure to them. The presence of A-type PAs in grape seeds is particularly interesting because, for instance in cranberries, these compounds have been proposed as active components which inhibit the adherence of *E. coli* to uro-epithelial cell surfaces, thus preventing bacterial colonisation and subsequent infection (Foo *et al.* 2000). A-type PAs have only been found in a few plants and fruits (Gu *et al.* 2003), e.g. ly-

Table 2

Signals of putative B-type and A-type proanthocyanidins identified in MALDI-TOF spectra of samples studied. G: gallate

Tannin ID	B-type			A-type		
	$[M+H]^+$	$[M+Na]^+$	$[M+K]^+$	$[M+H]^+$	$[M+Na]^+$	$[M+K]^+$
dimer	579	601	617	577	599	615
dimer 1G		753	769		751	767
dimer 2G		905	921		903	919
trimer	867	889	905	863, 865	887	903
trimer 1G		1041	1057		1039	1053, 1055
trimer 2G		1193	1209		1189, 1191	1205, 1207
trimer 3G		1345	1361		1341, 1343	1357, 1359
tetramer	1155	1177	1193	1149, 1151, 1153	1773, 1175	1189, 1191
tetramer 1G	1305	1329	1345	1303	1325, 1327	1341, 1343
tetramer 2G		1481	1497		1475, 1477, 1479	1491, 1493, 1495
tetramer 3G		1633	1649		1629, 1631	1645, 1647
tetramer 4G		1785	1801		1781, 1783	1795, 1797, 1799
pentamer		1465	1481		1461, 1463	1475, 1477, 1479
pentamer 1G		1617	1633		1613, 1615	1629, 1631
pentamer 2G		1769	1785		1763, 1765, 1767	1781, 1783
pentamer 3G		1921	1937		1917, 1919	1933, 1935
pentamer 4G		2073	2089		2069, 2071	2085, 2087
pentamer 5G		2225			2221, 2223	
hexamer		1753	1769		1747, 1749, 1751	1763, 1765, 1767
hexamer 1G		1905	1921		1901, 1903	1917, 1919
hexamer 2G		2057	2073		2053, 2055	2069, 2071
hexamer 3G		2209	2225		2205, 2207	2221, 2223
hexamer 4G		2361	2377		2357, 2359	2371, 2373, 2375
hexamer 5G		2513	2529		2509, 2511	2523, 2525, 2527
hexamer 6G		2665			2661, 2663	
heptamer		2041	2057		2037, 2039	2053, 2055
heptamer 1G		2193	2209		2189, 2191	2205, 2207
heptamer 2G		2345	2361		2339, 2341, 2343	2357, 2359
heptamer 3G		2497	2513		2493, 2495	2509, 2511
heptamer 4G		2649	2665		2645, 2647	2661, 2663
heptamer 5G		2801	2817		2797, 2799	
heptamer 6G		2953			2949, 2951	
octamer		2329	2345		2325, 2327	2339, 2341, 2343
octamer 1G		2481	2497		2477, 2479	2493, 2495
octamer 2G		2633	2649		2629, 2631	2645, 2647
octamer 3G		2785	2801		2781, 2783	2797, 2799
octamer 4G		2937	2953		2933, 2935	2949, 2951
octamer 5G		3089	3105		3087	
octamer 6G		3241	3257		3239	
octamer 7G		3393				
nonamer 1G		2769	2785		2765, 2767	2781, 2783
nonamer 2G		2921	2937		2919	2933, 2935
nonamer 3G		3073	3089		3071	3087
nonamer 4G		3225	3241		3223	3239
nonamer 5G		3377	3393		3375	
nonamer 6G		3529				
decamer 1G		3057	3073			
decamer 2G		3209	3225		3207	3223
decamer 3G		3361	3377		3357, 3359	3375
decamer 4G		3513	3529		3511	
decamer 5G		3665				
decamer 6G		3817				
undecamer 1G		3345	3361			
undecamer 2G		3497	3513			
undecamer 3G		3649	3665			

chee seeds (Xu *et al.* 2010), plums and cinnamon (Gu *et al.* 2003). Matching our findings, the presence of galloylated A-type PAs in grape seeds has recently been demonstrated (Passos *et al.* 2007). PAs with one or two interflavanoid A-type bonds were also identified in bark stem of *Pavetta owariensis* (Balde *et al.* 1995).

Seeds PA profile of the grape varieties: In some samples, the Boltzman-shaped curve of MALDI spectra was interrupted at low *m/z* values and the highest intensity was observed for the signals at *m/z* 559 ('Aramon', 'Terzi 100-31', 'Incrocio Bruni 624', 'Cabernet Sauvignon') and *m/z* 577 ('Clinton', 'Couderc 25', 'Seibel 10878', 'Seyve Villard 23-369' and '23-399', 'Terzi 1086', 'Terzi 9746'). For most samples the most abundant ion was at *m/z* 1193 corresponding to trimer 2G sodium adducts, in 'Seibel 8357' and 'Seyve Villard 12-357' at *m/z* 905 corresponding to dimer 2G sodium adducts. In the case of the accession under identification as 'Clinton' the MALDI spectrum was characterized by high chemical background and it was more complicated than the other samples. In particular the signals of A-type PAs were often higher than B-type ones. Moreover, other two series of ions starting from *m/z* 559 and *m/z* 711 (559 + 152), respectively, and including the signals at *m/z* 847, 1135, 1423, 1711, 1999 and 2287, and at *m/z* 999, 1287, 1863, 2151, 2439 and 2727, respectively, were observed (Fig. 4 of supplemental material). These two series differ from putative PAs A-type by 18 Da (Mouls and Fulcrand 2012).

Total polyphenol index of extracts was determined and data are reported in Tab. 1. This index is a parameter useful for a comparison of polyphenolic content of the samples. The results ranged from a minimum of 200 mg·kg^{-1} grape to a maximum of 2600 mg·kg^{-1} grape ('Terzi 108-6'), while those found in *V. vinifera* samples are between 1050-1670 mg·kg^{-1} grape. To compare the samples, also the number-average molecular weight (M_n) was calculated by using the signals intensity of MALDI spectra according to equation (3):

$$M_n = \sum [(m/z)_i \cdot I_i] / \sum I_i \quad (3)$$

where I_i is the absolute intensity of each tannin identified (Nonier *et al.* 2004, Manè *et al.* 2007), and are reported in Tab. 1. The average M_n for hybrid grapes was 1375 ± 97, while that of the three *V. vinifera* samples 1376 ± 18. These data match those in the literature for commercial tannin fractions of grape and apple (Manè *et al.* 2007), and highlight that the main peaks in MALDI spectra correspond to oligomers. Total polyphenols index and M_n of 'Chardonnay' extract are lower than the data reported in literature for the same variety after phoroglucinolysis (1,400 mg·kg^{-1} *vs* 2300 mg·kg^{-1}) (Manè *et al.* 2007), but this is probably due to the different analytical methods used and samples studied. It was observed that the higher the degree of polymerisation, the lower the ion peak intensity. M_n was higher than *V. vinifera* samples for 'Seyve Villard 12-390', 'Galibert 255-43' and 'Seibel 9110' ('1518', '1571' and '1541', respectively). In 'Seyve Villard 12-390', very high polyphenolic content was also found (1.1 g·kg^{-1} grape). Several studies showed a positive correlation between tannin chain length and organoleptic characteristics: the higher the MW, the lower the bitterness and the stronger the astringency (Vidal *et al.* 2003). M_n data of these three varieties show higher contents of high-MW tannins, as a consequence their extracts are particularly interesting for oenological uses.

Some hybrid grapes were richer of A-type PAs with respect to *V. vinifera* samples. In particular, 'Bacò 1', 'Bertille Seyve 4825', 'Couderc 25', 'Seibel 10878', 'Seyve Villard 12-390', 'Seyve Villard 23-369', 'Seyve Villard 23-399', 'Galibert 255-43', 'GF.84-21-9', 'Incrocio Bruni 624', 'Seibel 9110', and 'Seyve Villard 12-375' showed PAs with two or three A-type bonds up to nonamers and a profile partially similar to cranberry (Fig. 2) (Feliciano *et al.* 2012). The high content of A-type PAs, characterized by bacterial antiadhesion activity (Howell *et al.* 2005), may further increase the interest for nutraceutical uses of these grapes.

The large number of signals in Tab. 2 was reduced to 109 by summing the intensities of $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ species and those of PAs with at least one A-type bond. Further reduction of the variables was achieved by performing one-way ANOVA and Fisher's test using the mean value of the hybrid samples towards the mean value for *V. vinifera*. Variables with a high F statistic and p-value < 0.05 were considered significant in differentiating between hybrid and *V. vinifera* varieties. Fifteen PAs resulting more characteristics of hybrid grapes, in particular A-type $[M+Na]^+$ dimer digallate (or $[M+K]^+$ trimer), A-type $[M+Na]^+$ dimer dehydrated, and B-type $[M+Na]^+$ decamer tetragallate (Tab. 3).

Conclusions

In spite of the presence in the matrices of sodium and potassium salts, which may affect signal intensities, MALDI-TOF analysis has provided a rapid and complete characterization of grape seed extracts and allowed the identification 55 B-type and 93 A-type proanthocyanidins, respectively.

A high amount of antioxidant polyphenols makes the extract potentially interesting for uses in nutraceutical industry, and high-MW grape seed tannins are used for the clarification of must and wines. Seed PAs of these hybrid grapes were studied for the first time, and some of them present sources of natural compounds potentially very interesting for the industry. In particular, 'Terzi 108-6' seed extract showed high content of antioxidant polyphenols. Despite MALDI-TOF allowed to study PAs just until undecamers, the comparison with the other *V. vinifera* and hybrid grape samples evidenced that 'Seyve Villard 12-390' is characterized by a relevant presence of higher oligomers. If further studies performed by gel permeation chromatography will confirm relevant presence also of PAs with MW until 10000 (Kennedy *et al*. 2003), the seed extract of this grape variety can be an interesting source of oenological tannins. As a consequence, if these varieties will show also interesting organoleptic properties they can be used for production of grape juices and their byproducts as sources of PAs for industrial purposes.

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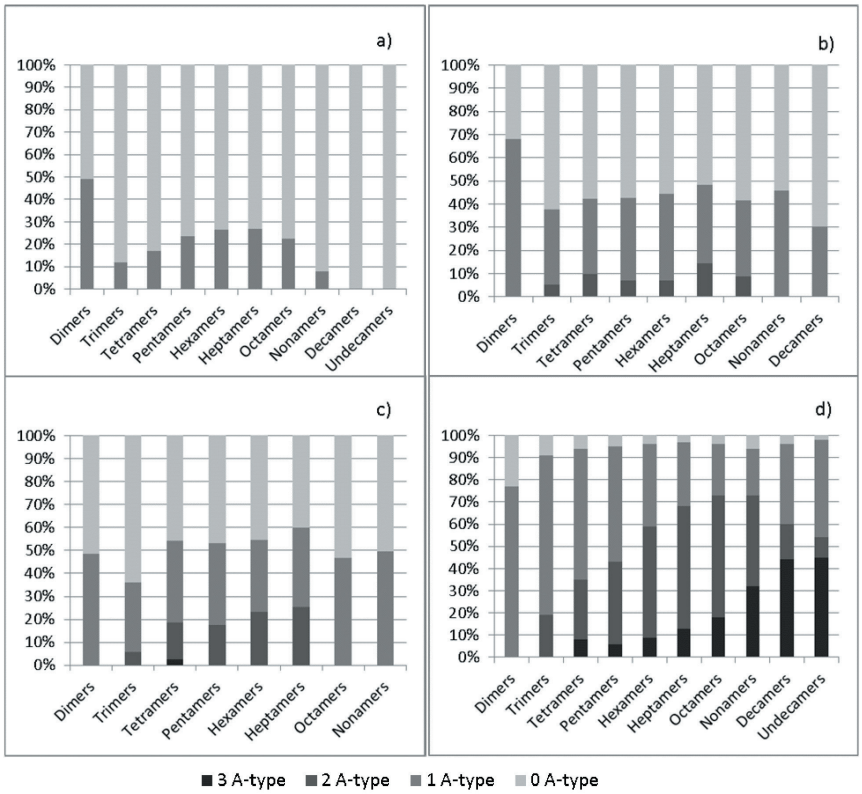

Fig. 2: Percentage of A-type and B-type PAs determined in: **a**) *V. vinifera* samples studied (mean data); **b**) 'Seyve Villard 23-399'; **c**) 'Seibel 10878'; **d**) cranberry extracts (Feliciano *et al.* 2012).

Table 3

Most significant PAs in differentiation between hybrid and *V. vinifera* grapes. Variables were selected by F-Test. *m/z*: masses of species attributed to each tannin. F: Fisher F statistic; P: *p*-value significant < 0.05; G: gallate

Variable	*m/z*	F	P
$[M+Na]^+$ dimer A 2G or $[M+K]^+$ trimer A	903, 919	13.10	0.001
$[M+Na]^+$ decamer B 4G	3513	12.99	0.001
$[M+Na]^+$ dimer A dehydrated	559	12.25	0.001
$[M+Na]^+$ trimer A 1G	1039, 1053, 1055	9.27	0.005
$[M+Na]^+$ hexamer B 3G or $[M+K]^+$ heptamer B 1G	2209	9.01	0.005
$[M+Na]^+$ nonamer B 4G or $[M+K]^+$ decamer B 2G	3225	8.97	0.005
$[M+Na]^+$ undecamer B 2G	3497	7.34	0.011
$[M+Na]^+$ tetramer A	1149, 1151, 1153, 1173, 1175	7.16	0.012
$[M+Na]^+$ trimer A	863, 865, 887	6.33	0.017
$[M+Na]^+$ tetramer B 3G or $[M+K]^+$ pentamer B 1G	1633, 1649	5.93	0.020
$[M+Na]^+$ trimer A 2G or $[M+K]^+$ tetramer A	1189, 1191, 1205, 1207	5.48	0.025
$[M+Na]^+$ pentamer B 3G or $[M+K]^+$ hexamer B 1G	1921, 1937	5.01	0.032
$[M+Na]^+$ pentamer B 4G or $[M+K]^+$ hexamer B 2G	2073	4.81	0.035
$[M+K]^+$ tetramer B 4G	1801	4.26	0.047
$[M+Na]^+$ tetramer A 4G or $[M+K]^+$ pentamer 2G	1781, 1783	4.19	0.049

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