Selective binding of potassium and calcium ions to novel cyclic proanthocyanidins in wine by HPLC-HRMS

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ABSTRACT

Rationale

Cyclic B-type (referred also as crown) proanthocyanidins were recently identified in wines. An HPLC-HRMS/MS method was applied to study the binding of cyclic and non-cyclic PAC to potassium and calcium ions, which affect the chemico-physical stability of wines. Different binding affinities suggest that cyclic and non-cyclic analogues, despite the equal number of monomer units, influence the colloidal stability of wine and are related to the grape variety or winemaking conditions.

Methods

Nineteen red and white wines were analysed by HPLC high resolution tandem mass spectrometry with positive electrospray ionization to investigate the distribution of novel cyclic proanthocyanidins and their calcium, potassium and sodium adducts. Principal Component Analysis was used to study the distribution of the wines and the relationships among proanthocyanidins with and without cation complexes.

Results

A dependence on specific isomers (and conformations) was found for the non-cyclic procyanidin (PC) trimer whereas the cyclic tetrameric PAC were shown to bind better to potassium than their non-cyclic analogues. The binding to these metals appeared to be influenced not only be the number of monomer units but also by the conformation assumed by the molecules. Statistical analysis evidenced that the adducts distribution in different wines is less influenced by the grape variety used for winemaking than their associated $[M+H]^+$ species studied earlier.

Conclusions

Wines from 19 grape varieties were investigated in order to identify potassium and calcium complexes of non- and cyclic B-type proanthocyanidins that were recently discovered. The results showed a dependence of the distribution of metal complexes according to the cyclic or non cyclic geometry of proanthocyanidins. The multivariate analysis of the mass spectrometric results showed a relationship with the grape variety, however not so straightforward as evidenced for the non-complexed species.

Keywords: cyclic proanthocyanidins; crown proanthocyanidins; wine; metal adducts; high resolution mass spectrometry.

Abbreviations: PAC, proanthocyanidins; PCA, principal component analysis.

1. INTRODUCTION

The prevention of tartrate salts precipitation in bottled wine is a fundamental oenological technique applied to preserve wine from the associated turbidity.[1] In fact, potassium hydrogen tartrate (KHT) is usually present at supersaturated concentrations in wines and it leads often to crystals precipitation. For example, the KHT saturation point at 20 ^{o}C in a 10% ethanol solution is 2.9 g·L^{-1}.[2] In reality, KHT is present at higher concentrations in wine. Therefore, precipitation is thermodynamically favoured at the temperatures at which wine is usually stored. Also the much less soluble calcium tartrate can precipitate, but still its concentration is much lower than KHT in wine. Calcium is often introduced incidentally, for example as bentonites (aluminium silicates hydrated with Ca^{2+} and Na^{+} counter-cations) which are used for preventing protein haze formation. Several approaches have been adopted in order to avoid the overtime KHT crystallization. An effective one is the addition of polymers able to provide protecting colloids, such as metatartaric acid or carboxymethyl cellulose.[2] When KHT aggregates in presence of metatartaric acid it tends to include the polymer in the growing crystals. This prevents the crystal growth and the precipitation. Carboxymethyl cellulose has been shown to be more effective in prolonging the wine stability, since metatartaric acid can be further hydrolysed to tartaric acid units, this way further increasing the probability of precipitation instead. Another strategy is cold treatment at temperatures close to the wine freezing point. This would force the salts above the saturation level to precipitate, therefore reducing the presence of long-term precipitation by direct filtration of the excess salt precipitate. Other approaches have been proposed, such as resin ion exchange (e.g. K^{+}, Ca^{2+} and Mg^{2+} for H^{+} or Na^{+}) or increasing the quantity of mannoproteins dissolution into wine from spent yeasts through the *batonnage* technique.[3–6] Besides, proanthocyanidins (PAC) are among those abundant polyphenol components of wine that have been shown to prevent tartrate instability.[2] PAC constitute a wide class of molecular oligomers, built up with flavan-3-ol monomer units. They bear all the possible substitutions that their constituent monomer units can possess, such as esterification (gallates) and variable substitutions at the flavanolic B-ring (e.g. catechol and pyrogallol). These compounds can have also very variable stereo-chemical configurations and inter-monomeric binding preferences. Therefore, PAC are a mixture of diverse components that represent one of the richest and most abundant sources of antioxidants in wine.[7] Besides, their size increases overtime through polymerizations until they become

insoluble and precipitate. Their precipitation at higher molecular weight due to lowered solubility is a cause for loss of antioxidants from wine during its ageing, too.

Recently, a series of reports have revealed the existence of a new class of more polar (more soluble) cyclic PAC.[8,9] Their structures have not been resolved yet since only one cyclic tetramer procyanidin (PC) has been purified and fully characterized. However, LC-MS studies have shown that these cyclic species are likely to be composed by one main isomer only, differently from their non-cyclic congeners that show many different isobaric species instead. Experimental evidences (NMR) proved that only one regio- (and stereo-) isomer was present at least for such tetramer (a definite bonds arrangement with two C*4*-C*6* and two C*4*-C*8* inter-monomeric bonds and (-)-epicatechin only as the constituent monomer).[8] Some unforeseen ambiguity required further analysis that led to the conclusion that this tetramer structure must be cyclic and that the cyclic PAC series (4-mer, 5-mer, 6-mer and etc.) must surely be extended to heavier analogues,[10] and to analogues containing other monomers than (-)-epicatechin, such as (epi)gallocatechin.[11] These studies showed also that this tetramer is present also in organisms (*Vaccinium* sp.) other than *Vitis vinifera* sp.[10]

The ability of phenolic antioxidants and polyphenols of binding metals has been studied.[12–21] The aim of the present work is a preliminary qualitative study of the binding properties of these novel PAC to metals in several red and white wines. Mass spectrometry is an established method for metal-polyphenol complexes analysis. However, methods involving RP-LC separations prior to the MS analysis are not employed extensively for the elucidation of such complexes.[18,22] Nonetheless, our aim is far from the estimation of binding constants or geometries, but is instead a qualitatively assessment of the presence of preferential bindings. The use of mass spectrometry coupled to liquid chromatography, although avoided usually for these metal complexes,[14,18] could allow to ascertain (only qualitatively) our hypothesis that the different isomers may exert different affinities for potassium and calcium.

Furthermore, the identification of new chemical markers of wine quality and authenticity is also a main aim of our investigation. The wide variety of these species allowed in recent reports to propose their relative abundances as tools for differentiating the wines by grape variety.[11,23] Another sought application would be the definition of suitable variables for monitoring the effects of technological applications, as for example the use of metatartaric stabilization and the use of bentonite.

2. EXPERIMENTAL

2.1 Material

All solvents and additives used (LC-MS grade) were purchased from Sigma-Aldrich Ltd. Wines from South Tyrol (Italy) were collected in a local winery (Kellerei Bozen/Bolzano, BZ, Italy) and an agricultural high school (Happacherhof, Auer/Ora, BZ, Italy). The wines were all of PDO/DOC grade (Table 1). Wines description is provided elsewhere in more detail.[23]

2.2 Samples' preparation

No extraction was applied to the samples. They were instead concentrated at reduced pressure (9-10 mbar) at 30 °C. Then, they were dried by 30 min of gentle N_2 flux. Finally, they were recovered in the mobile phase A (see HPLC-HRMS section for details) to a final concentration 10 times higher than the initial one. The samples were always filtered before column injection (0.2 µm, regenerate cellulose).

2.3 HPLC-HRMS/MS analysis

The HPLC-HRMS/MS method applied was presented earlier.[10,24,25] Briefly, the HPLC-HRMS system was composed of a Q Exactive HRMS instrument (Thermo Fisher Scientific, Rodano, Milano, Italy) coupled with a 16-channel DAD-provided Agilent 1260 HPLC (Agilent Technologies Italy S.p.A., Cernusco sul Naviglio, Milano, Italy). The separation was carried out at 1 mL min^{-1} on a ODS Hypersil C18 column (125 mm × 4.6 mm i.d., 5 µm, Thermo Sci.) equipped with a pre-column filter (ODS Hypersil, 5 µm pore size, 10 x 4 mm drop-in guards, Thermo Fisher Scientific). The mobile phase consisted of a combination of solvent A (0.1%v/v formic acid in 0.02 mol L^{-1} ammonium formate in water) and solvent B (0.1%v/v formic acid in saturated ammonium formate acetonitrile LC-MS grade). The gradient was set as follows: from 5% B at 0 min to 25% B (v/v) at 21 min, then to 95% B at 22 min until 27 min, to 5% at 28 min, followed by re-equilibration step (5% B) at 32 to 35 min. The DAD spectra were recorded from 210 to 600 nm and provided real-time monitoring at 280 nm, 320 nm, 365 nm, 420 nm and 520 nm (+/- 4 nm). The Q Exactive HESI source was operated in positive ionization mode using the following conditions: sheath gas = 20 (arbitrary units), aux gas = 5 (arbitrary units), aux temperature = 250 °C, spray voltage = +3.5 kV, capillary temperature = 320 °C and RF S-lens = 70. The mass range selected was from 500 to 2,000 m/z with a FullMS working resolution = 70,000 (@200 m/z), AGC target = $3 \cdot 10^6$, max. injection time = 300 ms. Data dependent HPLC-MS/MS experiments were run separately on the N_2 concentrated samples: Full-MS parameters were kept as shown,

MS/MS AGC = 10^6, max. injection time = 300, FT-MS set resolution = 35,000, loop count = 5, isolation window = 2 or 3 m/z with 1 m/z offset, normalized collision energy = 15 eV. For data dependent settings: minimum AGC target = $3 \cdot 10^3$, apex trigger = 2 to 8 sec, charge exclusion = 3 – 8 and higher, dynamic exclusion = 3 sec. LC-MS/MS experiments were tested also in negative ionization (spray voltage -3.5 kV). Lock masses were constantly employed to correct mass deviations across the FullMS acquisition range throughout the experiments. The HPLC-DAD data were collected and analyzed by OpenLab software while the MS data and results were collected and analyzed by Xcalibur 3.1 software (Thermo Fisher Scientific). XLStat (version 2016.02.28430, Addinsoft, Paris, France) was employed for the statistical analysis and The Unscrambler (version 10.4.43636.111, CAMO Software AS., Oslo, Norway) software was employed for the statistical analysis.

3. RESULTS AND DISCUSSION

The list of 19 wines is reported in Table 1. They were analysed for the presence of the metal adducts of proanthocyanidins, from dimeric to hexameric oligomers. The main cations investigated were potassium and calcium as their concentrations are important wine quality and stability markers. In addition, magnesium, zinc, lead, cadmium, iron and copper were however investigated but the re-concentration (ten times the native concentration) of wine was not yet sufficient for their identification, therefore no results are reported about them. Sodium adducts instead were observed and included in the list. In Table 2 the metal adducts identified are reported.

The relative abundances obtained for each PAC in all 19 samples are reported in Table SI 1(a-d) (**Supporting Information**). Firstly, three factors can be considered with respect to the distribution of these species in the samples: *i*) the total abundance of the specific metals; *ii*) the total abundance of the specific oligomers; *iii*) the affinity of the oligomers for the metals. An interesting evidence was the affinity of some metals for specific congeners (e.g. trimer-K^+). The PC trimer complex with potassium appeared to occur only with one trimer congener eluting at 3.3 min. This same effect was not observed for the dimer, since the correspondent dimer-K^+ adducts eluted in correspondence to all the dimer-H^+ adducts. This evidence indicates that the affinity of the trimer with potassium is not possibly only due to the number of monomer groups and it should depend instead on certain preferred conformations of the macromolecule, which is induced by the specific linkages of (+)-catechins and (-)-

epicatechins and their mutual binding (C4-C6 *vs* C4-C8) and ratio and location of (+)-catechins and (-)-epicatechins. The other trimer-H^+ isomers, although similar in intensity, did not display an associated K^+-adduct. Interestingly, this congener was precisely the most polar one among trimers (i.e. the first one, eluting at 3.3 min). However, this effect appears to be limited to this one case. Besides, other oligomers showed co-elution of certain adducts with their $[M-H]^+$ analogues. This was common for most of the identified adducts but not for all of them.

The origin of these adducts in wine itself is confirmed since not all wine samples possessed all adducts or in the same proportions (see Table SI 1 in **Supporting Information**): this excludes the formation of artefacts during the elution (also, LC-MS grade solvents were used). Moreover, it is interesting to note that these complexes must be relatively stable. In fact, the eluent had a relatively high concentration (phase A: 0.02 mol·L^{-1}, phase B: saturated) of ammonium formate whose ionic strength may displace other bound cations. Moreover, as exposed previously, the binding must be conformation-selective since the relative abundances were strongly influenced by the specific conformations and not by the number of catechol units only. This was confirmed by comparison between the cyclic and the linear oligomers (from the tetramer upwards). Whereas the proportion of cyclic procyanidins and cyclic prodelphinidins varied according to the grape variety used for wine, here the preferences for potassium and calcium complex formation had totally different profiles. For instance, in almost all wines, calcium bound the cyclic tetramer procyanidin almost 100% over the total (cyclic *plus* non-cyclic) tetramer procyanidins. Similarly, potassium appeared to favour entirely the cyclic over the non-cyclic pentamer in four red wines, namely Lagrein, Merlot, Pinot Noir and two St. Magdalener but quite the opposite for all white wines, namely Gewürztraminer, Sauvignon blanc and Chardonnay. In addition, sodium bound exclusively to the linear tetramer, and not the cyclic one. Statistical analysis was then applied to investigate the contribution of these variables (the relative abundances of metal-PAC adducts as measured with the current method) to the total variance. The variables that most accounted for the variance were selected by ANOVA and listed in Table 3.

In **Figure 1** and **2** the Principal Component Analysis for red and white wines respectively are shown, using the relative abundances of these species as variables. The processing was done separately for white and red wines due to their heterogenous nature.

The PCA separations of the wines by their grape variety worked worse here than in previous reports (where no metal complex variables had been applied),[11,23] still a neat separation was achieved by grape variety for red and for white wines alike. A particular exception was the St.Magdalener samples. Two St.Magdalener (Huck am Back) were neatly separated from a third one (Moar). This set of variables appeared to be less affected by the grape variety and some other effect (e.g. winemaking procedures) may be involved. In fact, these wines are produced by the same winery using grapes from different vineyards located in the same territory. Other variables employed previously did not show any significant specific separation for these samples in PCA of cluster analysis.[11,23]

An interesting aspect is the effect of different tartrate stabilization approaches (e.g. use of metatartaric acid or cold stabilization). The cold treatment should lead to an overall loss of potassium from the wine, which should have an effect on the potassium complex amount. The sample LG-2 underwent a cold treatment with no addition of metatartaric acid, but, according to PCA, LG-2 did not differ remarkably from LG-1, which was instead added with metatartaric acid to achieve tartrate stabilization. The addition of metatartaric acid did not alter the overall concentration of potassium in solution but just its rate of crystallization, therefore it is possible that the binding with PAC was not so affected as the rate of crystal formation/growth.

Besides, a neat distinction between red and white wines was observed, which was not so foreseeable this time,[10,11,23] for the similar aforementioned reasons. This could suggest that the relative abundance of specific oligomers may be again the most important variable to take into account, with a less defined contribution from metal binding.

4. CONCLUSIONS

Proanthocyanidins complex with potassium and calcium metals were screened in 19 wines from the South-Tyrolean region. An HLPC-HRMS/MS approach allowed to identify several candidates and to highlight the probable contribution from specific isomeric forms at several *n*-meric stages. Namely, potassium appeared to bind selectively to one non-cyclic trimer (the most polar one in particular). Then, calcium and potassium appeared to bind more to the single cyclic tetramer procyanidin than to the many non-cyclic tetramer procyanidin isomers. This is an example of how molecular geometry (cyclic *vs* non-cyclic) affected the selectivity. Similarly, potassium

bound preferentially to the cyclic pentameric procyanidin whereas calcium preferred the non-cyclic congener. In addition, calcium was found to bind more to the cyclic pentameric proanthocyanidin containing one (epi)gallocatechin than its non-cyclic congener.

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CONFLICT OF INTERESTS

The authors declare no conflict of interests

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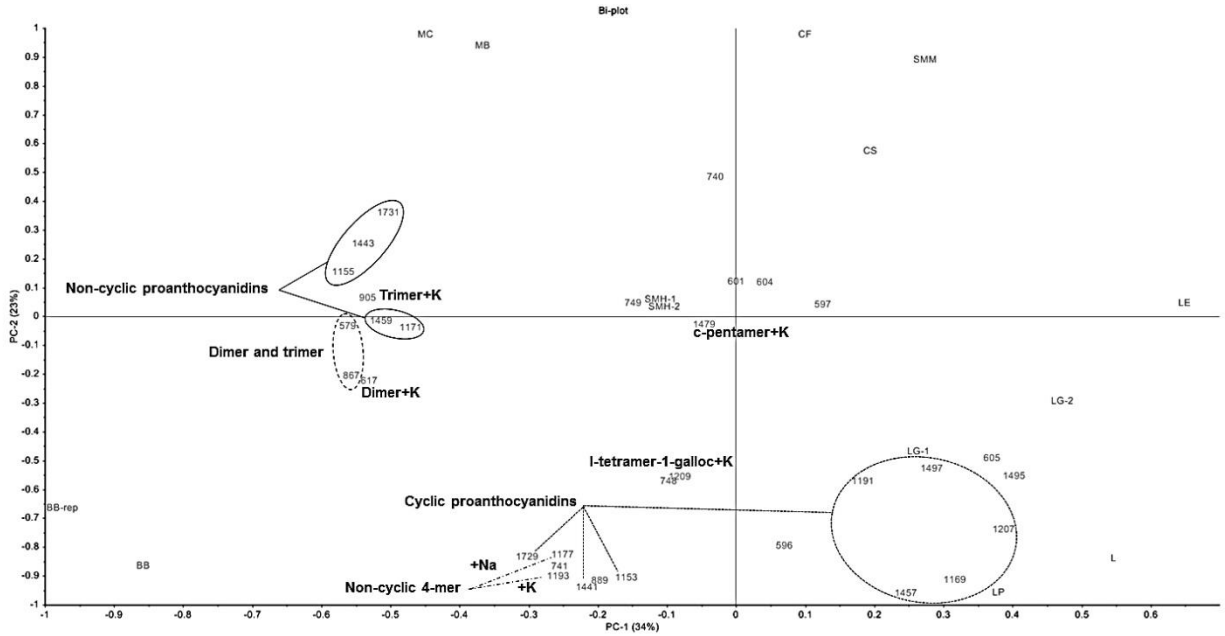

Figure 1 Principal Component Analysis (PC1 *vs* PC2) of red wine samples. Legend: 579 = dimer; 617 = dimer-K^+; 601 = dimer-Na^+; 867 = trimer; 905 = trimer-K^+; 889 = trimer-Na^+; 1155 = l-tetramer; 1193 = l-tetramer-K^+; 597 = l-tetramer-Ca^{2+}; 1177 = l-tetramer-Na^+; 1153 = c-tetramer; 1191 = c-tetramer-K^+; 596 = c-tetramer-Ca^{2+}; 1443 = l-pentamer; 1481 = l-pentamer-K^+; 741 = l-pentamer-Ca^{2+}; 1441 = c-pentamer; 1479 = c-pentamer-K^+; 740 = c-pentamer-Ca^{2+}; 1731 = l-hexamer; 1769 = l-hexamer-K^+; 885 = l-hexamer-Ca^{2+}; 1729 = c-hexamer; 1767 = c-hexamer-K^+; 884 = c-hexamer-Ca^{2+}; 1171 = l-tetramer-1-galloc; 1209 = l-tetramer-1-galloc-K^+; 605 = l-tetramer-1-galloc- Ca^{2+}; 1169 = c-tetramer-1-galloc; 1207 = c-tetramer-1-galloc-K^+; 604 = c-tetramer-1-galloc-Ca^{2+}; 1459 = l-pentamer-1-galloc; 1497 = l-pentamer-1-galloc-K^+; 749 = = l-pentamer-1-galloc-Ca^{2+}; 1457 = c-pentamer-1-galloc; 1495 = c-pentamer-1-galloc-K^+; 748 = = c-pentamer-1-galloc-Ca^{2+}.

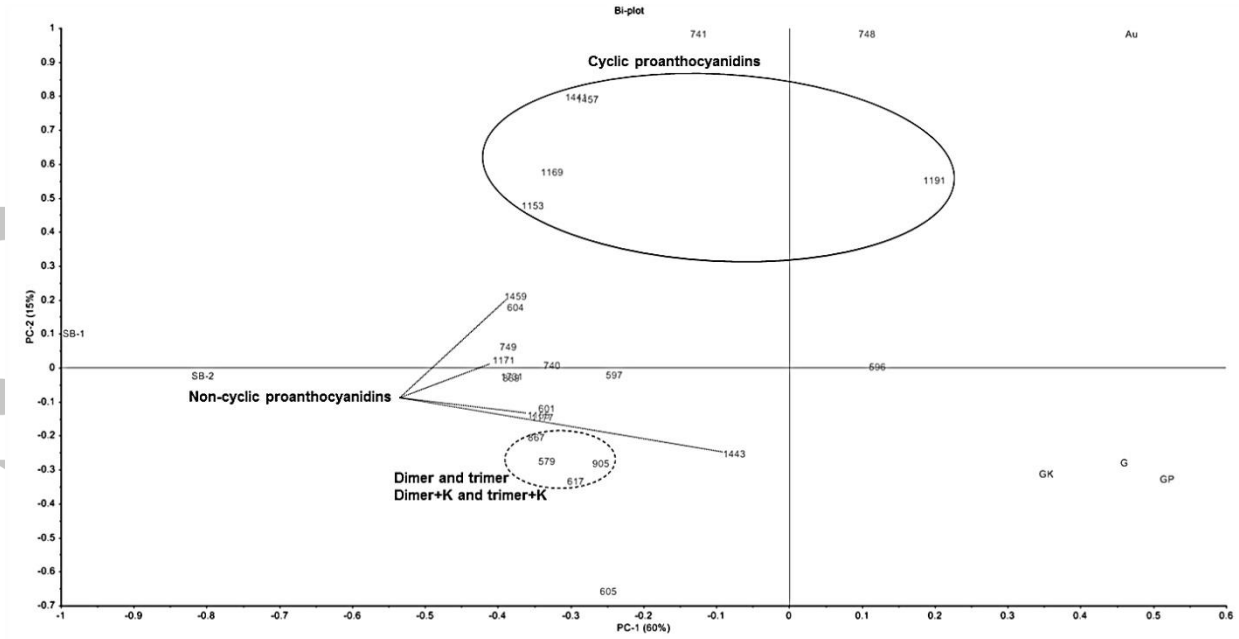

Figure 2 Principal Component Analysis (PC1 *vs* PC2) of white wine samples. Legend: 579 = dimer; 617 = dimer-K^+; 601 = dimer-Na^+; 867 = trimer; 905 = trimer-K^+; 889 = trimer-Na^+; 1155 = l-tetramer; 1193 = l-tetramer-K^+; 597 = l-tetramer-Ca^{2+}; 1177 = l-tetramer-Na^+; 1153 = c-tetramer; 1191 = c-tetramer-K^+; 596 = c-tetramer-Ca^{2+}; 1443 = l-pentamer; 1481 = l-pentamer-K^+; 741 = l-pentamer-Ca^{2+}; 1441 = c-pentamer; 1479 = c-pentamer-K^+; 740 = c-pentamer-Ca^{2+}; 1731 = l-hexamer; 1769 = l-hexamer-K^+; 885 = l-hexamer-Ca^{2+}; 1729 = c-hexamer; 1767 = c-hexamer-K^+; 884 = c-hexamer-Ca^{2+}; 1171 = l-tetramer-1-galloc; 1209 = l-tetramer-1-galloc-K^+; 605 = l-tetramer-1-galloc- Ca^{2+}; 1169 = c-tetramer-1-galloc; 1207 = c-tetramer-1-galloc-K^+; 604 = c-tetramer-1-galloc-Ca^{2+}; 1459 = l-pentamer-1-galloc; 1497 = l-pentamer-1-galloc-K^+; 749 = = l-pentamer-1-galloc-Ca^{2+}; 1457 = c-pentamer-1-galloc; 1495 = c-pentamer-1-galloc-K^+; 748 = = c-pentamer-1-galloc-Ca^{2+}.

Table 1 List of studied wines.

SAMPLE	WINE NAME	ABBREVIATION
6	Lagrein	L
7	Lagrein Prestige Klebelsberg	LP
8	Lagrein Eyrl	LE
9	Lagrein Grieser Collection I	LG-1
16	Lagrein Grieser, Collection 2	LG-2
2	Cabernet Franc	CF
3	Cabernet Sauvignon	CS
4	Merlot collection	MC
5	Merlot barrique	MB
10_1	Blauburgunder (Pinot noir)	BB
10_2	Blauburgunder (Pinot noir)	BB-rep
1	St.Magdalener Moar	SMM
17	St.Magdalener Classico Huck-I	SMH-1
18	St.Magdalener classico Huck-II	SMH-2
11	Gewürztraminer Kleinstein	GK
12	Gewürztraminer	G
14	Gewürztraminer Passito	GP
13	Sauvignon Blanc	SB-1
15	Sauvignon Blanc	SB-2
19	Aurum, Chardonnay Passito	Au

Table 2 List of metal-PAC adducts identified in wines. The corresponding MS spectra of sample 10 are shown in SI. Legend: l, non-cyclic (linear); c, cyclic.

Species	Elemental Composition	Found Mass (m/z)	δ (ppm)	Retention Time(s) (±0.1 min)	Figure	Comments
dimer-K^+	$[C_{30}H_{26}KO_{12}]^+$	617.1053	-0.5	4.2, 5.0, 7.6, 9.4, 10.3	**SI1**	eluting at most of the EIC r.t. of the main dimer-H^+ isomeric species
dimer-Na^+	$[C_{30}H_{26}NaO_{12}]^+$	601.1328	2.0	7.8, 10.6, 16.3, 23.6, 23.8	**SI10**	eluting at most of the EIC r.t. of the main dimer-H^+ isomeric species.
trimer-K^+	$[C_{45}H_{38}KO_{18}]^+$	905.1683	0.7	3.3	**SI2**	eluting only at the EIC r.t. of the more polar isomeric trimer-H^+ species
trimer-Na^+	$[C_{45}H_{38}NaO_{18}]^+$	889.1967	1.9	1.6, 23.8	**SI10**	eluting mostly at the latest EIC r.t. of the main isomeric dimer-H^+ species
l-tetramer-K^+	$[C_{60}H_{50}KO_{24}]^+$	1193.2338	1.2	1.0, 3.3, 3.9, 4.4, 7.4, 8.6, 9.4	**SI3**	only weak traces eluting at much anticipated r.t. than the correspondent main isomeric l-tetramer-H^+ species
l-tetramer-Na^+	$[C_{60}H_{50}NaO_{24}]^+$	1177.2593	0.8	9.4, 11.4, 23.7	**SI10**	eluting at some of the EIC r.t. of the main dimer-H^+ species and at 23.7 min as all the other Na^+ adducts
l-tetramer-1-galloc-Ca^{2+}	$[C_{60}H_{50}CaO_{25}]^{2+}$	605.1134 (z = +2)	1	3.9 4.3, 10.2	**SI4**	not all isobaric peaks are present in all the samples
c-tetramer-K^+	$[C_{60}H_{48}KO_{24}]^+$	1191.2160	-0.6	3.7	**SI5**	eluting at the same r.t. of the correspondent main c-tetramer-H^+ species. Absence of higher r.t. traces (associated to the a-type tetramer)
c-tetramer-Ca^{2+}	$[C_{60}H_{48}CaO_{24}]^{2+}$	596.1072 (z = +2)	-0.5	1.0,1.7, 6.4, 9.4, 11.0	**SI6**	much wider distribution of r.t. than the corresponding c-tetramer-H^+ species
c-tetramer-1-galloc-K^+	$[C_{60}H_{48}KO_{25}]^+$	1207.2111	-0.4	2.5	**SI7**	eluting at the same r.t of the correspondent c-tetramer-1-galloc-H^+
c-tetramer-1-galloc-Ca^{2+}	$[C_{60}H_{48}CaO_{25}]^{2+}$	604.1064 (z = +2)	1.8	2.8, 3.9, 4.3, 5.3, 14.8	**SI8**	traces

Table 3 ANOVA (for all observations): Means for variable Variety. All and only the significant variable are shown.

Species	Index used in tables and charts	Pr > F
l-Dimer $+H^+$	579	0.000
l-Dimer $+K^+$	617	0.001
l-Trimer $+H^+$	867	0.000
l-Trimer $+K^+$	905	0.000
l-Trimer $+Na^+$	889	0.002
l-Tetramer $+H^+$	1155	0.000
l-Tetramer $+K^+$	1193	0.010
l-Tetramer $+Na^+$	1177	0.003
c-Tetramer $+H^+$	1153	0.000
c-Tetramer $+K^+$	1191	0.026
l-Pentamer $+H^+$	1443	0.000
l-Pentamer $+K^+$	1481	
l-Pentamer $+Ca^{2+}$	741	0.000
c-Pentamer $+H^+$	1441	0.000
l-Hexamer $+H^+$	1731	0.000
l-Hexamer $+K^+$	1769	
c-Hexamer $+Ca^{2+}$	884	
c-Hexamer $+H^+$	1729	0.000
c-Hexamer $+K^+$	1767	
l-Tetramer-1-OH $+H^+$	1171	0.000
c-Tetramer-1-OH $+H^+$	1169	0.000
c-Tetramer-1-OH $+K^+$	1207	0.000
c-Tetramer-1-OH $+Ca^{2+}$	604	0.034
l-Pentamer-1-OH $+H^+$	1459	0.000
l-Pentamer-1-OH $+Ca^{2+}$	749	0.000
c-Pentamer-1-OH $+H^+$	1457	0.000
c-Pentamer-1-OH $+K^+$	1495	0.005
c-Pentamer-1-OH $+Ca^{2+}$	748	0.028