NOVEL CYCLIC PROANTHOCYANIDINS IN WINES FROM SOUTH TYROL: DEPENDANCE ON THE GRAPE VARIETY

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Introduction

Condensed tannins (proanthocyanidins, PAC) are secondary metabolites associated to the astringent taste in wine. PAC are made of condensed flavan-3-ol monomers, with variable monomers number and type. A large variety of these substituted flavan-3-ol monomers has been identified in proanthocyanidins. For example, galloylated flavanols such as (epi)catechin gallate (Tsai Su & Singleton, 1969, Lee & Jaworski, 1987) were identified in grape seeds, as well as the more abundant (epi)catechin and (epi)gallocatechin. Prodelphinidins are a class of proanthocyanidins observed in the grape berries skin containing (epi)catechin and (epi)gallocatechin constituents (Chira, Schmauch, Saucier, Fabre, & Teissedre, 2009). Despite the wide literature related to such oligo- and polymeric compounds, the variety of structures is wide and not completely unravelled.
A novel crown (macrocyclic) B-type tetrameric procyanidin in wines from the Bordeaux region (France) was recently identified and its structure has been determined by NMR. Moreover, this crown procyanidin tetramer appeared to be specifically localized in grape skin (Jouin, Rossetti, Teissèdre, & Jourdes, 2017; Jourdes et al., 2016; Zeng, Pons-Mercadé, Richard, Krisa, Teissedre, & Jourdes, M. 2019). More recently, an approach based on H/D isotope exchange coupled to HPLC-MS/MS was employed to confirm the structure of this crown tetrameric procyanidin by discriminating it from its isomeric A-type procyanidin analogue (Longo, Rossetti, Scampicchio, & Boselli, 2018). Two other crown congeners (a pentamer and a hexamer cyclic procyanidin) (hexamer in Figure 1) were also proposed.

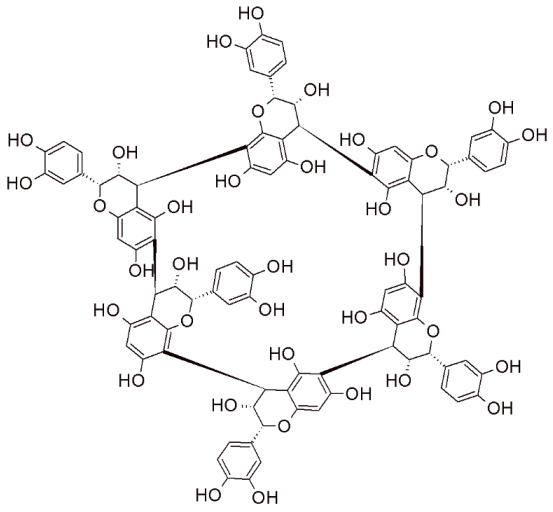

Figure 1. Structure attributed to the cyclic hexamer procyanidin identified by H/D exchange HPLC-HRMS. The stereogenic centres' configurations and the inter-flavanol linkage preferences are shown just for illustration, in analogy with the fully resolved structure of the crown procyanidin tetramer (Zeng et al., 2019), however they must not be regarded as resolved for the hexamer.

However, in the cited work only procyanidins (hence, with flavan-3-ols (+)-catechin and (−)-epicatechin constituent monomer units) were observed. Nonetheless, the list of cyclic proanthocyanidins was suspected to be much larger than the one initially investigated. In fact, beside (+)-catechin and (−)-epicatechin, many more possible monomer units with different substitutions should be considered for building up the wide class of crown proanthocyanidins. Therefore, our aim was to investigate the presence in wine of possible natural macrocyclic oligomers containing other monomer units as with their non-cyclic analogues, exploiting high resolution tandem mass spectrometry (HRMS/MS) and H/D exchange for confirmation (Longo et al., 2018a). Two prodelphinidins identified and confirmed by HRMS/MS and by H/D exchange are presented. Furthermore, the distribution of these compounds in wines obtained from different grape varieties is discussed. More details are reported in a previously published paper by the same research group (Longo et al., 2018b*).*

Experimental section

Material

Solvents and pure reagents (LC-MS grade) were obtained from Sigma-Aldrich Ltd, as well as deuterium oxide (D_2O) (99.9% D). White and red wines are the same as those reported in Longo et al. (2018a). They were donated by a local cooperative winery (Kellerei Bozen, BZ, Italy) and a local agricultural high school (Happacherhof, Auer/Ora, BZ, Italy). All wines were PDO/DOC grade from the South Tyrol region. The wines are listed in Table 1.

WINE NAME	ABBREVIATION
Lagrein	L
Lagrein Prestige Klebelsberg	LP
Lagrein Eyrl	LE
Lagrein Grieser (Collection I)	LG-1
Lagrein Grieser (Collection 2)	LG-2
Cabernet Franc	CF
Cabernet Sauvignon	CS
Merlot (collection)	MC
Merlot (barrique)	MB
Blauburgunder	BB
Blauburgunder	BB-rep
St.Magdalener Moar	SMM
St.Magdalener Classico Huck-I	SMH-1
St.Magdalener Classico Huck-II	SMH-2
Gewürztraminer Kleinstein	GK
Gewürztraminer	G
Gewürztraminer passito	GP
Sauvignon Blanc	SB-1
Sauvignon Blanc	SB-2
Chardonnay passito	Cp

Table 1. List of wines (vintage 2016) studied. Blauburgunder is the German name of Pinot noir.

Samples preparation

The samples were prepared according to a previous reported methodology (Longo et al., 2018a). Briefly, 10 mL of wine were concentrated at 30°C under vacuum followed by 30 min of gentle N_2 flux, then they were recovered in 1 mL of HPLC solvent A (see below). For MS/MS qualitative studies, the samples were concentrated 30-fold. For H/D isotopic exchange analysis, the deuterated solvent A was used instead of normal solvent A (see below) after several washing and evaporation steps with deuterated solvent B to ensure a complete removal of non-deuterated polar residues. All samples were filtered (0.2 µm, regenerate cellulose) prior to the HPLC analysis.

Instrumental method

The HPLC-HRMS/MS method applied was adapted from published reports (Longo et al., 2018a,b). The HPLC-HRMS system consisted of a Q Exactive HRMS instrument (Thermo Fisher Scientific, Rodano, Milano, Italy) coupled to an Agilent 1260 HPLC (Agilent Technologies Italia S.p.A., Cernusco sul Naviglio, Milano, Italy). The separation was carried out with an ODS Hypersyl C18 LC column (125 mm × 4.6 mm i.d., 5 µm, Thermo Sci.) protected with a HPLC pre-column filter (ODS Hypersil, 5 µm pore size, 10 x 4 mm drop-in guards, Thermo Fisher Scientific). The HPLC flow rate was 1 mL min^{-1}. The mobile phase consisted of solvent A (0.1% v/v formic acid in 0.02 mol L^{-1} ammonium formate in water or 0.1% v/v deuterated formic acid in 0.02 mol L^{-1} fully deuterated ammonium formate in D_2O) and B (0.1% v/v formic acid in saturated ammonium formate acetonitrile or 0.1% v/v deuterated formic acid in fully deuterated saturated ammonium formate acetonitrile. The data (only of the non-re-concentrated wine samples) were collected and analyzed by by Xcalibur 3.1 software.

Statistical analysis

XLStat (Addinsoft, Paris, France) and The Unscrambler (CAMO Software AS., Oslo, Norway) were the software packages employed for the statistical analysis.

Results

Identification of crown prodelphinidins

The list of the identified PAC species is reported in Table 2. The analysis aimed mainly at identifying all those PAC with a molecular weight ranging between the dimeric and the hexameric PAC. The reported compounds were identified in a Lagrein wine. No PAC with a number of momomeric units exceeding six has been found in wine so far.

PAC species (*)	detected EIC peaks	found ion (*m/z*)	av. Δ m/z (ppm)	related deuterated ion (*m/z*)	retention times in H_2O (±0.1 min)	retention times in D_2O (±0.1 min) (***)	MS/MS fragments {H_2O}	R.t. of MS/MS (±0.1 min)
c-tetramer (****)	1	1153.2604	-0.3	1174.3909	3.7	5.0	**1153.259**, 1001.213, 865.197, 695.140, 577.134, 451.102, 409.091, 289.070, 247.060	3.7
c-tetramer-1-galloc	1	1169.2546	-0.9	1191.3935	2.5	3.4	**1169.255**, 1153.557, 881.192, 713.146, 699.867, 669.031, 633.061, 575.116, 476.465, 425.086, 409.092, 407.076, 325.112, 289.070, 247.060	2.5
c-pentamer	1	1441.3213	-2.0	1467.4863	4.3	5.8	**1441.317**, 1423.306, 1289.270, 1271.260, 1263.249, 119.214, 1083.193, 967.167, 695.136, 577.132, 409.090, 407.074, 289.070, 247.060	4.3
c-pentamer-1-galloc	1	1457.3181	-0.7	1484.4891	2.8	4.0	**1457.315**, 1439.304, 1305.271, 1289.271, 1117.197, 947.173, 849.702, 577.134, 449.087, 425.087, 408.070, 407.076, 305.203, 289.071, 287.055, 271.060, 247.060	2.8
c-hexamer (****)	1	1729.3870	-0.2	*NA*	10.0	*NA*	**1729.381**, 1711.365, 1577.334, 1559.325, 1441.316, 1289.269, 1119.211, 865.197, 695.136, 577.132, 559.123, 517.111, 409.090, 331.080, 289.070, 247.060	10.0

Table 2. List of identified cyclic PAC species. (*) c = cyclic B-type; "tetramer", "pentamer" and "hexamer" terms indicate the number of monomer units; 1-galloc = one (epi)gallocatechin unit in the molecule. (**) arbitrarily taken at the highest peak of all isobaric TIC (MS/MS filter) peaks. (***)

The presented list includes tetrameric, pentameric and hexameric *crown procyanidin*. A more extended list was analysed in a previous publication (Longo et al., 2018b). Previously identified procyanidins were discussed in an early study (Longo et al., 2018a). Two prodelphinidins were observed and confirmed by H/D exchange and MS/MS analysis. According to their molecular masses (Table 2), these are respectively a cyclic B-type tetrameric prodelphinidins formed by one (epi)gallocatechin and three (epi)catechin units and a cyclic B-type pentameric prodelphinidin consisting in one (epi)gallocatechin unit and four (epi)catechin units, respectively. Their HRMS extracted ion chromatograms with water (EIC{H}), their measured MS/MS spectra and HRMS extracted ion chromatograms with deuterated solvent (EIC{D}) are shown in Figure 2 (for the tetramer) and 3 (for the pentamer), respectively.

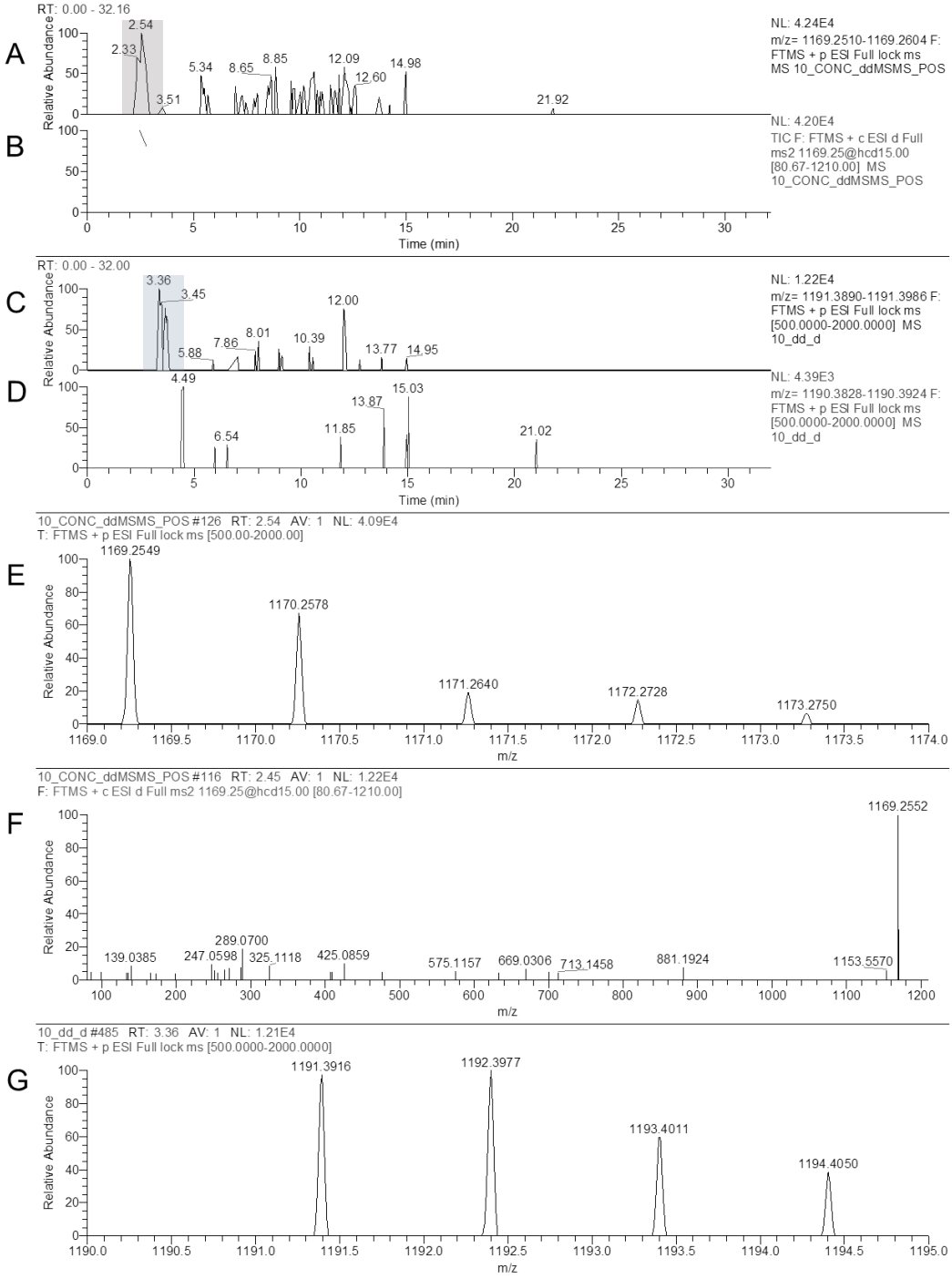

Figure 2. c-tetramer-1-galloc (R.t. = 2.5 min). A) EIC{H}; B) TIC{H} of performed MS/MS analysis; C) EIC{D}; D) EIC{D} of 1190.3876 m/z (corresponding to the isomeric a-tetramer-1-galloc); E) HRMS(H)

at 2.5 min; F) MS/MS(H) spectrum at 2.5 min; G) HRMS(D) at 3.4 min. The parts highlighted in green show the peak associated with the identified cyclic oligomers.

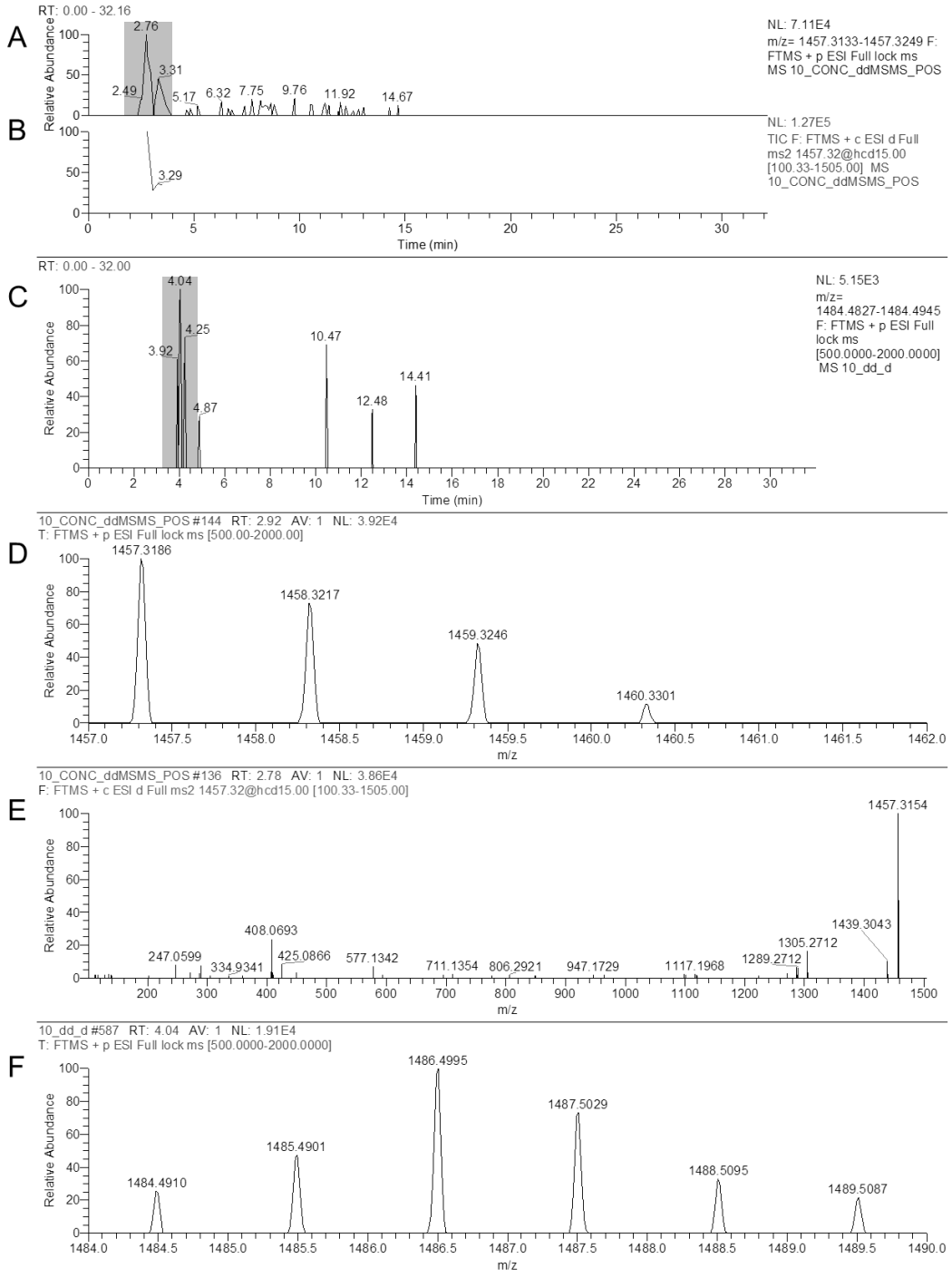

Figure 3. c-pentamer-1-galloc (R.t. = 2.8 min). A) EIC(H); B) TIC(H) of MS/MS; C) EIC(D); D) HRMS(H) at 2.9 min; E) MS/MS(H) spectrum at 2.8 min; F) HRMS(D) at 4.0 min. The parts highlighted in green show the peak associated with the identified cyclic oligomers.

Regarding the MS/MS spectra (**2E** and **3E**), the fragmentation pattern for cyclic prodelphinidins were analogous to those seen with procyanidins: cyclic analogues show the tendency of fragmenting less than their linear analogues at low collision energies (Longo et al., 2018a). As mentioned in previous reports (Zeng, Pons-Mercadé, Richard, Krisa, Teissedre, & Jourdes, M. 2019; Longo et al., 2018a,b), the increased resistance of the cyclic backbone to acidic depolimerizazion and collision-induced fragmentation (see MS/MS spectra in **2E** and **3E**) of these novel PAC is a peculiar feature that distinguished all the identified cyclic B-type PAC

from the non-cyclic ones (B- or A- type alike). Besides, the number of H/D exchanged hydroxylic protons corresponded to the predicted numbers for these two species (**2F** and **3F**). The same HRMS{D} peaks may also arise from interference from the non-cyclic A-type analogue (displaying a theoretical -1 Da than the cyclic B-type); in **2D** the contribution from the non-cyclic A-type c-tetramer-1-galloc prodelphinidins was investigated, showing no overlaps, hence no contributions to the peak outlined from its A-type analogue.

Distributions of crown prodelphinidins in wine

In order to obtain a fingerprint of the crown prodelphinidins in different red and white wines, all values were previously normalized applying the following equation.

$$RATIO = 100\% * \frac{[cyclic]}{[cyclic] + [non-cyclic]} = 100\% * \frac{[cyclic]}{[total]}$$

The reported ratio is based on ratios of relative abundances recorded for the cyclic ([cyclic]) and the sum of the cyclic and non-cyclic ([non-cyclic]) considering two analogues with the same number of monomers and monomer composition. Using this normalization, the percentage of cyclic compounds was reported, rather than their relative abundances which were much smaller than those of their non-cyclic analogues.

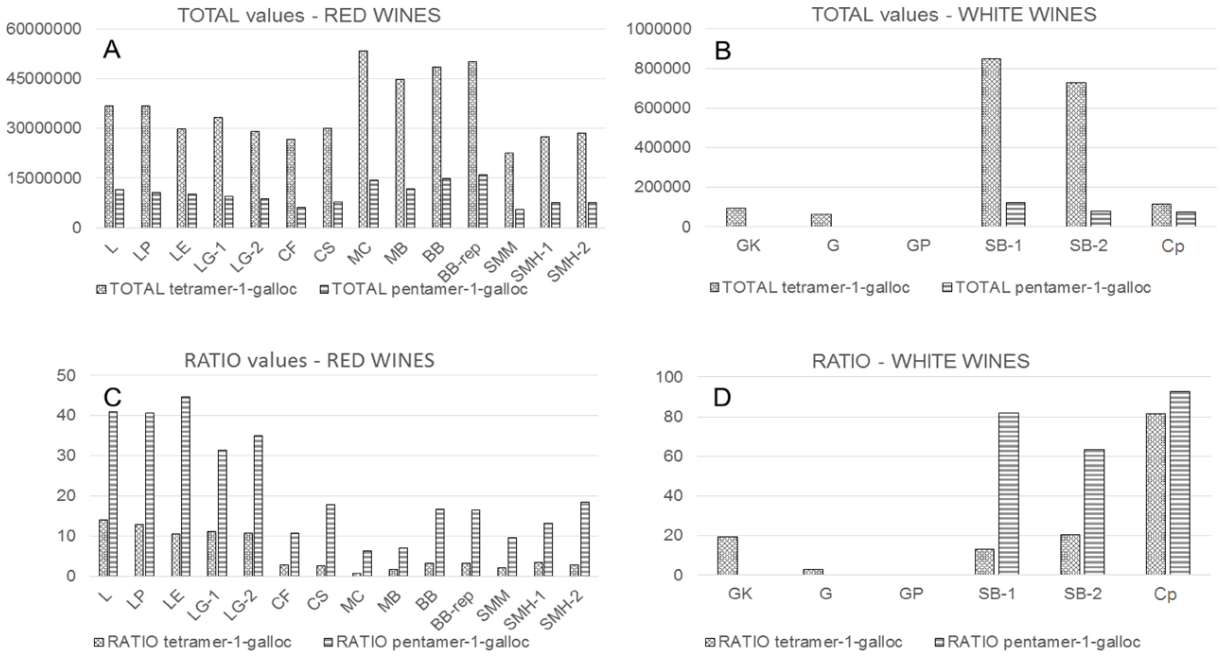

*Figure 4. Upper layer: sums of the relative abundances for cyclic and non-cyclic tetramer-1-galloc, and for cyclic and non-cyclic pentamer-1-galloc in **A**) red wines, **B**) white wines. Lower layer: ratios (see Equation) of relative abundances for c-tetramer-1-galloc over all tetramer-1-galloc, and for c-pentamer-1-galloc over all pentamer-1-galloc in **C**) red wines, **D**) white wines. Legend: L = Lagrein, LP = Lagrein Prestige, LE = Lagrein Eyrl, LG = Lagrein Grieser, CF = Cabernet Franc, CS = Cabernet Sauvignon, MC = Merlot collection, MB = Merlot barrique, BB = Blauburgunder (Pinot noir), SMM = St.Magdalener Moar, SMH-1 = St.Magdalener Huck-1, SMH-2 = St.Magdalener Huck-2, GK = Gewürztraminer Kleinstein, G = Gewürztraminer, GP = Gewürztraminer Passito, SB-1 = Sauvignon blanc-1, SB-2 = Sauvignon blanc-2, Cp = Chardonnay Passito.*

The most striking difference was observed in white varieties. Gewürztraminer showed no presence of cyclic pentamer prodelphinidins, whereas almost all pentamer prodelphinidins in the analysed Chardonnay were cyclic. Sauvignon blanc displayed ratios analogous to some red wines. Among red wines, the highest percentage of cyclic congeners was observed in Lagrein although Merlot and Pinot noir (Blauburgunder) had the highest total values.

Preliminarily, an analysis of variance (ANOVA) with Tukey's HSD tests was applied on the shown variables and on the total values. The results are summarized in Table 3.

	TOTAL tetramer-1-galloc	RATIO tetramer-1-galloc	TOTAL pentamer-1-galloc	RATIO pentamer-1-galloc
LAGREIN	33126724.713 b b	11.823 b bc	10141947.661 bc b	38.500 b c
PINOT NOIR	49270093.073 a a	3.262 b cd	15504135.469 a a	16.593 c d
CHARDONNAY	113344.286 c d	81.317 a a	74718.368 d d	92.910 a a
SAUVIGNON B.	788783.578 c d	16.871 b b	101364.996 d d	72.602 a b
CABERNET S.	30026845.625 b bc	2.647 b cd	7757045.407 c bc	17.829 bc d
MERLOT	48992501.651 a a	1.149 b d	13146379.475 ab a	6.624 c de
CABERNET F.	26692655.576 b bc	2.834 b cd	6245092.821 c c	10.754 c de
StMAGDALENER	26208874.417 b c	2.738 b cd	7007778.685 c c	13.767 c d
G.TRAMINER	52835.320 c d	7.382 b bcd	0.000 d d	0.000 c e
Pr > F	0.000	0.000	0.000	0.000
Significant	Yes	Yes	Yes	Yes

Table 3. Grouping obtained by analysis of variance on the variables' dataset. Letters displayed in italic shows the grouping on the basis on the chosen set of variables.

The four variables combined differentiated among all varieties, although not all Tukey's HSD tests between two pairs of variables identified significant differences when just including these two variables.

Conclusions

Two unconventional crown (cyclic) prodelphinidins were identified in wine by high resolution mass spectrometry, fragmentation experiments and isotopic exchange. They are a tetrameric and a pentameric cyclic congener both containing only one (epi)gallocatechin unit whose distribution was investigated in nineteen wines from South Tyrol, mostly produced with international grape varieties. The statistical analysis indicated that the profile of cyclic proanthocyanidins were related to the grape variety used to compose the investigated wines. Moreover, the number of cyclic proanthocyanidins identified so far is presumably only a small part of those naturally occurring, due to their structural complexity and variability. This can be an important starting point for future studies on the development of new wine quality markers based on the profile of these recently identified flavonoids.

Acknowledgement

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