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ORIGINAL ARTICLE

Coupling between high-resolution mass spectrometry and focalized data-analysis methods provides the identification of new putative glycosidic non-anthocyanic flavonoids in grape

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Abstract

Introduction The biochemical diversity of flavonoids is based on glycosylation, methylation, acylation, and many other modifications of the flavonoid backbone. Liquid chromatography coupled to high-resolution mass spectrometry demonstrated to be a powerful approach to gain new insights into the flavonoid composition of many plant species, including grapes.

Objectives Among different metabolomic approaches, suspect screening analysis relies on the construction of a specific database and on ultra-high performance liquid chromatography/quadrupole time-of-flight (UHPLC/QTOF) analysis to find new compounds of oenological interest.

Methods A homemade database containing mass data information retrieved from the literature specific for plant flavonoid derivatives (*GrapeFlavMet*) was constructed. Tandem mass spectrometry analysis of *V. vinifera* and hybrid grape extracts was performed, and MS/MS fragmentation allowed to assign the putative flavonoid chemical structure to various identification levels, as established by the Metabolomics Standard Initiative.

Results By this approach, putative flavonoid derivatives with different glycosylation and acylation patterns were identified. They include three pentoside derivatives of tetrahydroxy-flavone, tetrahydroxy-flavanone and myricetin isomers, a putative dihydrorhamnetin hexoside derivative, three cinchonain isomers (phenylpropanoid-substituted flavan-3-ols with antidiabetic properties), and two syringetin isomer derivatives (acetyl- and *p*-coumaroyl-hexoside). Two acetyl-hexoside derivatives of dihydrorhamnetin and pentahydroxy-methoxy-flavanone, and three derivatives of tetrahydroxy-dimethoxy-flavanone (acetyl, *p*-coumaroyl, and caffeoyl-hexoside) were tentatively annotated.

Conclusions Most of the compounds were identified in grape for the first time, while two putative syringetin derivatives previously proposed in the literature were confirmed. These findings deepen the current knowledge on grape flavonoids, suggesting more connections at the biochemical level.

Keywords Glycosidic flavonoids · Metabolomics · High-resolution mass spectrometry · QTOF · Grape

1 Introduction

Flavonoids are specialized plant metabolites with many biological functions. They act as plant signaling molecules, attract pollinators, protect against UV light, and perform as a defense against pathogens and herbivores (Winkel-Shirley, 2001). In grapes, they play a key role in terms of fruit quality and contribute to optical and organoleptic characteristics of wines, such as color intensity and stability, astringency, and wine aroma. The main classes of these secondary metabolites include anthocyanins, flavones and flavonols, flavanones and flavanonols, flavanols and proanthocyanidins (Flamini, 2003).

Flavonoids belong to the phenylpropanoid class, the third largest class of plant metabolites: with over 8000 metabolites they are characterized by great chemical diversity

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(Tohge et al., 2017). Although the aglycone biosynthesis tends to be conserved between species, glycosyltransferase, methyltransferase, and acyltransferase enzymes operate additional modifications, which are involved in the further decoration of the flavonoid backbone. This results in the large natural variety of flavonoid derivatives, commonly species-specific (Tohge et al., 2017, 2018). These modifications, known as tailoring reactions, are responsible for the final biological and physicochemical properties of flavonoid molecules accumulated in cells (Saito et al., 2013).

Which is the physiological role covered by these modifications remains in many cases unknown (Alseekh et al., 2020). In recent studies performed on the model plant *Arabidopsis* and Brassicaceae, UV-B stress experiments demonstrated the accumulation of phenyl acylated flavonoids, e.g., flavonols decorated with hydroxycinnamoyl units, suggesting a relationship between tolerance to enhanced irradiation conditions and specific decoration (Tohge et al., 2018; Alseekh et al., 2020).

New advances in chemical profiling by liquid chromatography coupled to high-resolution mass spectrometry (UHPLC-HRMS) techniques allow new insights into the flavonoid composition of many plant species, often revealing compounds described for the first time (Panighel et al., 2015; Piccolella et al., 2019; De Rosso et al., 2020). Suspect screening analysis is a high-resolution mass spectrometry (HRMS) metabolomic approach that relies on the structural information on the metabolites available in the literature (e.g., the molecular formula and structure) (Krauss et al., 2010). By construction of a specific enological database (*GrapeMetabolomics*) using the information found in the literature and performing ultra-high performance liquid chromatography/quadrupole time-of-flight (UHPLC/QTOF) analysis, this approach has increased the knowledge on grape metabolomics and the metabolites of oenological interest (Flamini et al., 2013a). Currently, *GrapeMetabolomics* includes more than 1.100 polar metabolites mainly belonging to the chemical classes of anthocyanins and pyranoanthocyanin derivatives, flavanols and proanthocyanidins, flavones and flavonols, flavanones, stilbenes, glycosidic grape aroma precursors (Flamini et al., 2015).

Soft ionization provided by coupling electrospray ionization (ESI) and LC/MS analysis produces stable ions of glycosidic flavonoids that are easily cleaved by MS/MS. Deciphering the MS/MS spectra by the study of fragmentation patterns and comparison with data reported in the literature and public databases allows for the putative assignment of chemical structures, or unequivocal assignment when the reference standard is available.

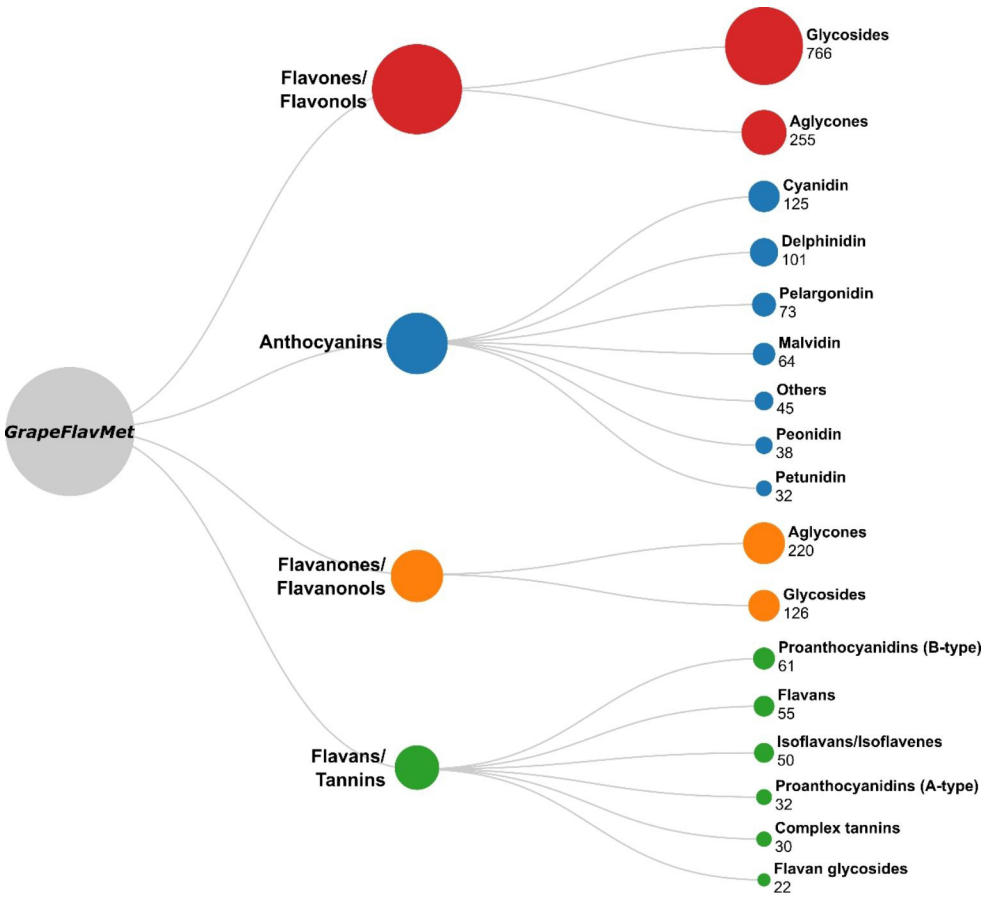

Fig. 1 Dendrogram representation of non-unique chemical formulas reported in *GrapeFlavMet* database. The number of formulas for each subclass is reported

By developing a targeted data analysis workflow many new grape compounds were identified: for example, some *p*-coumaroyl glycoside flavonols (Panighel et al., 2015), flavonoids such as dihydromyricetin-*O*-hexoside, taxifolin-di-*O*-hexoside, isorhamnetin, and a pinoquercetin isomer, the structures of some flavonoids before just proposed in grape were characterized (i.e., taxifolin-pentoside, tetrahydroxy flavanone hexoside, tetrahydroxy-dimethoxyflavanone hexoside, peonidin-*O*-pentoside) (De Rosso et al., 2020). By developing this approach also many glycoside terpenols and C13-norisoprenoids (grape aroma precursors), were characterized (Flamini et al., 2014; Godshaw et al., 2019; Caffrey et al., 2020; Song et al., 2020; Wei et al., 2021).

In this work, we applied this targeted metabolomics approach using a homemade database (*GrapeFlavMet*) constructed by using mass data reported in the specific literature of flavonoids diffused in the plant kingdom. Currently, *GrapeFlavMet* includes a total of 2095 flavonoid compounds divided into four main chemical classes: flavones/flavonols, flavanones/flavanonols, anthocyanins, flavans, and tannin derivatives (Fig. 1). The study aimed to uncover new flavonoid glycosides present in extracts by searching the putative structures in the HRMS profiles of both *V. vinifera* and hybrid grape varieties. New putative grape compounds were annotated at various identification (ID) levels according to the guidelines of the Metabolomics Standards Initiative (MSI) (Sumner et al., 2007; Schymanski et al., 2014) and findings are presented.

Finally, metadata associated with our experiment and MS/MS spectra were submitted to the open-source repository MetaboLights (Haug et al., 2020), allowing efficient sharing and reusability of mass spectra data (Savoi et al., 2021).

2 Methods and materials

Chemical standards, sample preparation methods, and UHPLC-QTOF conditions were the same described in the previous paper (De Rosso et al., 2020) and reported in detail in the protocol section of the open access study deposited in the Metabolights database with the identifier MTBLS4202. Briefly, twenty berries were homogenized with liquid nitrogen and extracted using pure methanol in ratio 2:1 (ml/g) under stirring for 20 minutes. The solution was added with 200 ml of internal standard (IS) 4',5,7-trihydroxy flavanone 500 mg/l solution and centrifuged at 10 °C for 20 min. The supernatant was filtered through Acrodisc GHP 0.22-µm filter (Waters) and collected in a vial.

Samples studied were Raboso Piave (Vitis International Variety Catalogue, VIVC, number 9864) withered grapes and the red hybrid grape varieties Clinton (*V. riparia* × *V. labrusca*; VIVC n. 2711), Seyve Villard 12–347 (Seibel 6468 × Seibel 6905; VIVC n. 11,587), Seyve Villard 12–390 (Seibel 6468 × Seibel 6905; VIVC n. 11,592), Seyve Villard 29–399 (VIVC n. 11,663), Seibel 19,881 (VIVC n. 11,461), and Seibel 8357 (Seibel 6150 × Seibel 5455; VIVC n. 2768). Grapes were harvested from CREA grapevine germplasm (Susegana, Treviso, Italy) in 2011, 2012, and 2013.

Analyses were performed using a UHPLC Agilent 1290 Infinity system coupled to Agilent 1290 Infinity Autosampler (G4226A) and Agilent 6540 accurate mass QTOF MS (nominal resolution 40.000) equipped with Dual Agilent Jet Stream Ionization source (Agilent Technologies, Santa Clara, CA). Chromatographic conditions: Zorbax reversed-phase column (RRHD SB-C18 3 × 150 mm, 1.8 µm) (Agilent Technologies, Santa Clara, CA), mobile phase composed by (a) 0.1% (v/v) aqueous formic acid and (b) 0.1% (v/v) formic acid in acetonitrile. Flow rate: 0.4 ml/min. Sample injection 10 µL; column temperature 35 °C. Analyses were performed in both positive and negative ionization modes by recording data in full scan acquisition using Agilent MassHunter version B.04.00 (B4033.2) software. MS/MS spectra were acquired in Data-Dependent Acquisition (DDA) mode using the Auto MS/MS mode of the instrument. Acquisition rate: 2 spectra/s. A maximum of two precursors in the *m/z* 60-1700 range were fragmented per cycle (1.6 s) using collision energies linearly variable between 20 and 70 eV.

2.1 Database construction

The in-house DB *GrapeFlavMet* containing flavonoid names and formulas was constructed as a spreadsheet in Excel (Excel 2019, Microsoft Office). Flavonoid derivatives were annotated mainly from the book "Flavonoids. Chemistry, Biochemistry and Applications" by Andersen & Markham (2005), which contains information on plant flavonoids updated until 2005. The idea is to update the flavonoid DB *GrapeFlavMet* with the most recent findings on flavonoid compounds retrieved from the latest literature, and to implement the original DB *GrapeMetabolomics* as new flavonoid derivatives are identified.

Compounds were annotated with their common names, when present (e.g., Almeidein), and with information regarding substitutions on the aglycone backbone and the glycosidic moiety (e.g., dihydroxy-methoxy-flavone C-dipentoside for Almeidein). Compounds with substituents in different positions or differing in stereochemistry were not distinguished but grouped under the same molecular formula. For each compound, molecular formula and monoisotopic mass of the neutral and the ion adducts ($[M+H]^+$ or $[M-H]^-$) were checked using the public databases PubChem (https://pubchem.ncbi.nlm.nih.gov/), KEGG (https://www.

genome.jp/kegg/), and Chemspider (https://www.chemspider.com/) and introduced in the DB.

GrapeFlavMet consists of 2095 flavonoid non-unique chemical formulas as a total, divided into four classes (Fig. 1). The largest class comprises 1061 flavones or flavonols, subdivided into aglycones (255) and glycosides (766), followed by 346 flavanones or flavanonols (220 aglycones and 126 glycosides). The class comprising 250 flavans and tannin derivatives includes monomeric flavans, glycosylated flavans, isoflavans and isoflavenes, dimeric and oligomeric proanthocyanidins A-type and B-type, and complex tannins (mostly non-proanthocyanidin derivatives and hydrolyzable gallo- or ellagi-tannins). For completeness and future analyses, 478 anthocyanin compounds were also reported in the DB, but they were not considered in the present study. Since anthocyanins in large part occur in grapes as glycosylated forms, they were subdivided by aglycone structure; the subclass "others" includes methylated, hydroxy, deoxy, and pyranoanthocyanins.

2.2 Data analysis workflow

The metabolomic workflow represented in Fig. 2 was used to identify new putative flavonoid glycosides in grapes. After MS data acquisition, features were extracted using the "*Find by Molecular Feature*" algorithm in Agilent MassHunter software. Then, the algorithm "*Find by Formula*" was used to perform a targeted search taking as input molecular formulas contained in two accurate mass libraries: *GrapeFlavMet*, as previously described, and *GrapeMetabolomics*, the first homemade database containing 1189 compounds (mostly putative) found in grape and wine extracts (Flamini et al., 2013a, 2015). To each match, a score based on accurate mass, isotope abundance pattern, and isotope spacing, was assigned.

Peaks identification was confirmed by performing two analytical repetitions of each sample according to criteria proposed for metabolomics. In our experience, because mass accuracy of some metabolites can be affected by a low signal intensity, a cut-off of i.d. score > 78% was applied to select the metabolites used for the following data analysis. Screening of compound masses and formulas in the two DBs produced a total of 269 matches: by using *GrapeMetabolomics* 101 possible compounds, corresponding to 52 unique formulas, were found; in *GrapeFlavMet* the targeted search produced 168 matches, corresponding to 88 unique formulas (Online Resource 1).

Each MS/MS spectrum was manually interpreted to verify the assignment of the proposed molecular structure to the fragmentation pattern. As a further confirmation, the putative structures and the MS/MS spectrum also underwent *in-silico* interpretation using the Molecular Structure Correlator (MSC) software of the instrument. The correlation score calculated for each candidate structure of the parent compound is reported in Online Resource 5.

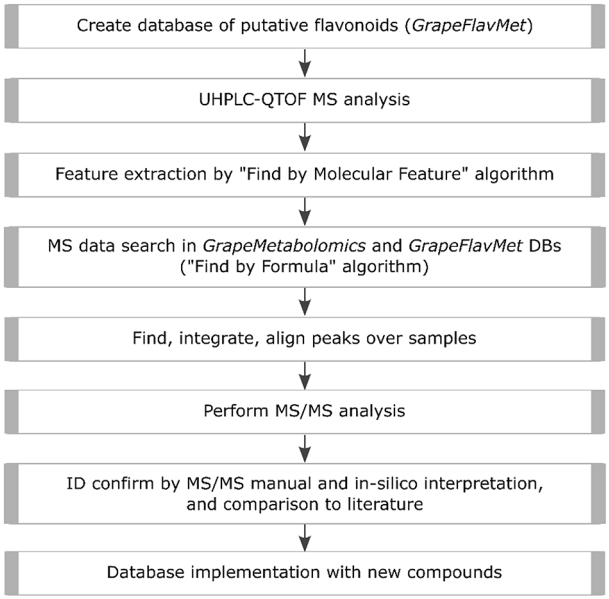

Fig. 2 Metabolomic workflow used for the identification of novel flavonoid glycosides in grape

Since the MS^2 analysis was performed in AutoMS mode, the MS/MS spectra were not available for all the compounds detected in MS1; or, in many cases, the MS/MS spectrum was not informative. Anyway, flavonoid compounds tentatively identified at lower ID levels (Sumner et al., 2007; Schymanski et al., 2014) are reported in Online Resource 1 as well. Compounds the which putative structure was supported with sufficient confidence by the MS/MS spectrum (ID level 2b) and by data reported in the literature (ID level 2a) were added to the native database *GrapeMetabolomics*. Elucidation of their fragmentation spectra is part of the present study.

3 Results and discussion

The flavonoids putatively identified belong to the chemical classes of flavones/flavonols and flavanones/flavanonols with different glycosylation and acylation patterns. The identification based on the study and manual interpretation of MS/MS spectra, along with other contextual information such as ionization mode, isotopic pattern, retention time, and parent compound information, allowed assignment of most compounds to putative ID levels 2 and 3.

The compounds identified at ID level 2a or 2b are three pentoside derivatives of tetrahydroxy-flavone, tetrahydroxy-flavanone and a myricetin isomer, dihydrorhamnetin

hexoside, two syringetin isomer derivatives, acetyl- and *p*-coumaroyl-hexoside, and three isomers of cinchonain, an interesting compound belonging to the class of flavonolignans here reported in grape for the first time. In the case of tetrahydroxy-flavone pentoside, a match was found with the MS/MS spectra reported in the Mass Bank of North America (MoNA, ucdavis.edu). The spectra were registered using similar analytical conditions (Spectrum VF-NPL-QTOF008178k or Spectrum VF-NPL-QTOF008179) and allowed to identify the compound as kaempferol-3-α-L-arabinopyranoside by increasing the identification level to 2a. In the case of cinchonain, the MS/MS fragmentation pattern was compared to that reported in previous studies performed in negative ionization mode (Zhang et al., 2016; Brahmi-Chendouh et al., 2019).

Mainly belonging to the flavanone/flavanonol chemical class, the compounds tentatively annotated at ID level 3 comprise two acetyl-hexoside derivatives of dihydrorhamnetin and pentahydroxy-methoxy-flavanone, and three tetrahydroxy-dimethoxy-flavanone derivatives, acetyl-, *p*-coumaroyl, and caffeoyl-hexoside.

MS/MS fragmentations of the putative compounds identified in the present study are reported in and the Online Resources 1, 4, and 5, and the identification of the fragments is discussed in Online Resource 2.

Pentoside moiety was found in grapes bound to small polyphenols (Barnaba et al., 2017) and anthocyanins (Mazzuca et al., 2005; Picariello et al., 2012; Wojdyło et al., 2018; Pérez-Navarro et al., 2019; De Rosso et al., 2020). Glycoside flavonols usually occur in grape as galactosides, rhamnosides, rutinosides, and glucuronides (Flamini et al., 2013b; Castillo-Muñoz et al., 2009, 2010), but recently quercetin pentoside has been found in skins extract of *V. vinifera* Sercial and Tinta Negra grapes (Perestrelo et al., 2012; Hilbert et al., 2015) coupled LC-MS and LC-NMR to investigate *V. vinifera* and wild grape varieties: in wild *Vitis* species, they found the presence of quercetin pentoside, along with the rhamnoside and diglucoside derivatives, and

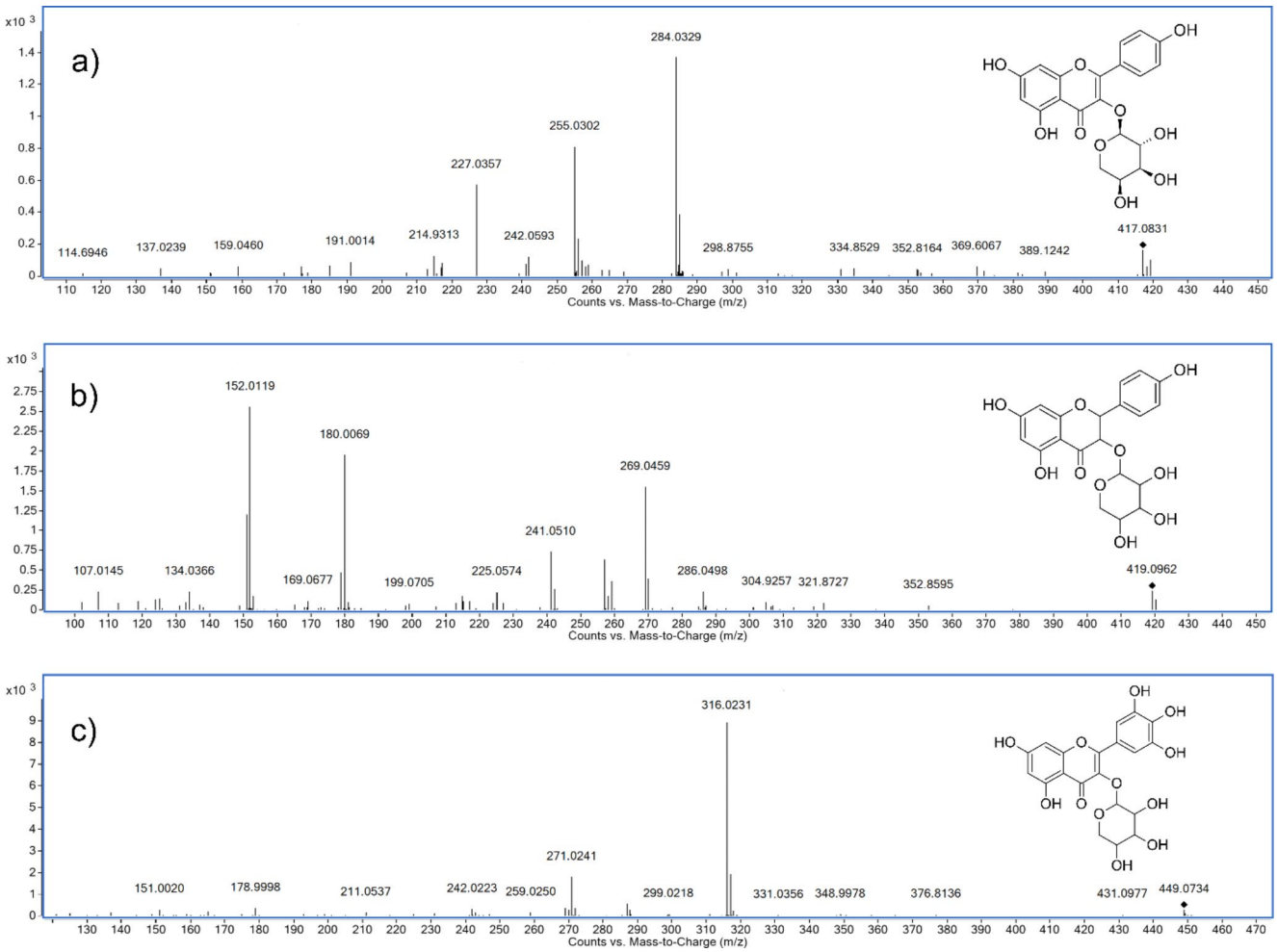

Fig. 3 MS/MS spectra of pentoside flavonoids identified in grape: (a) kaempferol-3-α-a-L-arabinopyranoside; (b) tetrahydroxy-flavanone pentoside; (c) hexahydroxy-flavone (putative myricetin) pentoside

hypothesized that, like for anthocyanins (Mazzuca et al., 2005), the presence of these unusual glycoside derivatives is a phenotypic character typical of hybrid grape varieties, useful for their chemotaxonomic differentiation.

In the present study, three pentoside flavonoids were identified: kaempferol pentoside, tetrahydroxy-flavanone pentoside, and hexahydroxy-flavone (putative myricetin) pentoside. The MS/MS spectra are shown in Fig. 3. In our study, the signals of kaempferol pentoside were found only in the hybrid grape variety Seyve Villard 12–347, tetrahydroxy-flavanone pentoside in *V. vinifera* grape Raboso, and hexahydroxy-flavone (putative myricetin) pentoside was found in the two hybrid grape varieties S. Villard 12–347 and Seibel 19,881 (Table 1).

The presence of tetrahydroxyflavanone *O*-deoxyhexoside, *O*-hexoside, and *C*-hexoside derivatives in *V. vinifera* withered grapes was previously proposed (Toffali et al., 2011) and the structures of two tetrahydroxy-flavanone hexoside isomers were recently characterized by HR-MS/MS (De Rosso et al., 2020). Myricetin-7-*O*-pentoside was recently identified in the skins of the *V. vinifera* variety Vranac (Šuković et al., 2020).

Search in *GrapeFlavMet* of the compound eluting at 13.2 min ($[M-H]^-$ precursor ion at *m/z* 479.1195) identified putative tetrahydroxy-methoxy-flavanone (dihydrorhamnetin) hexoside. The mass spectrum of this compound is shown in Fig. 4a, and the fragmentation pathway is proposed in Online Resource 3 (a).

The chemical formula $C_{24}H_{20}O_9$ identified in *GrapeFlavMet*, corresponding to the precursor ion $[M-H]^-$ at *m/z* 451.1034, was putatively assigned to cinchonain. The extract ion chromatogram of this precursor ion showed three different peaks at the retention times 15.8, 16.4, and 16.7 min, and all compounds showed an MS/MS spectrum similar that reported in Fig. 4b. Possibly, they correspond to three of the four putative cinchonain isomers shown in Online Resource 6. Previous studies (Zhang et al., 2016; Brahmi-Chendouh et al., 2019) performed in negative ion mode reported for cinchonain the same MS/MS fragmentation pattern we observed and proposed in Online Resource 3 (b). Cinchonains are phenylpropanoid-substituted flavan-3-ols or flavonolignans found in different plant species (Nonaka & Nishioka, 1982a; Nonaka et al., 1982b; Li et al., 2013). Numerous studies reported the bioactive properties of cinchonain and its derivatives. For example, extracts of the Japanese tree medlar (*Eriobotrya japonica*) leaves have been traditionally used as a treatment for diabetes mellitus type 2; the whole extract and the fraction containing only cinchonain Ib increased insulin level *in vitro* and *in vivo* (normal rats), demonstrating the anti-hyperglycemic-induced effect (Qa'dan et al., 2009; Patel et al., 2012). A mixture of cinchonain Ia and Ib isolated from the bark of *Trichilia catigua*, a medicinal plant widely distributed in Brazil, showed antibacterial activity against *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* strains (Pizzolatti et al., 2002). Extracts from the same plant demonstrated that cinchonain monomers had antioxidant activity similar to procyanidin B2 (Resende et al., 2011).

Recently, the presence of cinchonain was reported in grapevine leaves of hybrid varieties (Rizzato, 2018) but never observed before in grape berries. In addition, we

Table 1 Putative flavonoids identified in various grape varieties. Grape metabolite contents in withered Raboso Piave grape extract were estimated as mg IS/Kg on the $[M-H]^-$ ion signals; IS: Internal Standard

putative metabolite	mg IS/Kg grape	grape varieties in which the metabolite was identified
Kaempferol-3-α-L-arabinoside	0.010	non-*Vitis vinifera* (Seyve Villard 12–347)
Tetrahydroxy-flavanone pentoside	0.014	*V. vinifera* (Raboso)
Hexahydroxy-flavone (myricetin) pentoside	0.008	non-*Vitis vinifera* (S. Villard 12–347/Seibel 19,881)
Cinchonain isomers	0.044	*V. vinifera* (Raboso) non-*Vitis vinifera* (Seibel 19,881/S. Villard 29–399/ Clinton/S.Villard 12–347/S. Villard 12–390/Seibel 8357)
Tetrahydroxy-methoxy-flavanone (dihydrorhamnetin) hexoside	3.299	*V. vinifera* (Raboso) non-*Vitis vinifera* (Seibel 19,881/S. Villard 12–347/Seibel 8357/Clinton)
Tetrahydroxy-methoxy-flavanone (dihydrorhamnetin) acetyl-hexoside	0.107	*V. vinifera* (Raboso) non-*Vitis vinifera* (Seibel 8357)
Pentahydroxy-methoxy-flavanone acetyl-hexoside	0.069	*V. vinifera* (Raboso) non-*Vitis vinifera* (Seibel 8357)
Tetrahydroxy-dimethoxy-flavone (syringetin) acetyl-hexoside	0.007	non-*Vitis vinifera* (Seibel 8357)
Tetrahydroxy-dimethoxy-flavanone acetyl hexoside	0.602	*V. vinifera* (Raboso) non-*Vitis vinifera* (Seibel 8357/ Seibel 19,881)
Tetrahydroxy-dimethoxy-flavone (syringetin) *p*-coumaroyl-hexoside	0.013	non-*Vitis vinifera* (Seibel 19,881/Seibel 8357/S. Villard 12–347)
Tetrahydroxy-dimethoxy-flavanone *p*-coumaroyl-hexoside	0.589	*V. vinifera* (Raboso) non-*Vitis vinifera* (Seibel 19,881/S. Villard 12–347)
Tetrahydroxy-dimethoxy-flavanone caffeoyl-hexoside	0.007	non-*Vitis vinifera* (Seibel 8357/Seibel 19,881)

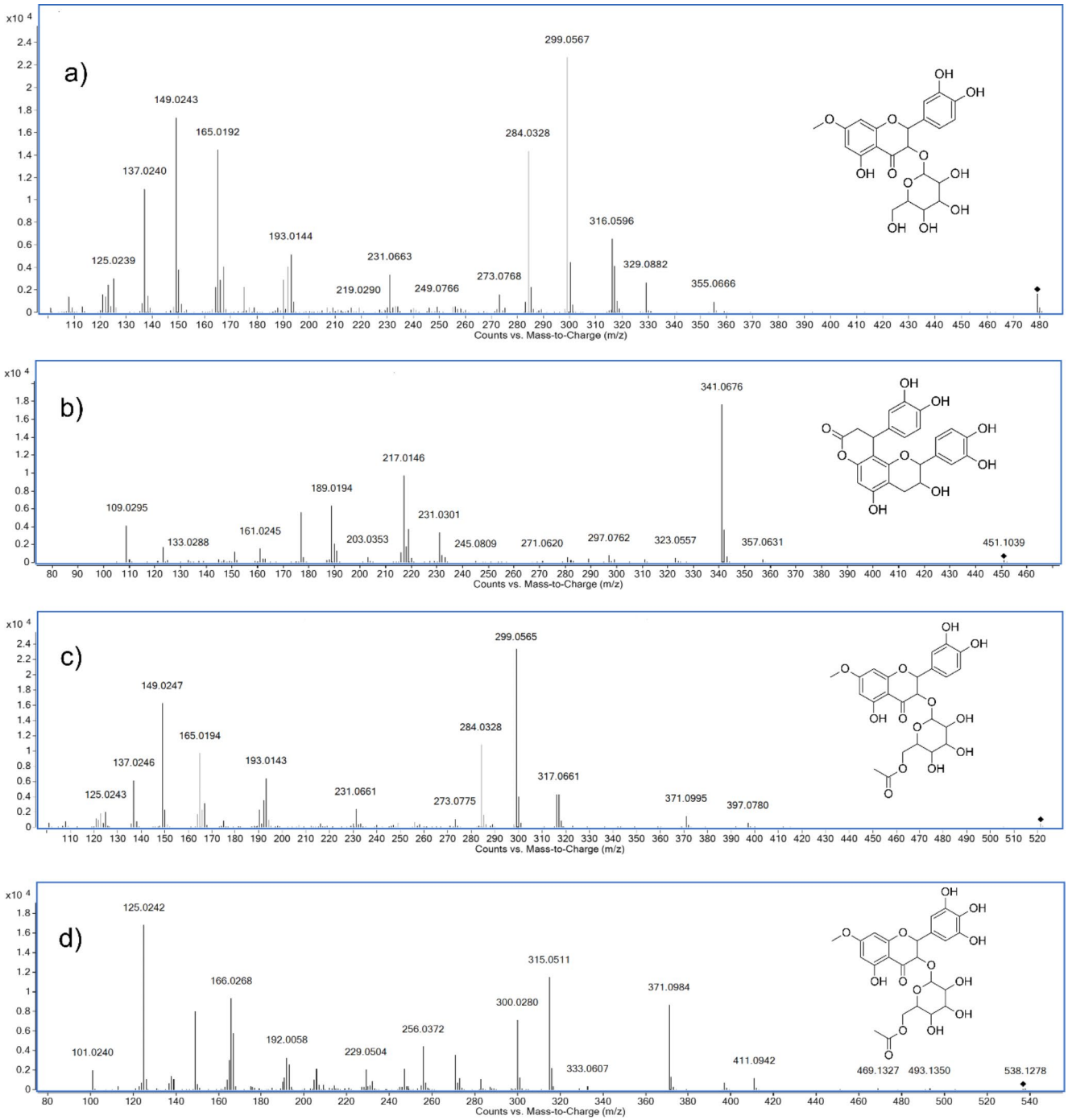

Fig. 4 MS/MS spectrum of (a) tetrahydroxy-methoxy-flavanone (putative dihydrorhamnetin) hexoside; (b) cinchonain isomer at Rt 15.8 min; (c) tetrahydroxy-methoxy-flavanone (putative dihydrorhamnetin) acetyl-hexoside; (d) pentahydroxy-methoxy-flavanone acetyl-hexoside

found this compound both in *V. vinifera* and non-*V. vinifera* grapes (Table 1).

Even though annotated only by their molecular formula (ID level 4), we found evidence for the presence of cinchonain related compounds: apocynin A-D, and cinchonain IIa/IIb (also named kandelin A-1/A-2) isomers (Online Resource 1). With one more hydroxyl group with respect to cinchonains, apocynin A-D isomers (Fan et al., 1999) are the (epi)gallocatechin-type analogs. Cinchonains, like proanthocyanidins, tend to polymerize with the consecutive addition of catechin/epicatechin units forming cinchonain IIa/IIb or kandelin A-1/A-2 (Nonaka et al., 1982b; Hsu et al., 1985). The radical scavenging activity of cinchonain dimers based on the DPPH (2,2-diphenyl-1-picryl-hydrazyl) essay

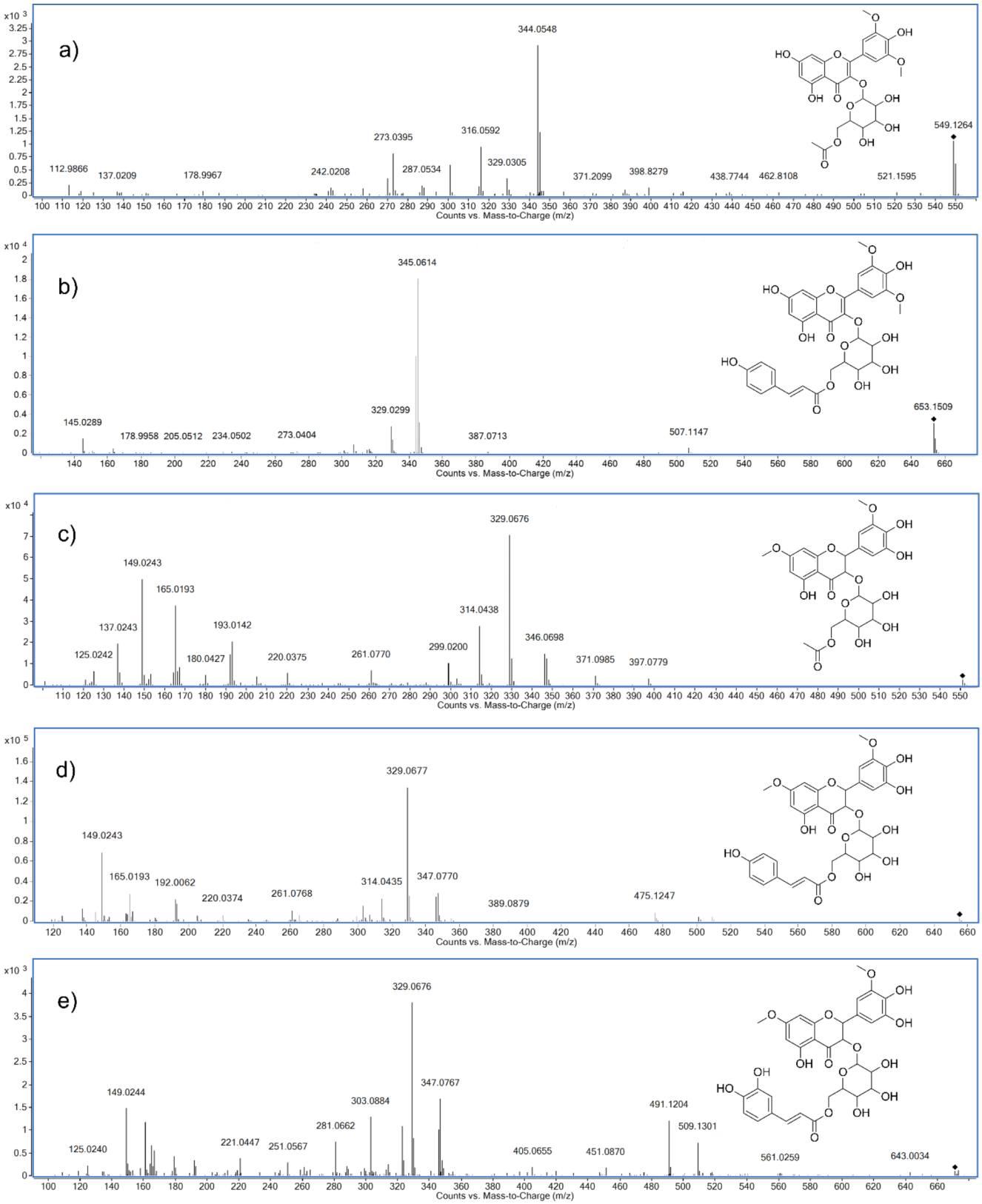

Fig. 5 MS/MS spectrum of (a) tetrahydroxy-dimethoxy-flavone (putative syringetin) acetyl-hexoside; (b) tetrahydroxy-dimethoxy-flavone (syringetin) *p*-coumaroyl-hexoside; (c) tetrahydroxy-dimethoxy-flavanone acetyl-hexoside; (d) tetrahydroxy-dimethoxy-flavanone *p*-coumaroyl-hexoside; (e) tetrahydroxy-dimethoxy-flavanone caffeoyl-hexoside

is not significantly different from that of procyanidin C1 (Resende et al., 2011).

Flavonoid acylation with aliphatic carboxylic acids (acetic, succinic, and malonic) and aromatic carboxylic acids (e.g., benzoic, *p*-coumaric, caffeic, ferulic, and sinapic) is a common modification reaction performed by flavonoid acyltransferase (FAD) (Saito et al., 2013) and phenylacyltransferase enzymes (Tohge et al., 2018). The major flavonoid substrates interested by these modifications are anthocyanins, isoflavones, flavones, and flavonols (Cunningham & Edwards, 2008).

In our compiled DB *GrapeFlavMet*, the highest acylation percentage is reached by glycosylated anthocyanins (70.5%), which present as most common substitutions *p*-coumaroyl, caffeoyl, and malonyl groups, often in combination. In anthocyanins, the esterification with phenylpropanoid groups promotes stacking and prevents the decomposition of the flavylium cation (Cunningham & Edwards, 2008; Alseekh et al., 2020). Among flavones/flavonols, 41% of the glycosylated derivatives are interested by acylation, mainly acetyl and *p*-coumaroyl. At least 10% of the glycosylated flavanones or flavanonols compiled in our DB present acylation, with acetyl and *p*-coumaroyl being the most common groups.

In our study, two acylated glycosyl flavonol derivatives were putatively identified at ID level 2b, and five glycosyl flavanones/flavanonols esterified by acetyl, *p*-coumaroyl at ID level 3, in one case caffeoyl groups were tentatively annotated. Previous evidence of non-anthocyanic acyl glycoside flavonoids in grape was provided by the identification of three *p*-coumaroyl hexosides (isorhamnetin, dihydrokaempferide, and chrysoeriol) (Panighel et al., 2014; De Rosso et al., 2020). This suggests that even though acylation and phenyl acylation is most common among anthocyanins and flavones/flavonols (Tohge et al., 2018), also flavanone/flavanonol subclass may be a substrate for which acylating enzymes compete.

The similarity of the MS/MS spectrum (Fig. 4c) to the one reported above for tetrahydroxy-methoxy-flavanone (dihydrorhamnetin) hexoside (Fig. 4a) and a mass difference of 204 Da consistent with the loss of acetyl-hexoside moiety, allowed us to tentatively annotate the compound eluted at 14.6 min ($[M-H]^-$ at *m/z* 521.1300) as tetrahydroxy-methoxy-flavanone (dihydrorhamnetin) acetyl-hexoside.

Search in *GrapeFlavMet* of the chemical formula $C_{24}H_{26}O_{14}$ (corresponding to the $[M-H]^-$ precursor ion at *m/z* 537.1250, peak retention time 14.2 min) was assigned to the putative pentahydroxy-methoxy flavanone acetyl-hexoside (MS/MS spectrum Fig. 4d). Pentahydroxy-methoxy-flavanone hexoside was previously found in *V. vinifera* withered grapes (Toffali et al., 2011); we found the acetyl hexoside derivative in both *V. vinifera* and hybrid grape varieties (Table 1).

The peak eluting at 16.7 min was assigned to the $[M-H]^-$ precursor ion at *m/z* 549.1250 corresponding to tetrahydroxy-dimethoxy-flavone (putative syringetin) acetyl-hexoside (MS/MS spectrum Fig. 5a). The fragmentation is similar to that already reported for syringetin isomers (Favre et al., 2018; van der Hooft et al., 2011). DB search putatively identified the compound eluting at 17.5 min ($[M-H]^-$ at *m/z* 653.1512) as tetrahydroxy-dimethoxy-flavonol *p*-coumaroyl-hexoside (MS/MS spectrum Fig. 5b). The finding of these two syringetin derivatives confirms previous results reported by Favre et al., (2018), who finds these compounds in *V. vinifera* grape skins and wines, along with acetyl and *p*-coumaroyl derivatives of laricitrin and isorhamnetin flavonols. Syringetin 3-*O*-acetyl glucoside was previously found in Cabernet Sauvignon skins (Wang et al., 2003) and in the red skins flavonol fraction of *V. vinifera* variety Petit Verdot (Castillo-Muñoz et al., 2007).

The database search putatively assigned the peak eluting at 14.4 min to the compound with $[M-H]^-$ ion at *m/z* 551.1406 to tetrahydroxy-dimethoxy-flavanone acetyl-hexoside (MS/MS spectrum Fig. 5c). Peak eluting at 15.7 min was assigned by the DB search to the $[M-H]^-$ precursor ion at *m/z* 655.1668 which corresponds to putative tetrahydroxy-dimethoxy-flavanone *p*-coumaroyl-hexoside (MS/MS spectrum Fig. 5d). Previously, the presence of tetrahydroxy-dimethoxy-flavanone hexoside in grape was proposed by Toffali et al., (2011) and the structure was confirmed by HR-MS/MS (De Rosso et al., 2020).

The peak exiting in the chromatogram at 15.1 min was assigned by search in the DB to the compound with $[M-H]^-$ ion at *m/z* 671.1617, corresponding to putative tetrahydroxy-dimethoxy-flavanone caffeoyl-hexoside (MS/MS spectrum Fig. 5e). This compound was found in two non-*V. vinifera* varieties: Seibel 19,881 and Seibel 8357 (Table 1).

4 Conclusions

Developing a specific database of flavonoids coupled to HR-MS/MS data provided the identification of new flavonoid glycosides at ID level 2 or 3. The identification of three pentoside flavonoids, added up quercetin, taxifolin, and peonidin pentosides previously found, confirming that biosynthesis of these glycoside derivatives in some varieties is not negligible. This approach also allowed the identification of unusual compounds, such as cinchonain and related flavonolignan compounds, introducing an interesting new class of grape flavonoids to investigate. Findings of the study and the identification of putative acylated glycosyl flavanones, enlarge the panorama of acylated glycosyl flavonoids by

inferring anthocyanins are not the only substrates for their biosynthesis in grape. These results enlarge the knowledge on grape flavonoids and the possibility to uncover additional connections in their biosynthetic pathways.

Metabolomics data associated with the identification of compounds have been deposited to the EMBL-EBI MetaboLights database (https://www.ebi.ac.uk/metabolights) with the identifier MTBLS4202.

The complete dataset can be accessed here https://www.ebi.ac.uk/metabolights/MTBLS4202.

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Author contribution statement RF conceived the study and edited the manuscript. MDR performed chemical analyses and data interpretation. FDM performed data collection, extraction, and interpretation, and wrote the manuscript. All authors read and approved the manuscript.

Data availability statement The metabolomics and metadata reported in this paper are available via Metabolights https://www.ebi.ac.uk/metabolights/ study identifier MTBLS4202.

Declarations

Author conflict of interest statement The authors declare no conflict of interest.

Compliance with ethical standards This article does not contain any studies with human and/or animal participants performed by any of the authors.

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