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ORIGINAL ARTICLE

An innovative approach to grape metabolomics: stilbene profiling by suspect screening analysis

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Abstract Suspect screening analysis is a targeted metabolomics approach in which identification of compounds relies on specific available information such as their molecular formula and isotopic pattern. This method was applied to the study of grape metabolomics with an UPLC/MS high-resolution Q-TOF mass spectrometer (nominal resolution 40,000) coupled with a Jet Stream ionization source. The present paper describes the detailed qualitative and quantitative study of grape stilbenes, the principal polyphenols associated with the beneficial effects of drinking wine. For identification of compounds, a new database was expressly constructed from the molecular information of potential metabolites of grape and wine from the literature and other electronic databases. Currently, *GrapeMetabolomics* contains about a thousand putative grape compounds. If untargeted analysis of a sample provides identification of a new compound with a sufficiently confident score, it is added to the database. Thus, by increasing the number of samples studied, *GrapeMetabolomics* can be expanded. This method is effective for identification of the molecular formulae of several hundred metabolites in two runs (positive and negative ionization) with minimal sample preparation, and can also be used to analyse some single classes of compounds involved in cell and tissue metabolism. With this approach, a total of 18 stilbene derivatives was identified in two grape samples (*Raboso Piave* and *Primitivo*) on the basis of accurate mass measurements and isotopic patterns, and identification was confirmed by MS/MS analysis. The approach can also potentially be applied to the metabolomics of other plant varieties.

Keywords Stilbenes · Grape · UPLC · Time of flight mass spectrometry · Metabolomics · Suspect screening analysis

1 Introduction

Stilbenes are vine phytoalexins also found in grape berries. These compounds are one of the classes of grape polyphenols associated with the beneficial effects of drinking wine. An epidemiological study carried out in the late 1970s showed that in France, despite the high consumption of foods rich in saturated fatty acids, the incidence of mortality from cardiovascular diseases was lower than in other comparable countries. This phenomenon, called the "French paradox", was correlated with the beneficial effects of consuming red wine as a major factor (Renaud and de Lorgeril 1992). In general, antioxidant activity is one of the main properties of polyphenols: in vitro studies show that they act as radical peroxyl scavengers in forming complexes with metals. In addition, their ability to cross the intestinal wall of mammals confers biological activities on them (although some have been shown to have low bioavailability), and their cellular signaling activity has

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been proven (Manach et al. 2005; Han et al. 2007; Flamini 2003, and references cited therein).

The main grape stilbenes are *cis*- and *trans*-resveratrol (3,5,4′-trihydroxystilbene) and their glucosides (*cis*- and *trans*-piceids), piceatannol (3,4,3′,5′-tetrahydroxy-*trans*-stilbene) and resveratrol dimers (viniferins) (Vitrac et al. 2005). Several in vitro studies show that resveratrol has anti-cancer, anti-oxidant and anti-inflammatory activities, confers cardioprotection, and inhibits platelet aggregation (Jang et al. 1997; Hung et al. 2000; Frankel et al. 1993; Frémont et al. 1999; Bertelli et al. 1995; Pace-Asciak et al. 1995; Bavaresco et al. 2012). Piceatannol blocks LMP2A, a viral protein-tyrosine kinase implicated in leukemia, non-Hodgkin's lymphoma and other diseases associated with the Epstein-Barr virus (EBV) (Geahlen and McLaughlin 1989; Swanson-Mungerson et al. 2003), and it also acts on human melanoma cells (Larrosa et al. 2004).

ε-Viniferins and ω-viniferins (isomers *E* and *Z*) and resveratrol trimers and tetramers (ampelopsin D, quadrangularin A, α-viniferin, *E*- and *Z*-miyabenol C, isohopeaphenol, ampelopsin H, vaticanol C-like) are synthesized in various parts of the plant such as leaves, roots, clusters and stems (Mattivi et al. 2011). In *Vitis* grapevine canes, *E*-ampelopsin E, Eamurensin B, *E*-resveratroloside, *E*-3,5,4′-trihydroxystilbene 2-C-glucoside, *Z*-ampelopsin E, scirpusin A, *E*- and *Z*-vitisin B have also been identified (Pawlus et al. 2013).

These compounds are formed by oligomerization of *trans*-resveratrol in grape tissues, a process induced as active defense of the plant against exogenous attack, or are produced by extracellular enzymes released from pathogens in an attempt to eliminate undesirable toxic compounds (Cichewicz et al. 2000; Sbaghi et al. 1996). The principal ones are ε-viniferins, δ-viniferins and pallidol (Bavaresco et al. 1997; Waffo-Téguo et al. 2001).

The methods commonly used for stilbene analysis are normal-phase or reverse-phase high performance-liquid chromatography (HPLC) and detection of *cis* and *trans* isomers at 285 and 325 nm wavelengths, respectively (Di Stefano et al. Di Stefano and Flamini 2008). In some studies, *trans*-resveratrol and piceatannol in wine were detected by liquid chromatography/mass spectrometry (LC/MS) operating in negative-ion mode and by Selected Ion Recording (SIR) of $[M-H]^-$ ion (Gamoh and Nakashima 1999; Buiarelli et al. 2007; Monagas et al. 2005; Stella et al. 2008). Some LC/MS positive-ion mode methods for resveratrol analysis have also been proposed (Flamini et al. Flamini and Dalla Vedova 2004; Careri et al. 2004).

'Metabolomics' is the comprehensive quantitative and qualitative study of all the metabolites within a cell, tissue, or organism. The major limitation of this approach is its current inability to describe the whole 'metabolome' profile exhaustively. LC, direct-injection and ESI mass spectrometry are powerful tools with high selectivity and sensitivity, allowing detection of non-volatile and labile components in samples (Mattoli et al. 2006). In general, 'untargeted metabolomics' provides high sensitivity, good resolution and high-throughput capacity, and in wines can reveal several thousand signals of candidate biomarkers in a single run (Arapitsas et al. 2012). Instead, targeted metabolomics is performed for quantitative studies on specific compounds, and most of the metabolome information of complex samples, such as wines, is missed (Cuadros-Inostroza et al. 2010; Vaclavik et al. 2011).

Suspect screening analysis is a mid-way approach. This method of identifying metabolites relies on the availability of specific information on compounds, e.g., their molecular formula and structure (Krauss et al. 2010). Currently, we are applying this approach to the study of grape metabolomic. A new database containing the putative metabolites of grape and wine (called *GrapeMetabolomics*) has been constructed, and includes information on the molecular formulae of compounds present in the literature and found in electronic databases.

In the present work, this approach was applied to stilbenes and their derivatives in some grape varieties. Analyses were performed by using an ultra-high performance-liquid chromatography (UPLC) high-resolution Q-TOF Mass Spectrometer (nominal resolution 40,000) coupled with a Jet Stream ionization source. Compounds were identified by *GrapeMetabolomics* on the basis of accurate mass measurements and isotope patterns, and multiple mass spectrometry (MS/MS) confirmed identification.

2 Materials and methods

2.1 Chemicals and sample preparation

Standard of *trans*-resveratrol, piceatannol, *E*-piceid and 4′,5,7-trihydroxy flavanone were purchased from Sigma-Aldrich (Milan, Italy); *Z*-piceid was produced by photo-isomerization of *E* isomer.

About 100 berries of the *V. vinifera* red grape varieties Raboso Piave and Primitivo were harvested in 2011 at full ripeness from the CRA-VIT grapevine Germoplasma Collection (Susegana, Veneto, Italy). Berries were picked randomly from five different plants and immediately frozen at −20 °C.

For sample preparation, twenty berries were weighed, homogenized using liquid nitrogen and the resulting powder was immediately extracted with pure methanol in ratio 2:1 v/w under stirring for 20 min. After addition of 200 μL of 4′,5,7-trihydroxy flavanone 500 mg/L solution as internal standard, the sample was centrifuged at 10 °C for 20 min. The solution was filtered with Acrodisc GHP

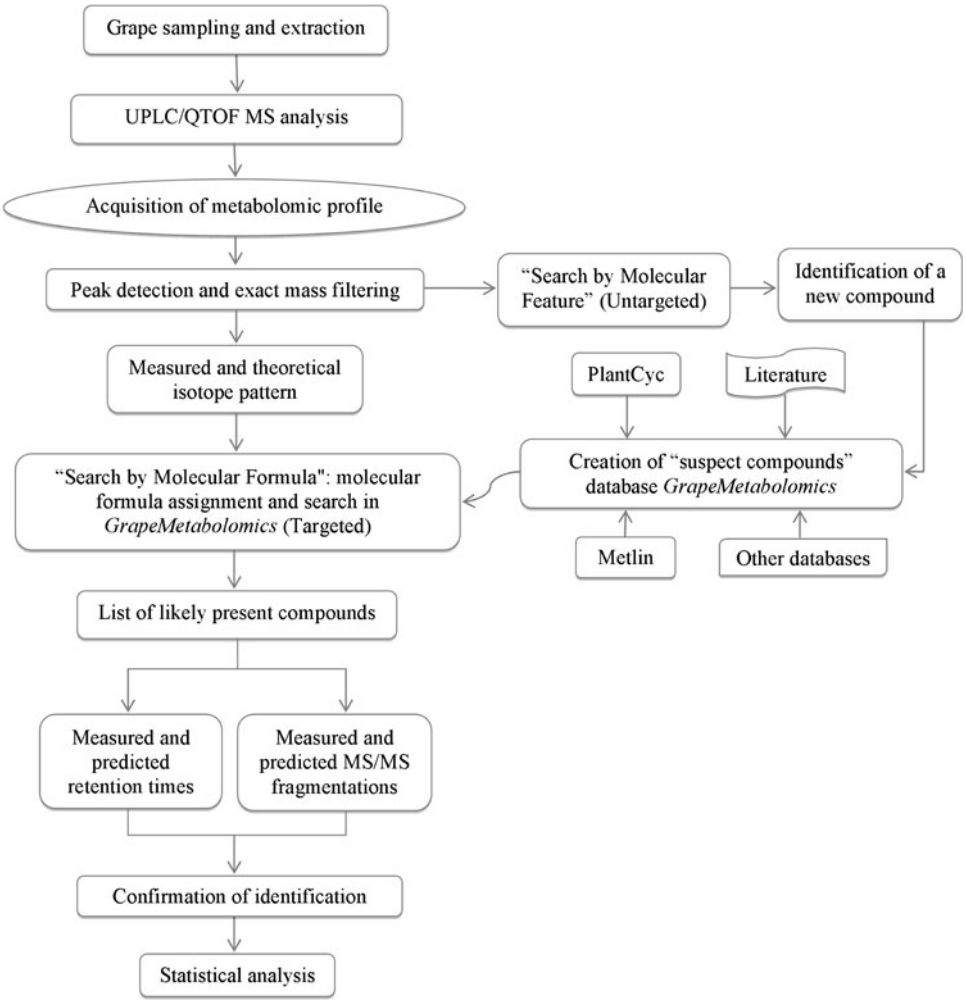

Fig. 1 Workflow used for suspect screening metabolomics. MS/MS fragments were manually used to confirm molecular formulae of stilbene compounds assigned by targeted analysis according to accurate mass and isotopic pattern. Tentative assignments of isomeric compounds were made on column elution sequences and comparisons with literature data. Retention times of compounds assigned were then introduced in GrapeMetabolomics

0.22 μm filter (Waters) and collected in a vial for LC/MS analysis. For each sample two replicate analyses were performed.

Contents in grape of viniferins and resveratrol oligomers were calculated on the analytical response of *trans*-resveratrol and were expressed as μg/kg grape of *trans*-resveratrol, those of glucoside derivatives as μg/kg grape of *trans*-piceid.

2.2 LC/QTOF mass spectrometry

The analytical system used was Agilent UHPLC 1290 Infinity coupled to Agilent 1290 Infinity Autosampler (G4226A) and Agilent 6540 accurate-mass Q-TOF Mass Spectrometer (nominal resolution 40,000) with Jet Stream Ionization source (Agilent Technologies, Santa Clara, CA). Two repeated analyses of each sample with full scan acquisition mode in both positive and negative ionization mode, were performed. A blank was run between each pair of analyses. The data acquisition software was Agilent MassHunter version B.04.00 (B4033.2). Chromatographic separation was performed using a Zorbax reverse-phase column (RRHD SB-C18 3 × 150 mm, 1.8 μm) (Agilent Technologies, Santa Clara, CA). The mobile phase was composed of (A) 0.1 % v/v aqueous formic acid and (B) 0.1 % v/v formic acid in acetonitrile. Gradient elution program: 5 % B isocratic for 8 min, from 5 to 45 % B in 10 min, from 45 to 65 % B in 5 min, from 65 to 90 % in 4 min, 90 % B isocratic for 10 min. Flow rate 0.4 mL/min. Sample injection 10 μL. Column temperature 35 °C.

QTOF conditions: sheath gas nitrogen 10 L/min at 400 °C; drying gas 8 L/min at 350 °C; nebulizer pressure 60 psig, nozzle voltage 1 kV, capillary voltage 3.5 kV. Signals in the *m/z* 100–1,700 range were recorded. Negative mass calibration was performed with standard mix G1969-85000 (Supelco Inc.) and had residual error for the expected masses between ± 0.2 ppm. Lock masses: TFA anion at *m/z* 112.9856 and HP-0921(+formate) at *m/z* 966.0007 in negative-ion mode, purine at *m/z* 121.0509 and HP-0921 at *m/z* 922.0098 in positive-ion mode. MS/MS

Table 1 Stilbene derivatives identified in studied grape samples according to accurate mass and isotopic patterns

Peak	RT (min)	Compound assignment	Formula	[M−H]$^-$		Δppm	ID. score
				Experimental mass	Theoretical mass		
1	13.94	*E*-astringin (piceatannol glucoside)**	C20H21O9	405.1198	405.1191	1.7	98.4
2	14.94	*E*-piceid*	C20H21O8	389.1249	389.1242	1.8	98.2
3	15.12	*Z*-astringin**	C20H21O9	405.1190	405.1191	−0.2	99.0
4	15.92	Piceatannol*	C14H11O4	243.0666	243.0663	1.2	99.5
5	15.97	*Z*-piceid*	C20H21O8	389.1244	389.1242	0.5	99.8
6	16.50	Dimer 1 (paffidol)**	C28H21O6	453.1347	453.1344	0.7	99.1
7	17.01	Paffidol-3-O-glucoside**	C34H31O11	615.1869	615.1872	−0.5	96.9
8	17.16	Dimer 2**	C28H21O6	453.1345	453.1344	0.2	98.2
9	17.29	*Trans*-resveratrol*	C14H11O3	227.0716	227.0714	0.9	99.7
10	17.91	Caraphenol B**	C28H21O7	469.1292	469.1293	−0.2	97.3
11	18.10	Tetramer 1**	C56H41O12	905.2602	905.2604	−0.2	98.6
12	18.49	Tetramer 2**	C56H41O12	905.2602	905.2604	0.3	99.5
13	18.61	Dimer 3 (*Z*-ε-viniferin)**	C28H21O6	453.1346	453.1344	0.4	99.5
14	18.81	Dimer 4 (*E*-ε-viniferin)**	C28H21O6	453.1346	453.1344	0.4	99.4
15	18.84	Trimerl (*Z*-miyabenol C)**	C42H31O9	679.1976	679.1974	0.3	98.2
16	18.93	Trimer 2 (*E*-miyabenol C)**	C42H31O9	679.1977	679.1974	0.4	98.7
IS	19.24	Trihydroxy flavanone	C15H12O5	271.0616	271.0612	1.5	98.1
17	19.56	Dimer 5 (δ-viniferins)**	C28H21O6	453.1342	453.1344	−0.4	99.2
18	20.24	Resveratrol methyl ether**	C15H13O3	241.0869	241.0870	−0.4	84.4

Tentative assignments of isomeric resveratrol dimers, trimers and tetramers based on column elution sequences and comparison with literature data

IS internal standard, *RT* column retention time, *ID. Score* maximum overall identification score percentage measured for compounds

* Identification based on accurate mass/isotope pattern and accurate mass/tandem MS of standard compound

** Putative assignment also based on MS/MS data and column sequence elution information from the literature

conditions: collision energies between 20 and 60 eV were used to fragment the parent ions in the *m/z* 100–1,700 range. Acquisition rate 2 spectra/s.

2.3 Data analysis

Data processing was performed with Agilent MassHunter Qualitative Analysis software version B.05.00 (5.0.519.0). The database of putative grape and wine metabolites (*GrapeMetabolomics*) was constructed by including the information on their molecular formulae found in the literature and electronic databases. Target identification of compounds was performed with algorithm "Find Compounds By Formula". Untargeted analysis was performed with algorithm "Find Compounds By Molecular Feature".

Confidence of compound identification based on accurate mass and isotope pattern was expressed by "overall identification score", computed as a weighted average of the isotopic pattern signals of compound, such as exact masses, relative abundances, and *m/z* distances (spacing). The weights of these parameter were: $W_{mass} = 100$, $W_{abundance} = 60$, $W_{spacing} = 50$, mass expected data variation 2.0 mDa + 5.6 ppm, mass isotope abundance 7.5 %, mass isotope grouping peak spacing tolerance 0.0025 *m/z* + 7.0 ppm.

3 Results and discussion

Accurate mass spectrometry is commonly used in plant metabolomics. Identification of compounds relies on raw data processing with specific algorithms which provide molecular formulae on the basis of: (a) the mono-isotopic mass measured; (b) relative abundances and *m/z* distances (spacing) of isotopic patterns (Kueger et al. 2012; Sana et al. 2008). Metabolites are identified by searching in available databases (Hanhineva et al. 2008; Brown et al. 2009; De Vos et al. 2007).

Unfortunately, although several databases containing plant metabolites are available, they are not specific for a particular matrix such as grape. When we tried to identify metabolites in grape extracts using some of these databases (e.g., PlantCyc, Flavonoid Viewer), we were only able to find a few of the compounds among the many we knew

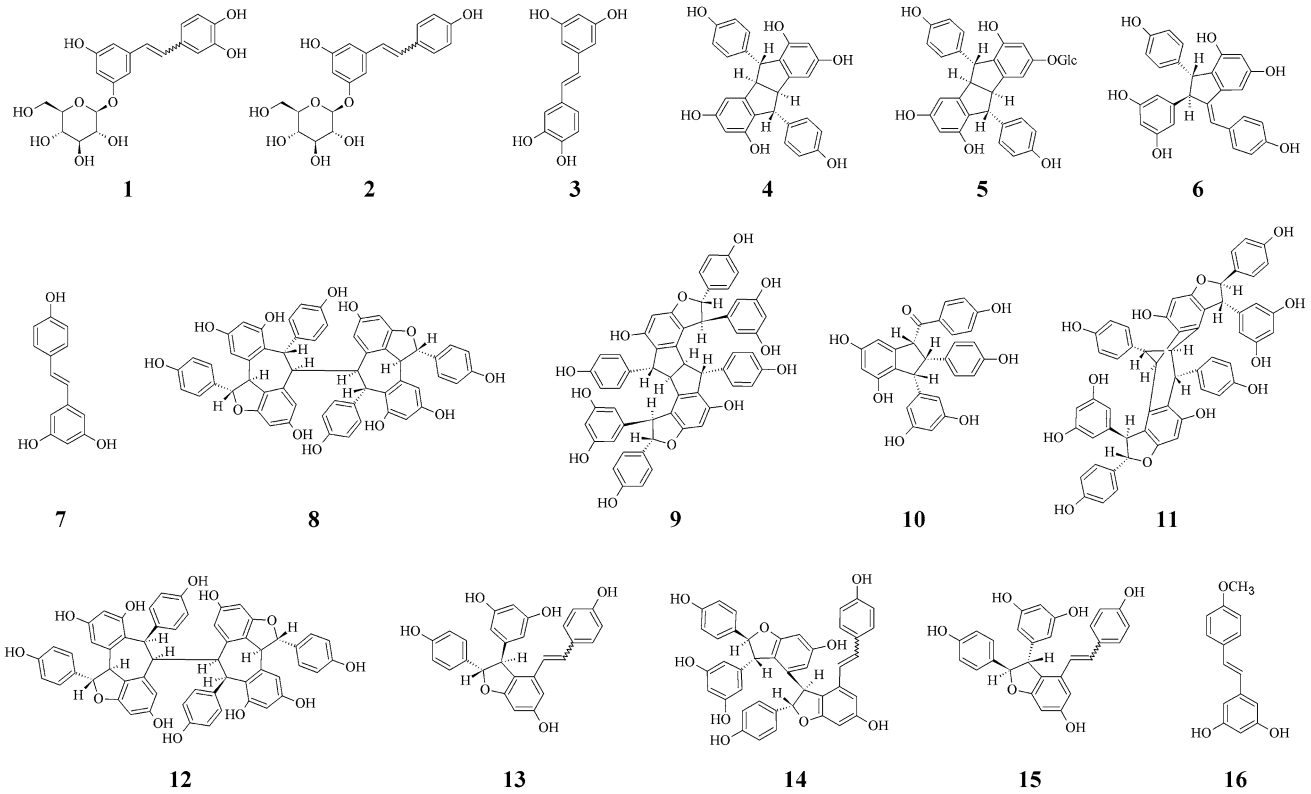

Fig. 2 Structures of stilbene derivatives identified in the grape samples studied. **1** *Z*- and *E*-astringin, **2** *Z*- and *E*-piceid, **3** piceatannol, **4** pallidol, **5** pallidol-3-O-glucoside, **6** parthenocissin A, **7** *trans*-resveratrol, **8** hopeaphenol, **9** ampelopsin H, **10** caraphenol B, **11** vaticanol C isomer, **12** isohopeaphenol, **13** *E*- and *Z*-ε-viniferin, **14** *E* and *Z* miyabenol C, **15** *E* and *Z* δ-viniferin, **16** *trans*-resveratrol-4′-methyl ether

occurred in the samples. To overcome this problem, we developed a targeted metabolomics approach by *suspect screening analysis*, in which identification of metabolites is based on knowledge of the molecular formula of the compounds examined.

The experimental elemental formulae of compounds were calculated with the algorithm "Find Compounds By Formula", which performs deconvolution of chromatograms and searches for compounds by comparing theoretical and experimental isotopic patterns extrapolated from raw data. The compounds were then identified by the home-made database of putative grape and wine metabolites, *GrapeMetabolomics,* expressly constructed by introducing the molecular formula of compounds of enological interest and generally identified in plant extracts, present in the literature and found in other electronic databases (PlantCyc, Flavonoid Viewer, Metlin).

The suitability of the method was evaluated by analysis of two grape variety extracts taken as models, due to their peculiar chemical characteristics: *Raboso Piave*, for richness of polyphenol and glycoside norisoprenoids, and *Moscato bianco* for glycoside monoterpenes (De Rosso et al. 2010; Flamini et al. 2001). The method identified most of the compounds expected in the samples. Currently, *GrapeMetabolomics* contains about a thousand putative grape compounds with molecular weights between 100 and 1,700 Da. After targeted search, the raw data are processed by an untargeted method with the algorithm "Find Compounds By Molecular Feature" which contains the algorithm Molecular Feature Extraction (MFE), followed by the algorithm Molecular Formula Generator (MFG) which provides a list of molecular formulae not present in *GrapeMetabolomics*. MFE is a compound finding algorithm which locates individual sample components, even when chromatograms are complex and the compounds are not well resolved. The algorithm uses the accuracy of mass measurements to group related ions-related by charge-state envelope, isotopic distribution, and/or the presence of adducts and dimers. It assigns ions which are related to the same neutral molecule to a single compound, referred to as a feature. MFG takes advantage of both mass accuracy and mass spectral information to apply additional constraints to the list of candidate molecular formulae detected. This is achieved by incorporating monoisotopic mass, isotope abundances, and spacing between isotope peak information into its calculations (Sana et al. 2008).

If the search for resulting molecular formulae in other databases provides identification of a new compound

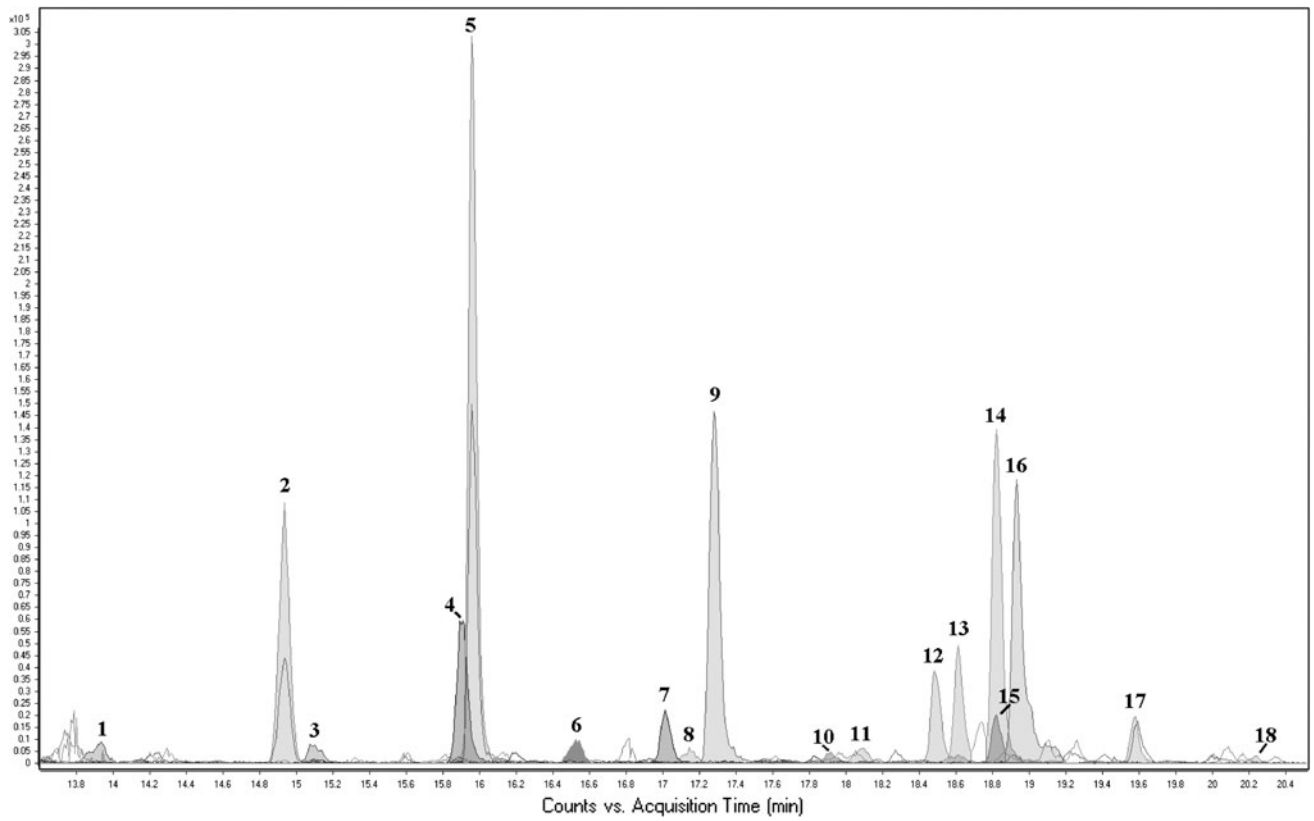

Fig. 3 Extract-ion chromatogram of stilbene derivatives in Raboso Piave grape extract. LC/QTOF negative-ionization mode analysis performed by reverse-phase column and gradient elution, with binary solvent composed of (a) aqueous formic acid 0.1 % (v/v) and (b) acetonitrile—formic acid 0.1 % (v/v). Compounds identified are listed in Table 1

compatible with a vegetable matrix with a sufficiently confident score, it is added to *GrapeMetabolomics,* together with its chromatographic retention time. Thus, by increasing the number of samples studied, the database itself can be expanded. Figure 1 illustrates the workflow used.

Sample extraction was performed in cold conditions with minimal sample handling, in order to limit artifacts and to minimize the possibility of false negatives. The latter may occur due to the lower source ionization efficiency of some metabolites in the sample. The absence of false positives was checked by analyzing blanks between each pair of samples.

In general, with both positive- and negative-ion mode analysis, the molecular formulae identified were assigned by the algorithm "Find Compounds By Formula" to 320–450 putative grape compounds, with overall identification scores higher than 60 % (calculated on accurate mass and isotope pattern). This number ranged according to grape variety, and mainly included polyphenols and antioxidant compounds such as anthocyanins, flavones and flavanones, flavanols and procyanindins, stilbenes, phenolic acids, and glycoside aroma precursors. Between 30 and 60 compounds showed identification scores higher than 99 %, and more than 100 higher than 95 % (see supporting supplementary information).

Overall, 18 stilbene derivatives were identified. They are listed in Table 1 and their structures shown in Fig. 2. Figure 3 shows the chromatogram for the Raboso Piave extract. Identification of *trans*-resvetatrol, piceatannol, *Z*- and *E*-piceid was performed according to the criteria given in the Proposed Minimum Reporting Standards For Chemical Analysis for non-novel metabolites and putatively annotated compounds (Sumner et al. 2007). For these compounds, two sets of independent and orthogonal data, accurate mass/isotope pattern and accurate mass/tandem MS, were used. For putative identification of the other stilbenes, in particular of isomeric compounds, MS/MS data and column sequence elution information from the literature were also used.

All stilbenes in Table 1 were identified by a targeted search in *GrapeMetabolomics* according to the algorithm "Find Compounds By Formula". The first identification of molecular formulae was based on the accurate mass and isotopic pattern. The overall identification scores (ID scores) ranged between 84.4 % (for resveratrol methyl ether, Table 1) and 99.8 % (for *cis*-piceid). Scores were generally affected by signal intensity (which mainly influenced the score parameters of isotopic patterns) and matrix

Table 2 MS/MS fragments used to identify stilbene derivatives

Compound	Precursor ion	MS/MS fragment mass	Formula	Theoretical fragment mass	Δppm	References
E-astringin (piceatannol glucoside)	405.1198	243.0665	C14H11O4	243.0663	0.8	Buiarelli et al. (2007) and Stella et al. (2008)
		201.0556	C12H9O3	201.0557	−0.5	Buiarelli et al. (2007)
		159.0446	C10H7O2	159.0452	−3.8	Buiarelli et al. (2007)
E-piceid	389.1249	227.0712	C14H11O3	227.0714	−0.9	Buiarelli et al. (2007) and Stella et al. (2008)
		185.0604	C12H9O2	185.0608	−2.2	Stella et al. (2008)
		143.0503	C10H7O	143.0502	0.7	Stella et al. (2008)
Z-astringin	405.1190	243.0663	C14H11O4	243.0663	0.0	Buiarelli et al. (2007) and Stella et al. (2008)
		201.0554	C12H9O3	201.0557	−1.5	Stella et al. (2008)
Piceatannol	243.0666	225.0554	C14H9O3	225.0557	−1.3	Buiarelli et al. (2007) and Stella et al. (2008)
		201.0556	C12H9O3	201.0557	−0.5	Stella et al. (2008)
		173.0600	C11H9O2	173.0608	−4.6	Stella et al. (2008)
		159.0448	C10H7O2	159.0452	−2.5	Stella et al. (2008)
		143.0506	C10H7O	143.0502	2.8	Stella et al. (2008)
Z-piceid	389.1244	227.0720	C14H11O3	227.0714	2.6	Buiarelli et al. (2007) and Stella et al. (2008)
		185.0611	C12H9O2	185.0608	1.6	Stella et al. (2008)
		143.0506	C10H7O	143.0502	2.8	Stella et al. (2008)
Dimer 1 (pallidol)	453.1347	359.0919	C22H15O5	359.0925	−1.7	Püssa et al. (2006)
		265.0504	C16H9O4	265.0506	−0.8	Püssa et al. (2006)
Pallidol-3-O-glucoside	615.1869	453.1343	C28H21O6	453.1344	−0.2	Püssa et al. (2006)
		359.0934	C22H15O5	359.0925	2.5	Püssa et al. (2006)
Dimer 2	453.1345	411.1230	C26H19O5	411.1238	−1.9	Püssa et al. (2006)
		359.0920	C22H15O5	359.0925	−1.4	Püssa et al. (2006)
Trans-resveratrol	227.0716	185.0606	C12H9O2	185.0608	−1.1	Stella et al. (2008)
		183.0812	C13H11O	183.0815	−1.6	Stella et al. (2008)
		159.0818	C11H11O	159.0815	1.9	Stella et al. (2008)
		143.0504	C10H7O	143.0502	1.4	Stella et al. (2008)
Caraphenol B	469.1292	451.1181	C28H19O6	451.1187	−1.3	Püssa et al. (2006)
		281.0819	C17H13O4	281.0819	0.0	Püssa et al. (2006)
		227.0717	C14H11O3	227.0714	1.3	Stella et al. (2008)
Tetramer 1	905.2602	811.2173	C50H35O11	811.2185	−1.5	Püssa et al. (2006)
		717.1766	C44H29O10	717.1766	0.0	Püssa et al. (2006)
		451.1198	C28H19O6	451.1187	2.4	Püssa et al. (2006)
		359.0912	C22H15O5	359.0925	−3.6	Pezet et al. (2003), Püssa et al. (2006) and Jerkovic et al. (2007)
Tetramer 2	905.2607	811.2185	C50H35O11	811.2185	0.0	(Püssa et al. 2006)
		717.1766	C44H29O10	717.1766	0.0	(Püssa et al. 2006)
Dimer 3 (*Z*-ε-viniferin)	453.1346	435.1228	C28H19O5	435.1238	−2.3	(Püssa et al. 2006 and Jerkovic et al. 2007)
		411.1230	C26H19O5	411.1238	−1.9	(Pezet et al. 2003 and Püssa et al. 2006)
		347.0928	C21H15O5	347.0925	0.9	(Pezet et al. 2003, Püssa et al. 2006 and Jerkovic et al. 2007)
		333.0761	C20H13O5	333.0768	−2.1	(Pezet et al. 2003 and Püssa et al. 2006)
		253.0497	C15H9O4	253.0506	−3.6	(Pezet et al. 2003 and Püssa et al. 2006)
		225.0559	C14H9O3	225.0557	0.9	(Stella et al. 2008)
		185.0603	C12H9O2	185.0608	−2.7	(Stella et al. 2008)

Table 2 continued

Compound	Precursor ion	MS/MS fragment mass	Formula	Theoretical fragment mass	Δppm	References
Dimer 4 (*E*-ε-viniferin)	453.1346	435.1238	C28H19O5	435.1238	0.0	Pezet et al. (2003), Püssa et al. (2006) and Jerkovic et al. (2007)
		411.1238	C26H19O5	411.1238	0.0	Pezet et al. (2003) and Püssa et al. (2006)
		359.0929	C22H15O5	359.0925	1.1	Pezet et al (2003) and Jerkovic et al (2007)
		347.0932	C21H15O5	347.0925	2.0	Pezet et al (2003) and Jerkovic et al. (2007)
		253.0512	C15H9O4	253.0506	2.4	Pezet et al. (2003) and Püssa et al. (2006)
		225.0552	C14H9O3	225.0557	−2.2	Stella et al. (2008)
Trimer 1 (*Z*-miyabenol C)	679.1976	661.1854	C42H29O8	661.1868	−2.1	Püssa et al. (2006)
		573.1543	C35H25O8	573.1555	−2.1	
		227.0726	C14H11O3	227.0714	5.3	Stella et al. (2008)
Trimer 2 (*E*-miyabenol C)	679.1977	661.1872	C42H29O8	661.1868	0.6	Püssa et al. (2006)
		573.1545	C35H25O8	573.1555	−1.7	
		359.0918	C22H15O5	359.0925	−1.9	Püssa et al. (2006)
		227.0717	C14H11O3	227.0714	1.3	Stella et al. (2008)
Dimer 5 (δ-viniferins)	453.1342	411.1242	C26H19O5	411.1238	1.0	Pezet et al. (2003)
		435.1257	C28H19O5	435.1238	4.4	Pezet et al. (2003)
		369.1134	C24H17O4	369.1127	1.9	Pezet et al. (2003)
		347.0923	C21H15O5	347.0925	−0.6	Pezet et al. (2003), Püssa et al. (2006) and Jerkovic et al. (2007)
		225.0559	C14H9O3	225.0557	0.9	Püssa et al. (2006) and Stella et al. (2008)
Resveratrol methyl ether	241.0869	225.0557	C14H9O3	225.0557	0.0	Mazzotti et al. (2010)
		195.0446	C13H7O2	195.0452	−3.1	Mazzotti et al. (2010)

Right: references with MS/MS data of compounds

background in the chromatograms (data not shown) (Krauss et al. 2010).

Identifications were confirmed manually by MS/MS with accurate mass measurements of the fragments formed, which conformed to practically all the compounds identified, and generally matched literature data (see Table 2).

In addition, the Primitivo chromatogram showed a signal at *m/z* 677.1818, found at retention time 19.38 min, which was identified as α-viniferin, a stilbene derivative previously found in *V. vinifera* leaves (Mattivi et al. 2011). Despite low mass error (0.1 ppm), MS/MS fragments (data not shown) and the low overall identification score (supporting supplementary information) did not confirm this compound.

This is the first time that these resveratrol trimers and tetramers have been found in grape, and that accurate mass data of their MS/MS fragments have been reported. Of course, accurate mass measurements could not distinguish between isomeric compounds, and tentative assignments were based on column elution sequences and comparisons with data in the literature (Romero-Pérez et al. 1999; Baderschneider and Winterhalter 2000; Vitrac et al. 2001; Pezet et al. 2003; Püssa et al. 2006; Jean-Denis et al. 2006; Pawlus et al. 2012; Mattivi et al. 2011; Takaya et al. 2003; Guebailia et al. 2006; Mazzotti et al. 2010; Adrian et al. 2000a, b; Douillet-Breuil et al. 1999; Jerkovic et al. 2007).

In particular, the MS/MS of peaks **1** and **3** (piceatannol glucoside), **2** and **5** (resveratrol glucoside) and **7** showed the ions formed by sugar residue loss (−162 Da) at *m/z* 243.066, *m/z* 227.071 and *m/z* 453.134, respectively, as main peaks in the mass spectrum. According to the column elution sequence, these signals were tentatively assigned to *Z*- and *E*-astringin, *Z*- and *E*-piceid (stilbenes already found in grape and wines) (Buiarelli et al. 2007; Romero-Pérez et al. 1999) and pallidol glucoside (reported in Riesling wine) (Baderschneider and Winterhalter 2000), respectively.

Dimers (precursor ion at *m/z* 453.134) showed a signal at *m/z* 359.092 as one of the main product ions, corresponding to the loss of phenol molecule. According to the elution sequence, peaks **6** and **8** were tentatively assigned to pallidol and parthenocissin A, respectively, two compounds previously reported in red wines (Vitrac et al. 2001). At retention time 17.91 min (peak **10**) a signal at *m/z* 469.129 was found, which may correspond to dimer caraphenol B-type previously found in *V. vinifera* stem (Püssa et al. 2006; Choi et al. 2010). MS/MS showed the

ions at *m/z* 451.118 and 281.082, matching results reported by Püssa et al. (2006), and the ion at *m/z* 227.072 corresponding to resveratrol molecule.

Peaks **13**, **14** and **17** showed a characteristic water loss, with formation of product ions at *m/z* 435.123 and *m/z* 347.092 (due to a 106-Da fragment loss), and a loss of 42 Da with formation of the signal at *m/z* 411.123. These product ions have previously been found in the fragmentation of δ- and ε-viniferins. The MS/MS of peak **17** also showed formation of the product ion at *m/z* 369.113, matching results reported for δ-viniferin (Pezet et al. 2003; Püssa et al. 2006; Jean-Denis et al. 2006). These three peaks were thus assigned to *Z*-ε-viniferin, *E*-ε-viniferin, and one or two overlapping δ-viniferins, respectively.

Peaks **15** and **16** with precursor ion at *m/z* 679.197 were identified as trimers. MS/MS fragmentation showed water loss (product ion at *m/z* 661.187) and 106 Da loss with formation of the ion at *m/z* 573.154. As no trimers have been previously reported in grapes or wines (Pawlus et al. 2012), these signals were assigned to *E*- and *Z*-miyabenol C, two compounds found in *V. vinifera* leaves (Mattivi et al. 2011). The trimer (+)-viniferol D was reported in *V. vinifera* stems (Takaya et al. 2003).

Peaks **11** and **12** (precursor ion at *m/z* 905.260) were identified as tetramers. MS/MS showed formation of the product ion at *m/z* 811.218 due to phenol loss. According to the elution sequence, peak **11** was tentatively assigned to hopheapenol, a compound found in red wine (Guebailia et al. 2006), and peak **12** to ampelopsin H, vaticanol C-like or isohopeaphenol, three compounds recently found in *V. vinifera* leaves (Mattivi et al. 2011).

Lastly, a $[M-H]^-$ ion with experimental mass at *m/z* 241.087 and retention time 20.24 min was found in the Raboso Piave extract (peak **18**). MS/MS showed a 16-Da fragment loss and formation of product ion at *m/z* 225.055. This fragmentation is similar to that of pterostilbene, present in grapes, wines and vine leaves (Mazzotti et al. 2010; Adrian et al. 2000a, b; Douillet-Breuil et al. 1999). These results suggested that this compound is a resveratrol methyl ether. Such compounds have not been reported in grape before, but some resveratrol methyl ethers have been found in other natural extracts (Kerem et al. 2003). The low overall ID score of this compound (Table 1) was probably due to low signal abundance (data not shown).

Table 3 lists the average semiquantitative data of compounds identified in the two grape varieties. Contents in grape of viniferins and resveratrol oligomers are expressed as μg/kg of *trans*-resveratrol and those of glucoside derivatives as *E*-piceid.

Due to the different ionization yields in the experimental conditions used, the response factors of piceatannol, *trans*-resveratrol and *E*-piceid were about 1, 6 and 17 times that of the internal standard, respectively. The response of *Z*-piceid was about half that of the *trans* isomer.

Table 3 Average quantitative data of stilbenes identified in Raboso Piave and Primitivo grape varieties in two replicate analyses

Compound	Formula	*Raboso Piave* μg/kg grape	*Primitivo*
E-astringin (piceatannol gbcoside)	C20H21O9	106.0 ± 5.8	884.2 ± 3.4
E-piceid	C20H21O8	395.3 ± 3.3	2,332.1 ± 48.9
Z-astringin	C20H21O9	101.7 ± 3.3	121.4 ± 1.0
Piceatannol	C14H11O4	41.8 ± 0.5	281.5 ± 10.2
Z-piceid	C20H21O8	1,476.8 ± 68.7	1,776.2 ± 47.4
Dimer 1 (pallidol)	C28H21O6	21.7 ± 0.2	356.2 ± 2.6
Paffidol-3-O-glucoside	C34H31O11	277.3 ± 4.4	187.5 ± 1.6
Dimer 2	C28H21O6	16.8 ± 1.2	131.0 ± 1.1
Trans-resveratrol	C14H11O3	1,134.8 ± 33.8	1,136.4 ± 53.6
Caraphenol B	C28H21O7	36.8 ± 1.7	104.2 ± 1.4
Tetramer 1	C56H41O12	40.3 ± 2.1	101.0 ± 2.3
Tetramer 2	C56H41O12	100.6 ± 0.0	536.6 ± 12.9
Dimer 3 (*Z*-ε-viniferin)	C28H21O6	214.6 ± 5.7	380.4 ± 7.0
Dimer 4 (*E*-ε-viniferin)	C28H21O6	592.5 ± 11.6	702.1 ± 3.4
Trimerl (*Z*-niyabenol C)	C42H31O9	253.9 ± 10.9	236.2 ± 7.8
Trimer 2 (*E*-miyabenol C)	C42H31O9	371.1 ± 28.3	1,357.8 ± 11.9
Dimer 5 (δ-viniferins)	C28H21O6	67.8 ± 0.1	67.9 ± 1.1
Resveratrol methyl ether	C15H13O3	37.3 ± 4.9	

Contents in grape of viniferins and resveratrol oligomers were expressed as μg/kg of *trans*-resveratrol, those of glucoside derivatives as μg/kg of *E*-piceid

This analytical method showed good repeatability, and only small differences between the two repeated analyses were observed in both samples. The data generally match the concentrations reported in the literature for some compounds, and piceids and *trans*-resveratrol were generally confirmed as the principal stilbenes in grape (Gatto et al. 2008; Bavaresco et al. 2003). The Primitivo grape had higher stilbene values with respect to the other variety, with total content greater than 10 mg/kg.

4 Concluding remarks

Suspect screening analysis is an effective approach in grape metabolomics, as it can identify several hundred metabolites in two runs (positive and negative ionization) with minimal sample preparation. It shows good reliability and correctly identified all stilbene derivatives found, as confirmed by MS/MS, although ID scores may be affected by signal intensity. The main difficulty of the method is to have available a specific database of grape metabolites which must be explicitly constructed, as recommended by Scalbert et al. (2009). One advantage of this reiterative approach is that, by increasing the number of samples studied, the database can be expanded.

The present study showed that this approach can also be used to analyse some single classes of compounds involved in cell and tissue metabolism. To the best of our knowledge, this is the first time that such detailed qualitative and quantitative profiling of grape stilbene derivatives has been reported, and studies focusing on other classes of grape compounds are in progress.

Although GrapeMetabolomics is not an open-source database specific for the enological field, the above approach also has potential application to the metabolomics of other plant varieties.

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