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ORIGINAL ARTICLE

High-resolution mass spectrometry metabolomics of grape chemical markers to reveal use of not-allowed varieties in the production of Amarone and Recioto wines

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Abstract

Introduction Grape varieties allowed to produce Amarone della Valpolicella and Recioto DOCG wines are strictly regulated by their disciplinary of production. These are Corvina Veronese and Corvinone grapes, to a lesser extent also Rondinella can be used. The use of other varieties, is not allowed.

Objectives To identify chemical markers suitable to reveal addition of two not allowed grape varieties to the Corvina/Corvinone blend, such as Primitivo or Negro Amaro.

Methods The identification of the secondary metabolites of the four grape varieties was conducted by high-resolution mass spectrometry (HRMS) metabolomics. By using the signals of these metabolites the indexes able to identify the presence of Primitivo or Negro Amaro grapes in the Corvina/Corvinone 1:1 blend were calculated.

Results Indexes of laricitrin (Lr), delphinidin (Dp), and petunidin (Pt) signals were effective to identify the use of 10% Primitivo, while α-terpineol pentosyl-hexoside and linalool pentosyl-hexoside reveal the presence of Negro Amaro in the grape blend.

Conclusions Varietal markers useful to detect the presence of Primitivo and Negro Amaro in the grape blend were identified by HRMS metabolomics, a method suitable to check the identity of grapes on arrival at the winery, as well as the fermenting musts. The effectiveness of the identified markers in the final wines have to be confirmed. Potentially, a similar approach can be used to reveal analogous frauds performed on other high-quality wines.

Keywords Amarone, Recioto · Metabolomics · High-resolution mass spectrometry · Primitivo · Negro Amaro · Wine · Grape

1 Introduction

Amarone della Valpolicella and Recioto are two famous DOCG red wines produced in the Verona province (Veneto region, North East of Italy). Typically, these wines are made by using a blend of autochthonous red grape varieties, such as Corvina Veronese and Corvinone, and Rondinella grape to a lesser extent. The disciplinary of production of Amarone della Valpolicella and Recioto wines, approved by Ministerial Decree March 24 (2010a, b), indicates the types and percentages of grape varieties that can be used in the blend, defines the municipalities in which their cultivation is allowed, the maximum yield per hectare and the winemaking practices allowed. Precisely, the grape blend has to be composed by 45–95% Corvina Veronese and maximum 50% Corvinone, and Rondinella between 5 and 30%. Additionally, up to 25% of other non-aromatic red grape varieties

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cultivated in the Verona province and listed in the National Register of Vine Varieties can be used (D.M. May 7 2004). In the case of Amarone della Valpolicella wines there is the additional requirement of minimum 2 years of barrel aging.

Despite the measures in place to regulate and guarantee the authenticity and geographical traceability of wines, the addition of not allowed grape varieties to enrich the wines in color, aroma or sugar content has been reported (Holmberg 2010).

Primitivo and Negro Amaro are non-aromatic red grape varieties cultivated in Southern Italy. Because of their high sugar and polyphenolic contents these grapes are suitable to be used to increase the alcohol, color and aroma of wines and in the past were often used to reinforce the wines (Del Gaudio and Nico 1960; Del Gaudio and Panzera 1960). The option of using or adding not allowed grapes in a wine blend increases in seasons characterized by unfavorable climatic conditions, thus resulting in yield losses or production of grapes with lower phenolic content.

Over the last years, there has been a growing interest in developing analytical methods for wine authentication (Versari et al. 2014; Villano et al. 2017). The chemical characterization of the products is generally performed through the study of their polyphenolic profiles, such as anthocyanins, flavones, flavonols, and hydroxycinnamic acids, as well as aroma compounds, such as terpenols, norisoprenoids, and benzenoids (De Rosso et al. 2012; Figueiredo-González et al. 2012; Flamini et al. 2001; Ghaste et al. 2015; Mattivi et al. 2006; Nasi et al. 2008).

For the characterization of the wine origin and variety, as well as the cultural and winemaking practices used, methods by nuclear magnetic resonance (NMR), were proposed (De Pascali et al. 2014; Hong 2011; Lee et al. 2009). In addition, liquid chromatography coupled with high resolution mass spectrometry (HRMS) has been shown to be a very effective tool to study grape and wine metabolomes, as it allows the identification of several hundred metabolites in just one analysis (Arapitsas et al. 2016a, b; Arbulu et al. 2015; Rubert et al. 2014; Vaclavik et al. 2011).

Recently, by performing HRMS-*suspect screening* metabolomics using an homemade database containing over 1000 grape and wine compounds, new stilbenes, flavonols, anthocyanins, and glycoside terpenes have been identified in grape (Flamini et al. 2013, 2014, 2015).

In general these studies focused on the characterization of a single variety of grapes or wines (e.g., De Rosso et al. 2016), while to our knowledge there are no studies attempting the identification of different grape varieties in blends.

In the present study, HRMS *suspect screening* analysis was applied to identify the variety markers suitable to detect not allowed use of Primitivo and Negro Amaro grapes in the production of Amarone della Valpolicella and Recioto wines. Main aims of the work were preliminarily to identify the chemical markers characteristic of each grape variety (Corvina, Corvinone, Primitivo and Negro Amaro). Then, by using these chemical markers, to develop a method to reveal the presence of Primitivo or Negro Amaro in the Corvina/Corvinone grape blend.

2 Materials and methods

2.1 Samples and standards

Grape samples of *V. vinifera* Corvina Veronese, Corvinone, Primitivo and Negro Amaro were harvested in 2016 from the vine Germoplasm Collection CREA-VE sited in Susegana (Veneto, Italy). For each variety, 100 berries were collected from five different plants using randomized criteria. The berries were collected at their at the technological maturity of the grapes (maximum soluble solid content in the juice) and kept frozen at − 20 °C until analysis.

Standards of kaempferol-3-*O*-glucoside, quercetin-3-*O*-glucoside, myricetin-3-*O*-glucoside, malvidin-3-*O*-glucoside, kaempferol-3-*O*-glucuronide, (−)-epicatechin, (+)-catechin, procyanidin B1, procyanidin B2, tamarixetin, syringetin and rutin were purchased from Extrasynthese (Genay, France); quercetin, myricetin, kaempferol, *trans*-resveratrol, *trans*-piceid, piceatannol, *E*-piceid, heptyl glucoside and 4′,5,7-trihydroxy flavanone from Sigma-Aldrich (Milan, Italy). δ-viniferin was provided by CT Chrom (Marly, Switzerland). *Z*-piceid was produced by photoisomerization of *E* isomer as reported for the isomerization of *trans*-resveratrol (conversion yield 83%) (Di Stefano and Flamini 2008). *E*-ε-viniferin was extracted from lignified vine cane according to the method by Pezet et al. (2003).

2.2 Sample preparation

Sample preparation for UHPLC–Q/TOF analysis by using 20 grape berries, was performed. After seeds were removed, pulps and skins were ground under liquid nitrogen using an ultraturrax (IKA, Staufen, Germany). Pure methanol was added to the resulting powder ratio 2:1 (v/w) and extraction was carried out for 20 min. After addition of 200 µL of 4′,5,7-trihydroxyflavanone solution 520 mg/L as internal standard, the sample was centrifuged (4000 rpm, 18 °C, 12 min). The supernatant was filtered by using an Acrodisc GHP 0.22 µm filter (Waters, Milford, MA, USA). The filtrate was then collected into a vial for LC-MS analysis. For each variety, two grape samples were studied.

By using the extracts of the four grape varieties, two samples of the following blends were prepared and analyzed in duplicate: (a) Corvina/Corvinone 1:1 (v/v); (b) 90% Corvina/Corvinone 1:1 + 10% Primitivo (v/v); (c) 90% Corvina/Corvinone 1:1 + 10% Negro Amaro (v/v).

2.3 UHPLC–Q/TOF analysis

The analytical system was composed of an Ultra-High Performance Liquid Chromatography system (Agilent 1290 Infinity and G4226A autosampler) coupled with an accurate-mass Quadrupole/Time of Flight (Q/TOF) Mass Spectrometer (Agilent 6540) with mass resolving power of 40,000 (defined at *m/z* 2722 when operated in 4 GHz High Resolution mode) and equipped with the Dual Agilent Jet Stream Ionization source (Agilent Technologies, Santa Clara, CA).

Data acquisition software: Agilent MassHunter version B.04.00 (B4033.2). Chromatographic separation: Zorbax reverse-phase column (RRHD SB-C18 3 × 150 mm, 1.8 µm) (Agilent Technologies, Santa Clara, CA); solvent A 0.1% (v/v) aqueous formic acid, solvent B 0.1% (v/v) formic acid in acetonitrile; gradient 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 5 µL; column temperature 35 °C.

False positives were checked by recording a blank between each pair of samples. For each sample two repeated analyses in both positive and negative ionization mode, were performed.

Q/TOF conditions: sheath gas nitrogen 10 L/min at 400 °C; drying gas nitrogen 8 L/min at 350 °C; nebulizer pressure 60 psig, nozzle voltage 0 kV (negative ionization mode) and 1 kV (positive ionization mode), capillary voltage ± 3.5 kV in positive and negative ion modes, respectively. Signals in the *m/z* 100–1700 range at acquisition rate 2 spectra/s, were recorded. Mass calibrations were performed with standard mix G1969-85000 (Supelco Inc.), residual error for the expected masses between ± 0.2 ppm. An example of tune files performed in two ionization modes is reported in Figure S1 supplemental material.

Negative ionization lock masses: TFA anion at *m/z* 112.9856 and HP-0921 at *m/z* 966.0007 (ion $[M+HCOO]^-$); positive ionization lock masses: purine at *m/z* 121.0509 and HP-0921 at *m/z* 922.0098.

Data analysis performed by Agilent MassHunter Qualitative Analysis software version B.05.00 (5.0.519.0). Coefficient of variance (SD × 100/mean) calculated as on the IS $[M–H]^-$ signal intensity in five extracts of Corvina grape was Cv = 4.0%. For putative identification, score > 60% calculated on the isotope pattern and minimum signal intensity of 10,000 counts confirmed in the two sample analyses was used. The achieved identifications based upon accurate mass calculations of the possible MFs were at level 2 of Metabolomics Standards Initiative (MSI) (Sumner et al. 2007), the number of candidates was then reduced by fitting to the isotopic MS patterns. Deconvolution method applied was Agile. The parameters used for compounds identification are reported in Figure S2.

Targeted data analysis was performed by using the algorithm *Find by Molecular Formula*. Compounds were identified by using the homemade HRMS-database *GrapeMetabolomics*. Identifications were confirmed by performing autoMS/MS of precursor ions in the *m/z* 100–1700 range (collision energy 20–60 eV, acquisition rate 2 spectra/s) and using the standards available. MS/MS spectra of tamarixetin, syringetin, *cis*-piceid, *trans*-piceid, kaempferol-3-*O*-glucuronide, kaempferol-3-*O*-glucoside, quercetin-3-*O*-glucoside, myricetin-3-*O*-glucoside, rutin, procyanidin B1 and B2 were compared to which of the standards (MS/MS spectra of tamarixetin and syringetin available in MetaboLights). For these compounds MSI i.d. level 1, was achieved (Tables S1, S2 supplemental material).

Quantitative analysis of α-terpineol pentosyl-hexoside, linalool pentosyl-hexoside and geraniol pentosyl-hexoside were performed on the signal intensity of $[M+HCOO]^-$ ions and the compounds were expressed as µg heptyl glucoside/kg grape (Flamini et al. 2014). Laricitrin glucoside was quantified on the $[M–H]^-$ signal intensity and expressed as µg 4′,5,7-trihydroxy flavanone/kg grape.

2.4 Statistical analysis

Statistical analysis was performed by PAST 3.01 software (Paleontological statistics software package for education and data analysis; Hammer, Ø., Harper, D.A.T., Ryan, P.D. 2001) using the intensity of the normalized signals recorded. The mean intensity of each metabolite was calculated from two biological samples and two analytical repetitions. Multivariate analysis was performed by using the $[M–H]^-$ or $[M]^+$ ion peak area normalized to the internal standard. Data were normalized (sum), transformed (log), and scaled (mean-centered by SD of each variable). The significance of the parameters used for calculation of the indexes was checked by Tukey's tests ($p < 0.001$).

Heat maps (Euclidean, ward) were calculated by MetaboAnalyst, version 3.0 (http://www.metaboanalyst.ca, last visited at the 15.03.2018, Xia and Wishart 2016). Data were scaled, centered and represented as red (high content), black (medium content), and green (low content) color. Sample clustering (Ward method, Euclidean distance) was performed on the margins of the matrices.

3 Results and discussion

3.1 Identification of metabolites in the four grape varieties

High-resolution MS (HRMS) identifies the molecular formulae of compounds on the basis of two orthogonal data, such as accurate mono-isotopic mass and isotope pattern

(spacing) (Sumner et al. 2007). In negative HRMS, by performing raw data processing using the targeted algorithm *Find by Molecular Formula* and the in-house constructed database of grape and wine metabolites (*GrapeMetabolomics*), around 360 compounds were identified. Extract ion chromatogram (EIC) of the metabolites identified in a Corvina extract is shown in Figure S3a supplemental material.

Within the list of putative metabolites, 91 compounds were selected as potential descriptors of the four grape varieties. In order to have sufficient confidence in their identification, they were chosen on the basis of the following criteria: (i) had an identification score > 90% and (ii) were non-novel compounds already identified in grape, (iii) moreover, are relevant compounds from a technological point of view as they are responsible for organoleptic or nutraceutical characteristics of the products.

The list of selected compounds is reported in Tables S1, S2, they include primary (e.g., aminoacids, phytohormones) and secondary (e.g. flavonols, flavanols, flavanones, stilbenes, glycoside aroma precursors) metabolites. Figure S3b shows the EIC of their $[M–H]^-$ ions. Additional orthogonal data, such as MS/MS fragmentation and the column elution order, confirmed their identification.

Positive MS showed the M^+ signals of the main 16 anthocyanins in grape, such as delphinidin (Dp), cyanidin (Cy), petunidin (Pt), peonidin (Pn), and malvidin (Mv) in the glucoside, acetylglucoside and *p*-coumaroylglucoside forms, and of malvidin caffeoylglucoside. The identification of the anthocyanins by HRMS was performed as previously described (Flamini et al. 2015).

Including these anthocyanins to the above list of selected descriptors yielded a total of 107 potential markers.

The peculiar compounds characteristic of Primitivo and Negro Amaro were highlighted by comparing the four varieties. In particular, glycoside monoterpenols differed significantly. These secondary metabolites are important aroma precursors responsible for the characteristics in particular of aromatic and semi-aromatic grape varieties, such as Muscat, Glera, and Riesling (Flamini et al. 2014), and are also studied for chemotaxonomic aims (Ghaste et al. 2015; Nasi et al. 2008). Figure 1a and Table S3 show that α-terpineol, linalool, and geraniol pentosyl-hexosides were detected in Negro Amaro (gray peaks), a result in agreement with the previous studies conducted on Negro Amaro grape (Tamborra and Esti 2010), while in Corvina/Corvinone grape extract just a weak geraniol pentosyl-hexoside signal (dark peak in Fig. 1a) was observed. In particular, only Negro Amaro showed the presence of α-terpineol pentosyl-hexoside and linalool pentosyl-hexoside, whereas low level of geraniol pentosyl-hexoside were also found in Corvina, Corvinone, and Primitivo extracts. Negative ionization of monoterpene glycosides produces high signals of both $[M–H]^-$ and $[M+HCOO]^-$ ions (Flamini et al. 2014). Also heptyl glucoside showed a high signal of the $[M+HCOO]^-$ adduct, therefore glycoside monoterpenols were quantified on this signal and expressed as μg/kg of heptyl glucoside.

Laricitrin (Lr) glucoside stood out as it showed a much higher signal in Primitivo with respect to Corvina and Corvinone. The content of this flavonol was by far the highest in Primitivo and particularly low in Corvina and Negro Amaro (Fig. 1b and Table S3).

Visualization of the heatmaps provides an immediate overview of the differences among the samples (Xia et al. 2012). The heatmaps in Fig. 2 (negative ionization MS) and Fig. 3 (anthocyanins detected in positive ionization) were used for comparison of the metabolite profiles of Corvina, Corvinone and Primitivo. In Figures S2 and S3 Corvina and Corvinone are compared to Negro Amaro.

Figure 2 shows that in Primitivo Lr and myricetin (Mr) derivatives were significantly higher than in Corvina and Corvinone (red bars), instead quercetin (Q), isorhamnetin and procyanidins were lower (green bars). Significant

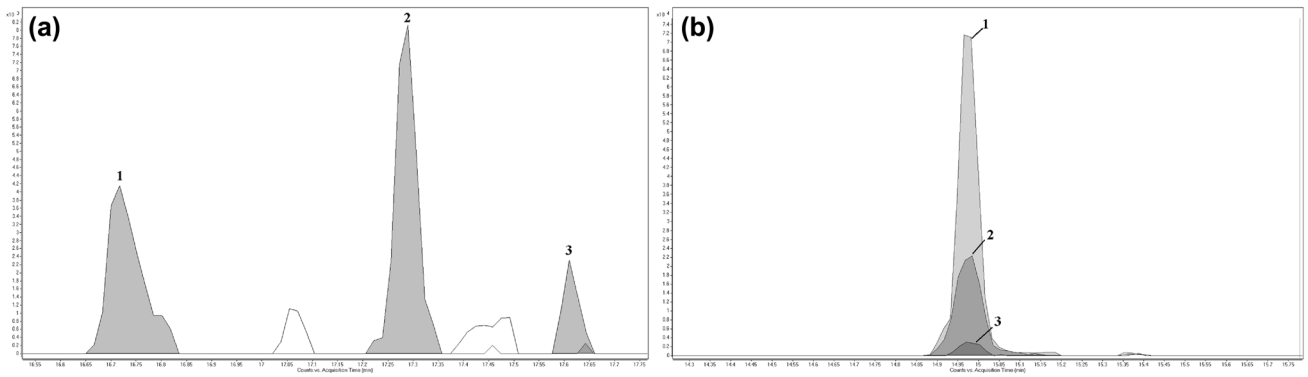

Fig. 1 EIC of some chemical markers identified as characteristic of the grape varieties studied. **a** Signals of glycoside terpenols in Negro Amaro (gray) and Corvina/Corvinone 1:1 blend extract (dark grey). (*1*) α-terpineol pentosyl-hexoside, (*2*) linalool pentosyl-hexoside, (*3*) geraniol pentosyl-hexoside. **b** EIC of laricitrin-3-*O*-glucoside signals in Primitivo (*1*), Corvinone (*2*), and Corvina (*3*) extracts

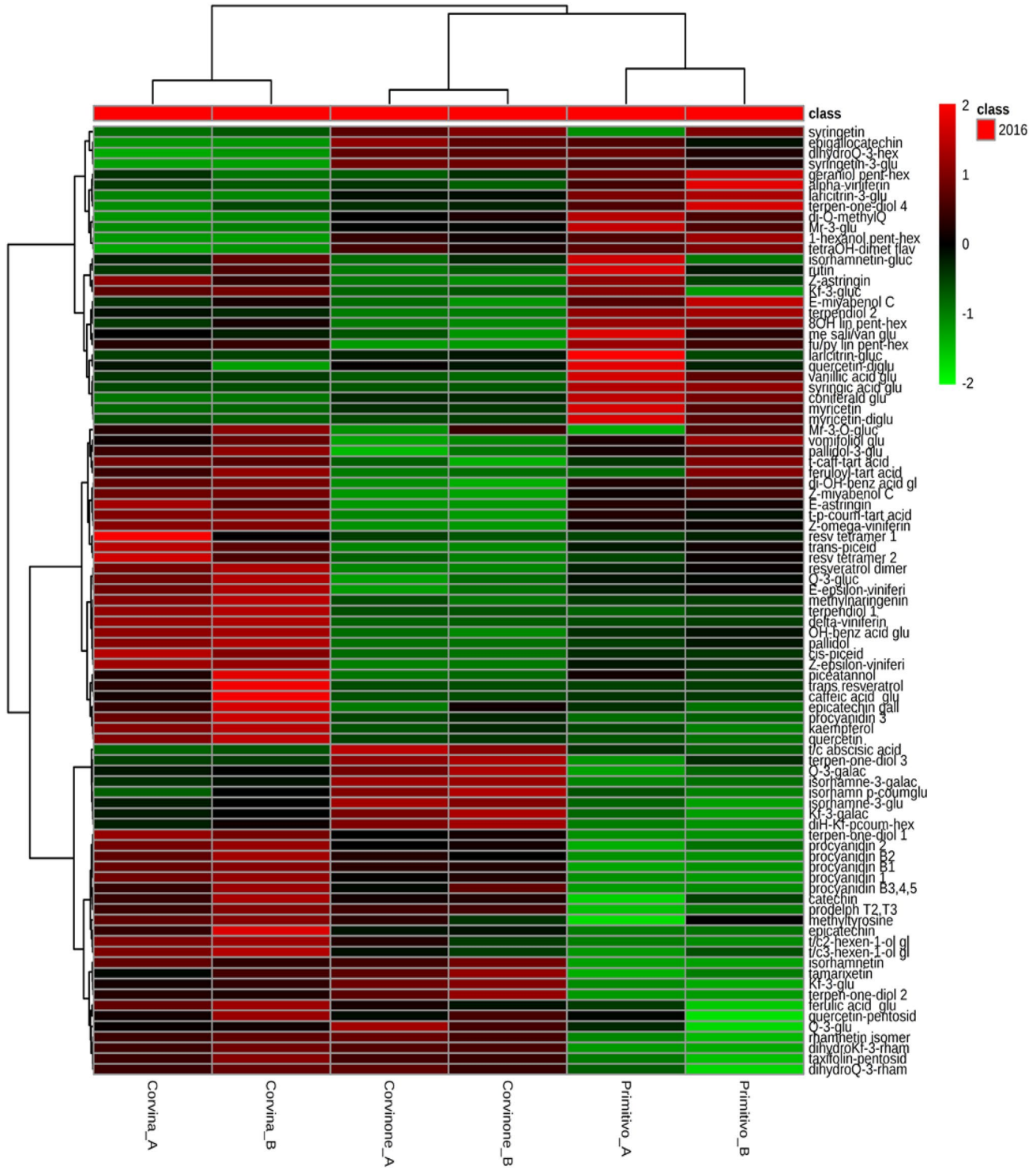

Fig. 2 Heatmap of the 91 metabolites detected in negative-ion mode analysis used to characterize Corvina/Corvinone/Primitivo grape varieties (n = 2, A and B). Compound abbreviations are reported in Table S2

differences among the samples for resveratrol and its derivatives were observed, with the concentration in Primitivo grapes laying in the middle. However, because these compounds are phytoalexins whose biosynthesis can be influenced by external factors, such as biotic and abiotic stresses, they were not assumed as variety markers.

Figure 3 shows that the contents of malvidin (Mv), delphinidin (Dp), petunidin (Pt) and their derivatives were

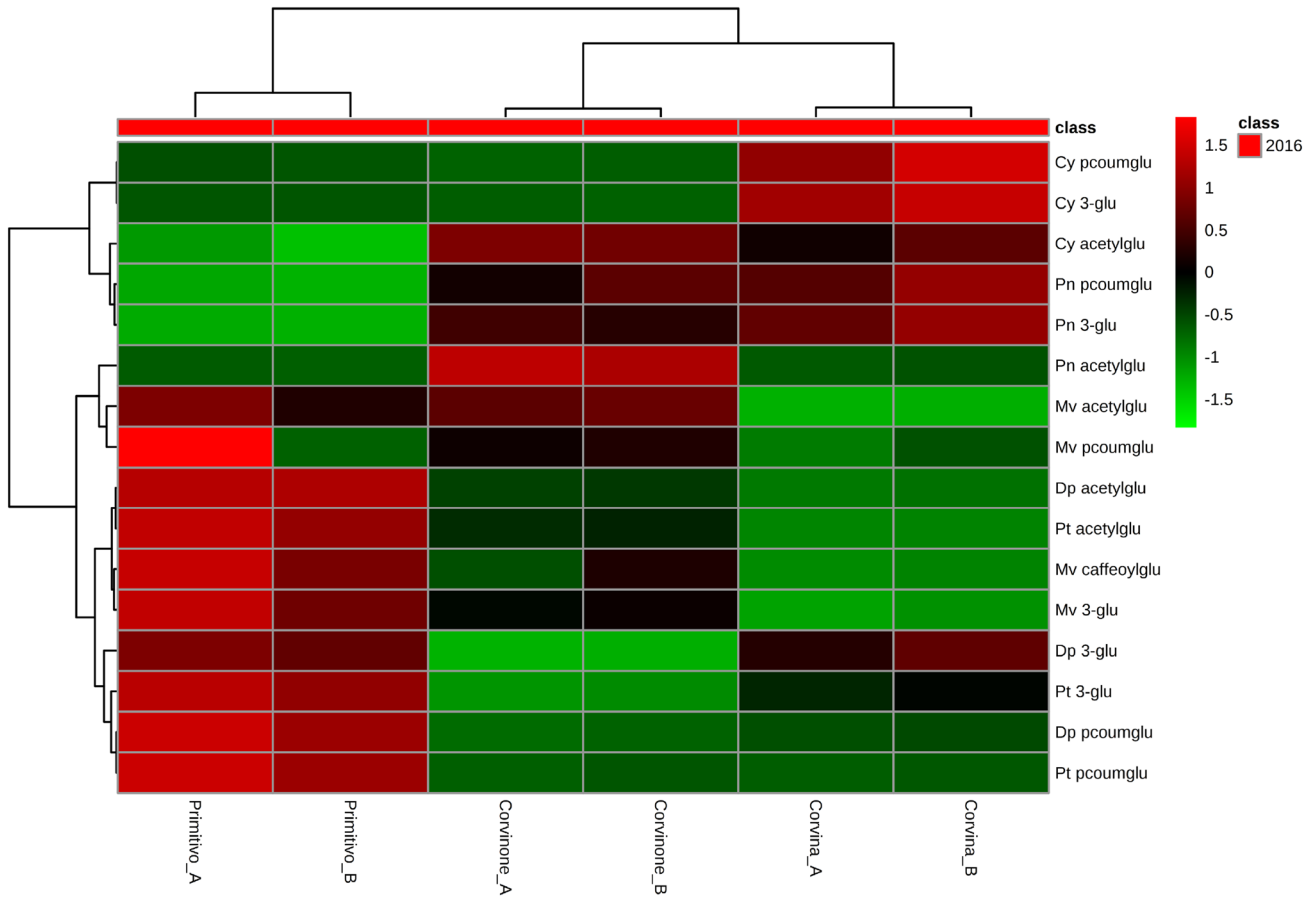

Fig. 3 Heatmap of anthocyanins detected in positive-ion analysis used to characterize Corvina/Corvinone/Primitivo grape varieties (n = 2, A and B). Compound abbreviations are reported in Table S2

significantly higher in Primitivo with respect to Corvina and Corvinone (red bars), instead peonidin (Pn) and cyanidin (Cy) were lower.

In Negro Amaro, beside the relevant presence of glycoside terpenols, Mr content was significantly higher than Corvina and Corvinone, as well as Cy-3-*O*-monoglucoside in agreement with a previous study (Tamborra and Esti 2010). Also Dp is high and in general the contents of *p*-coumaroyl and acyl glucoside anthocyanins are lower (Figures S4 and S5).

3.2 Study of secondary metabolite indexes as variety markers

Previous investigations have shown that the anthocyanin and flavonol profiles can differentiate the grape varieties and therefore can be used as authenticity markers (Ortega-Regules et al. 2006). Because of environmental and agronomical factors their absolute content in grape may vary in different vintages, but their qualitative profiles and ratios are relatively stable and linked to the variety (Cacho et al. 1992; Carreno et al. 1997; Ortega-Regules et al. 2006; Squadrito et al. 2007). The polyphenolic biosynthesis is regulated by genetic factors and the distribution of these compounds varies considerably among different grape cultivars (Fernández-López et al. 1998). In agreement with the findings for anthocyanin profiles, Downey et al. (2003) reported that the pattern of flavonol accumulation over three season had very little variation. Both the flavonol and anthocyanin profiles were successfully studied to discriminate among the grape varieties (Mattivi et al. 2006; Figueiredo-Gonzalez et al. 2013). It was observed, that the flavonoid 3′5′hydroxylase (F3′5H) enzyme is regulated by the variety, differently from F3′H, which is always active (Squadrito et al. 2007). For example, to discriminate between Carménère and Merlot wines the Q/Mr ratio was studied (von Baer et al. 2005).

To identify the variety markers lowly affected by external factors and representing the individual varieties, we grouped the compounds and calculated the indexes, as shown in Table 1. The parameters were calculated as the sum of the normalized chromatographic signal intensities

Table 1 Indexes studied as markers to identify the presence of Primitivo or Negro Amaro grapes in the Corvina/Corvinone 1:1 blend

Index	Parameter	Metabolite signals sum
Mr/Q	Mr	Myricetin, myricetin-3-*O*-glucuronide, myricetin-3-*O*-glucoside, myricetin-diglucoside
Mr/Kf	Q	Quercetin, quercetin-diglucoside, quercetin-pentoside, quercetin-3-*O*-glucuronide, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, rutin
Mr/dihydroKf	Kf	Kaempferol, kaempferol-3-*O*-glucuronide, kaempferol-3-*O*-galactoside, kaempferol-3-*O*-glucoside
Mr/dihydroQ	dihydroKf	Dihydrokaempferol-3-*O*-rhamnoside
Q/dihydroQ	dihydroQ	Dihydroquercetin-3-*O*-rhamnoside, dihydroquercetin-3-*O*-hexoside, taxifolin-pentoside
Lr/Q	Lr	Laricitrin-glucuronide, laricitrin-3-*O*-glucoside
Lr/Kf	Iso	Isorhamnetin, isorhamnetin glucuronide, isorhamnetin-3-*O*-galactoside, isorhamnetin-3-*O*-glucoside
Lr/Iso	Dp	Delphinidin acetylglucoside, delphinidin-*p*-coumaroylglucoside, delphinidin-3-*O*-glucoside
Dp/Cy	Cy	Cyanidin acetylglucoside, cyanidin-*p*-coumaroylglucoside, cyanidin-3-*O*-glucoside
Pt/Cy	Pt	Petunidin acetylglucoside, petunidin-*p*-coumaroylglucoside, petunidin-3-*O*-glucoside
Dp/*p*-coum anthocyanins	*p*-Coum anthocyanins	Dp, Cy, Pt, Pn and Mv *p*-coumaroylglucosides, Mv caffeoylglucoside
Glu anthocyanins/acyl anthocyanins	Acyl anthocyanins	Dp, Cy, Pt, Pn, and Mv *p*-coumaroylglucoside and acetylglucoside derivatives
	Glu anthocyanins	Dp, Cy, Pt, Pn and Mv 3-*O*-monoglucosides

Parameters used for calculation of the indexes are the sums of normalized chromatographic signals of the corresponding metabolites reported on the right

of the corresponding metabolites. Indexes calculated for the four single varieties are shown in Figures S6–S8.

Primitivo had the highest Mr/Q and Mr/Kf ratios in respect to the other varieties, being driven by the higher Mr as well as the lower Q and Kf (Figure S6), and the maximum values of the Lr indexes (Figure S7). Also the Mr/dihydroKf and Mr/dihydroQ indexes were higher in Primitivo and Negro Amaro, in particular Mr/dihydroKf was 10-fold than in Corvina and Corvinone. The high quercetin level in Negro Amaro was evidenced by the higher Q/dihydroQ ratio.

Primitivo also stood out for the highest Dp/Cy and Pt/Cy ratios which was due its high Dp and Pt contents. Higher Dp/*p*-coumaroyl anthocyanins and monoglucoside/acyl anthocyanins ratios were found in Negro Amaro (Figure S8), in agreement with the fact that this variety is characterized by high Dp content (Tamborra and Esti 2010).

In the biosynthesis of anthocyanins the enzymes 3′methyltransferase (3′OMT) and flavonoid-3′,5′-hydroxylase (F3′5′H) transform Cy into Pn and into Dp, respectively. Different activity of F3′5′H affects the ratios of dihydroxy/trihydroxy anthocyanins (Carreno et al. 1997), 3′OMT induces methylation of Dp into Pt, and Pt into Mv (Heller and Forkmann 1988). The higher levels of trihydroxylated anthocyanins found in Primitivo highlights a stronger activity of F3′5′H in this variety. Moreover, a study on genes expression of F3′H and F3′5′H showed that the biosynthetic pathways of flavonols and anthocyanins are closely related (Jeong et al. 2006). Our findings are in agreement with these observations, as the high hydroxylation activity in Primitivo is observed in the increase of tri-substituted anthocyanins (Dp and Pt) as well as the higher Mr, and higher 3′5′OMT activity during the veraison.

3.3 Identification of Primitivo and Negro Amaro in Corvina/Corvinone 1:1 blend

The calculated indexes were effective in discriminating Primitivo and Negro Amaro from Corvina and Corvinone when analyzed in single varieties. However, when dealing with this particular type of illegal practice, the situation is different, as the makers need to be applicable to analyze grape mixtures. Therefore, to investigate if these indexes could also be effective in grape blends, Corina/Corvinone (1:1) was added with 10% Primitivo and 10% Negro Amaro extract, respectively. It has to be noted that the quantities of Negro Amaro and Primitivo grapes added to the blend were quite small to test whether the method is effective in identifying also low presence of these grapes, but in the case of wine frauds the illegal addition would certainly be higher in order to have an effective economical advantage. Therefore, also a projection of the theoretical data for additions of the grapes at 15% and 20% to the Corvina/Corvinone 1:1 blend, was calculated (C/C + Prim15%, C/C + Negr15%,

C/C + Prim20%, C/C + Negr20%) and the results are shown in Figures S9, S10, and S11.

Study of the indexes related to Mr could reveal the presence of both Primitivo and Negro Amaro in the grape blend already at 10%, with increasing values for higher additions. The curves of Mr/Kf, Mr/dihydroKf, and Mr/Q indexes were characterized by high slopes, which clearly showed an addition of Primitivo. The Mr/dihydroQ and Mr/dihydroKf ratios revealed the presence of Negro Amaro in the grape blend (Figure S9).

Figure S10 shows that the Lr indexes increased with addition of Primitivo to the grape blend (in particular Lr/Q and Lr/Kf), but not for Negro Amaro. Addition of Primitivo at 10% can be revealed also by the increase of Dp/Cy and Pt/Cy ratios. Indexes of anthocyanins, such as Dp/*p*-coumaroyl anthocyanins and monoglucoside/acyl anthocyanins, increase for the addition of Negro Amaro, but not of Primitivo (Figure S11).

Comparison between the theoretical and experimental indexes for the 10% additions of Primitivo and Negro Amaro to the grape blend showed good agreement. Only in the case of the Mr/dihydroKf ratio the theoretical data resulted higher than the experimental ones for both varieties (Table S4).

PCA (Fig. 4) and Cluster analysis (Figure S12) were performed by using as variables the indexes in Table 1 measured in two samples of Corvina/Corvinone 1:1 (CC), and the blend added of 10% Primitivo (CCPrim) and 10% Negro Amaro (CCNegr). The first two PCA components grouped 92.3% of the total variance (first component 55.7% and second component 36.6%, respectively). Samples were clearly separated by variety: Dp/*p*-coum anthocyanins and monoglucoside/acyl anthocyanins are the main factors which separated the CCNegr samples from the others (Figure S5) due to the higher content of monoglucoside anthocyanins with respect to Corvina and Corvinone. The blends containing Primitivo were separated from the others mainly by the Lr indexes and by Dp/Cy and Pt/Cy ratios which reflect the high content of hydroxylated and methylated compounds in this variety. In general, the Mr indexes separate the blends containing Primitivo and Negro Amaro from Corvina/Corvinone 1:1 (Fig. 4).

4 Conclusions

HRMS-*suspect screening* metabolomics allowed to identify some varietal markers useful to characterize the four grape varieties studied, and to propose a method based on the calculation of secondary metabolite indexes to identify the not-allowed use of Primitivo and Negro Amaro grapes in

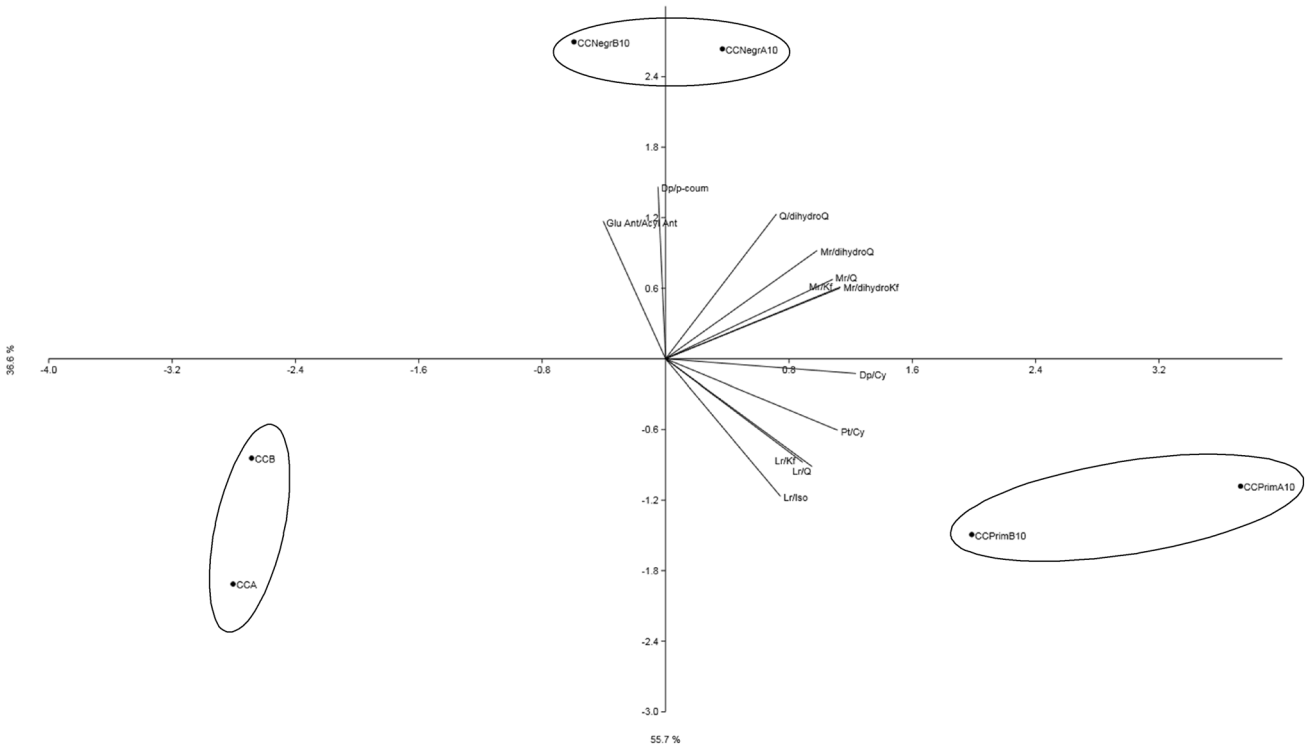

Fig. 4 Principal component analysis of the indexes calculated in the grape blends. CCA, CCB: Corvina/Corvinone 1:1 extracts; CCPrimA10, CCPrimB10: extracts Corvina/Corvinone 1:1 + 10% Primitivo; CCNegrA10, CCNegrB10: extracts Corvina/Corvinone 1:1 + 10% Negro Amaro; Grouping provided by Cluster Analysis, is shown (Figure S12)

the production of Valpolicella wines. This is the first method proposed for the identification of single varieties in a grape blend. The method allows to detect additions of the two varieties at 10% to the Corvina/Corvinone blend. In particular, the addition of Primitivo increases the indexes related to Lr, Dp, and Pt; the presence of α-terpineol pentosyl-hexoside and linalool pentosyl-hexoside signals in the chromatogram could reveal the use of Negro Amaro. Given that additions of at least 20–30% are expected for yielding a significant economic advantage, this method represent an effective tool to reveal the frauds carried out during grape blend preparation and in fermenting musts.

It has to be noted that the samples studied came from the same collection and were harvested in one vintage only. Consequently the findings do not take into account variables such as vineyard and vintage. However, the proposed approach represents an interesting model of study as it can potentially be applied to identify illegal additions of not-allowed grapes and musts to other high-quality wines.

Future investigations will be aimed at verifying whether it can be applied with success also to the wines. As a matter of fact, despite that the fermentation greatly impacts the metabolite profile of a wine, generally the products still partially maintain their varietal profiles. Therefore, it is expected that some of the identified indexes can be applied to the finished wines. For example, for aged wines the study could be extended to new compounds formed by transformation of the metabolites characteristic of the variety, such as 3′5′-substituted pyranoanthocyanidins in the case of Primitivo and free monoterpenes for Negro Amaro.

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Author contributions MDR performed the analyses and data acquisition. CMM performed the data interpretation, data analysis and wrote the paper. GG and ADV performed the experiments. RF conceived and designed the experiment and wrote the paper.

Compliance with ethical standards

Accession number Meta data and metabolite profiles associated with the research are available through the EBI MetaboLights database under the following accession number: MTBLS732.

Conflict of interest The authors declare that they have no conflict of interest.

Research involving animal and human rights This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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