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Ultrafast Polyphenol Metabolomics of Red Wines Using MicroLC-MS/MS

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Abstract

The taste and quality of red wine are determined by its highly complex mixture of polyphenols and many other metabolites. No single method can fully cover the full metabolome, but even for polyphenols and related compounds, current methods proved inadequate. We optimized liquid chromatography resolution and sensitivity using 1 mm i.d. columns with microLC pumps and compared data-dependent to data-independent (SWATH) MS/MS acquisitions. A high-throughput microLC-MS method was developed with a 4 min gradient at 0.05 mL/min flow rate on a Kinetex C18 column and Sciex TripleTOF mass spectrometry. Using the novel software MS-DIAL, we structurally annotated 264 compounds including 165 polyphenols in six commercial red wines by accurate mass MS/MS matching. As proof of concept, multivariate statistics revealed the difference in the metabolite profiles of the six red wines, and regression analysis linked the polyphenol contents to the taste of the red wines.

Keywords

red wines; polyphenols; metabolomics; 1 mm i.d. column; microLC-MS/MS

INTRODUCTION

Red wine is a prime example of how food quality is determined by its highly complex mixture of metabolites. While all wines are characterized by their abundance of alcohol, sugar, and simple organic acids, red wine is specifically known for its diversity of phenolic compounds, including phenolic acids, flavonoids, anthocyanins, and tannins.[1] For example, 111 polyphenols were reported in red wines according to Phenol-Explorer,[2] an online database of polyphenol contents in food. All of those complex components contribute

together to the color, aroma, taste, and mouthfeel of red wines. For example, vanillin and 4-allyl-2-methoxyphenol were reported to contribute to the *wood–vanillin–cinnamon* aroma in aged red wines.[3]

The polyphenol components in red wines have drawn a lot of interest due to their high antioxidant potential.[4] One well-known example is resveratrol, which has been studied intensively as an antiaging and anticarcinogenic agent.[5] The phenolic composition also plays a great role in the color stability and taste of red wines.[6] To qualitatively and quantitatively analyze the polyphenols and other components in red wine, reversed-phase high-performance liquid chromatography (HPLC) methods were developed with either ultraviolet (UV) or mass spectrometry (MS) detection.[7,8] However, most of the current methods measured only a few abundant components of red wines[9,10] and were inadequate for giving full coverage of these complex metabolites. Hence, we set out to develop and apply a suite of advanced metabolomic methods to utilize comprehensive profiling of polyphenols and related compounds and test these methods by chemometrics.

Miniaturization of column and instrumentation is one of the leading trends in the field of liquid chromatography. Ultrahigh-pressure liquid chromatography (UHPLC) columns with internal diameters (i.d.) of 2.1 mm have long taken over conventional HPLC columns of 4.6 mm for their higher sensitivity, faster separation, and lower consumption of organic solvents.[11] For the same reasons, 1 mm i.d. columns have been developed to further improve the sensitivity compared to 2.1 mm i.d. columns.[12] Consequently, 1 mm i.d. columns have been applied to a variety of studies of small molecules such as amino acids,[13] flavonoids,[14] and antibacterial drugs.[15] However, practical use of 1 mm i.d. columns is still limited due to instrumental constraints, because both the flow rate and the extra-column dispersion source need to be scaled down for optimal performance as the column volume decreases.[16] Recently, commercial instruments designed for microLC performance have become available, such as the Eksigent MicroLC 200 system and Dionex UltiMate 3000 Quaternary Micro LC system. A previous study showed that the microLC instrument provided significantly higher efficiency for 1 mm i.d. columns compared to common UHPLC instrument.[17] At the same time, more small-sized columns have been developed.[18,19] We here evaluated different columns and optimized microLC methods for comprehensive polyphenol profiling in red wines.

MATERIALS AND METHODS

Comparison of 1 mm i.d. Columns Using Polyphenol Standards.

An Eksigent MicroLC 200 was coupled to a SCIEX TripleTOF 5600 (SCIEX, Redwood City, CA, USA) for MS measurements from m/z 60 to 1250 in electrospray positive mode with spray voltage 5.5 kV at scan rate of 10 spectra per second. Three reversed-phase C18 columns of 1 mm i.d. and 5 cm length were examined, including a Kinetex C18 2.6 μm (Phenomenex, Torrance, CA, USA), an InertSustain C18 2.0 μm (GL Sciences, Tokyo, Japan), and an Acquity CSH 1.7 μm (Waters, Milford, MA, USA). The Kinetex and InertSustain columns were prototype columns, whereas the Acquity was a commercially available column. The standard mixture for LC-MS experiments contained (1) umbelliferone, (2) rutin, (3) naringin, (4) quercetin, (5) naringenin, (6) 6-hydroxy-flavone,

(7) genistein, (8) genistin, (9) daidzein, (10) daidzin, (11) glycitein, (12) glycitin, and (13) chrysin at 1.0 μg/mL each, in 10:90 methanol/water. Mobile phase A was water with 0.1% acetic acid, whereas mobile phase B was acetonitrile with 0.1% acetic acid. The LC gradient was from 10 to 90% B with different flow rates (0.025, 0.05, 0.075, 0.1 mL/min) and gradient times or gradient volumes (4 min, 8 min, and 0.6 mL). Injection volume was 0.2 μL. All experiments were carried out in duplicate. Data were processed by SCIEX MultiQuant 2.1 software.

LC Gradient Optimization Using a Red Wine Example.

To optimize LC gradients for total peak discovery, metabolomic tests were performed with wine extracts from a 2012 Petite Sirah (Concannon Vineyard, Livermore, CA, USA). Five hundred microliters of raw wine was added to an ice-cold premixed extraction solvent consisting of 500 μL of water and 500 μL of ethyl acetate. Samples were shaken for 5 min and centrifuged for 3 min at 16100 rcf, and then 300 μL of the ethyl acetate phase was transferred to a second vial and evaporated to dryness using a speed vacuum concentrator. As method blank control, instead of raw wine, 500 μL of water was added to the extraction solvent, and then the same protocol was applied. On the day of the experiment, samples were reconstituted in 150 μL of 90:10 acetonitrile/water, briefly vortexed, sonicated for 5 min, centrifuged for 3 min at 16100 rcf, and then transferred to HPLC vials with inserts. The same gradient series and mass spectrometry measurements were applied as to the polyphenol standards. The raw WIFF data files were converted to ABF files with Reifycs Analysis Base File Converter (Reifycs Inc., Tokyo, Japan) and processed by the freely available MS-DIAL[20] software (ver. 1.58) (http://prime.psc.riken.jp/Metabolomics_Software/).

Metabolome Composition Analysis of Six Red Wines Using the Optimized MicroLC-MS/MS Method.

Six bottles of red wines were opened and extracted fresh, including a 2009 Syrah (Putah Creek Winery, Solano county, CA, USA), a 2012 Pinot noir (Black Mountain Vineyard, Sonoma County, CA, USA), a 2010 Cabernet Sauvignon (Mosaic Winery, Alexander Valley, Sonoma County, CA, USA), a 2012 Zinfandel (Cline Cellars Winery, Sonoma County, CA, USA), a 2012 Petite Sirah (Blue Fin Winery, Napa, CA, USA), and a 2012 Merlot (Blue Canyon Wine Co., Greenfield, CA, USA). A panel of 14 nontrained adults tasted the remaining wines directly afterward. Overall likeability[21] (averaging aroma, flavor, and finish) was scored from 1 (worst) to 10 (best) for each wine with blinded labels. Raw wines were extracted in the similar way as the preliminary test, but instead of reconstituting the extracts with 150 μL of solvent, 75 μL was used to concentrate the samples. Six technical replicates were prepared for each wine as well as the method blank. As a result of the microLC-MS method optimization, a gradient of 4 min at a flow rate of 0.05 mL/min on a Kinetex C18 column was selected for data-dependent MS/MS acquisition for these wines. To compare the data-dependent and data-independent (SWATH) method, a 12 min gradient was used at a flow rate of 0.05 mL/min. MS/MS data were acquired in both positive and negative mode at a collision energy of 30 $\pm$ 15 V. For data-dependent acquisition of MS/MS, precursor ions from m/z 60 to 1250 were fragmented with a cycle time of 0.125 s. For data-

independent acquisition (SWATH), precursor ions from m/z 60 to 800 were fragmented, with a cycle time of 0.4 s. A SWATH window of 50 Da was used.

Data Processing, Statistics Analysis, and Visualization.

Peak picking and alignment were performed by MS-DIAL (ver. 1.58). Representative MS/MS spectra were exported from MS-DIAL in MGF format for compound identification. NIST MS PepSearch was used to search the MGF file against MS/MS libraries, including NIST14, MassBank,[22] ReSpect,[23] and METLIN.[24] For the remaining unknown compounds, the freely available MS2Analyzer[25] software (https://sourceforge.net/projects/ms2analyzer/) was used to search for characteristic neutral losses for glycosides, including pentosides (132.063 Da), hexosides (162.053 Da), deoxyhexosides (146.058 or 164.068 Da), acetylhexosides (204.063 Da), and hexuronide (194.043 Da). An m/z window of 0.005 Da and a relative intensity threshold of 0.5 were selected as input parameters. Each hit from MS2Analyzer output was queried by its neutralized molecular mass in Phenol-Explorer and by its precursor m/z value in the online METLIN database to extract potential structure candidates.

Principle component analysis (PCA) was performed for all the annotated compounds (reverse-dot score > 800) in positive ionization mode with MetoboAnalyst.[26] Before PCA, samples were normalized by sum, followed by log transformation and pareto scaling. Orthogonal signal correction partial least-squares (O-PLS) modeling was performed between the average tasting scores and peak intensities of all annotated compounds using Devium (https://github.com/dgrapov/devium). Two latent variables (LVs) were determined on the basis of the criteria of maximum Q^2 in the range from LV1 to LV10. Twenty permutation tests were used to validate the model, and 10 top features were selected for each sign. Finally, a chemical structural (Tanimoto > 0.9) and mass spectral similarity (similarity > 0.4) network was created for all annotated compounds in positive ionization mode with MetaMapR[27] and Cytoscape.[28] MS/MS spectra were pretreated (m/z rounded up to 0.1, intensity filter > 5% of base peak) before the analysis to improve the similarity score.

RESULTS AND DISCUSSION

Method Optimization: Concepts and Methods.

A good chromatographic method is essential to LC-MS experiments to minimize ion suppression effects, to help distinguish isomers, and to increase the number of features detected and annotated in untargeted metabolomics. In this work, we used two parameters, peak capacity and peak height, to quantify the performance of chromatographic separations. Peak capacity is defined as the number of peaks that can be separated with unit resolution within the gradient time with a one-channel detector. Peak intensity, here defined as peak height, is a measure of method sensitivity. It is noteworthy that peak intensity is not only related to sample concentration, injection volume, and detector sensitivity but also strongly influenced by chromatographic performance. For example, changing the column internal diameter from 2.1 to 1 mm can increase peak intensity by a factor of 4.41.[12]

However, chromatography is a process involving many factors, including stationary phase, particle size, column size, mobile phase, flow rate, gradient, etc. To optimize the microLC-MS method for phenolic compounds, we used different stationary phases, particle sizes, flow rates, and gradients to separate 13 standard phenol compounds as well as a preliminary red wine sample and evaluate the performance of chromatography on the basis of peak capacity, peak height, and peak numbers extracted from red wine sample.

Method Optimization: Column Selection with Phenol Standards.

First, we compared the performance of three 1 mm i.d., 5 cm length columns in terms of peak capacity and intensity: the Kinetex C18 (particle size = 2.6 μm), the InertSustain C18 (2.0 μm), and the Acquity CSH (1.7 μm) columns. To test the effects of various liquid chromatography conditions on those columns, experiments were performed under 11 gradient conditions at flow rates of 0.025–0.1 mL/min. Averaged peak widths and peak capacities were calculated for all of the standard peaks as in eqs 1 and 2.

$$\text{peak width} = (\text{Peak width at half} - \text{height} / 2.35) \times 4 \tag{1}$$

$$\text{Peak capacity} = \text{gradient time} / \text{peak width} \tag{2}$$

Figure 1 shows extracted ion chromatograms (EICs) of the phenol standards separated by the Kinetex column in a 4 min gradient at 0.05 mL/min, with an average peak width of around 2 s.

Figure 2 shows peak capacities and average peak intensities for the three columns under one representative gradient condition (see all results in Table S1). In general, the tested polyphenols were eluted at later retention times using the InertSustain column in comparison to the Acquity column and, hence, showed increased ionization efficiency and wider peaks because they eluted at higher organic/water solvent ratios. Taking the Kinetex column as an example to investigate the chromatography under different LC conditions, we observed the following trends: (1) peak capacity increased as the gradient time and flow rate increased; (2) peak height, by contrast, decreased as the gradient time and flow rate increased; (3) when the gradient volume (flow rate × gradient time) was constant, peak capacity and peak height also remained relatively constant. Here we estimated the optimum conditions by calculating and comparing the product of the peak capacity and peak height for each gradient condition. On the basis of the calculation (Table S1), an optimum condition for the 1 mm i.d. column was found using a 4 min gradient at 0.05 mL/min for the given polyphenol standards. Overall, under the conditions employed here, the Kinetex column provided the highest peak capacity and intensity among the three columns. We therefore selected this column in all following wine polyphenol studies. Other columns may perform better under optimized conditions for each column.

Method Optimization: Gradient Finalization with Red Wine Sample.

The same gradient experiments were repeated with an extracted test red wine sample to evaluate the effect of the LC parameters under realistic conditions. The data were processed

by MS-DIAL with an intensity threshold of 4000 counts. The numbers of detected *m*/*z*–retention time (rt) features is shown in Table 1. The trend was similar to the trend of peak intensities, indicating that peak intensity contributed more to the overall *m*/*z*–rt count than peak capacity. With a given intensity threshold for the data processing, higher peak intensities generally led to more detected peaks even if peaks were a little broader, on average, than comparative methods. Hence, the peak capacity parameter was less important to overall feature detections because mass spectrometers are very effective multichannel detectors, enabling selective quantification of many compounds even if these are coeluting (except for isomers). We observed that separation of isomers was related to the peak capacity, but it was also affected by the selectivity of the stationary phase and mobile phase. In conclusion, the 4 min gradient at flow rate of 0.05 mL/min that was selected by polyphenol standards was also confirmed to be highly suitable in the comprehensive metabolomics analysis of red wine samples.

MicroLC-MS/MS Analysis of Six Red Wines: Compound Identification.

Six red wines from different grape varieties and brands were analyzed to showcase the application of the optimized microLC-MS/MS method. Six replicates of each red wine were extracted with ethyl acetate[29] and measured in positive and negative electrospray ionization mode. Data-dependent MS/MS spectra were acquired to facilitate structural annotation of metabolites. MS-DIAL was used for peak detection and alignment, whereas NIST MS PepSearch was used to search the MS/MS spectra against libraries including NIST14, MassBank, ReSpect, and METLIN. Two hundred and sixty-four compounds were annotated with a weighted reverse-dot score[30] >800. Among the 264 compounds, 165 metabolites were classified as phenol compounds. The full list of annotated compounds is presented in Table S2 including detailed metadata such as retention times, precursor *m*/*z*, InChIKeys, and intensity tables. To further validate the annotation results, we confirmed 18 compounds by injecting authentic reference standards (see Table 2).

For many peaks, MS/MS library searches assigned the same compound name to multiple *m*/*z*–rt features. By definition, compounds that show different physicochemical properties (such as retention time) but have the same exact mass are isomers. We further defined here close positional isomers if peaks were well separated and MS/MS library queries yielded reverse-dot score >800. Among the 264 annotated compounds, 133 were found with one or more isomers, due to the complexity of polyphenol structures and high possibility for positional isomers. Figure 3 demonstrates the detection of a novel resveratrol isomer in addition to the well-known *cis*- and *trans*-forms. Other abundant isomers include, for example, isodihydroquercetin, isoprocyanidin B2, and isocaffeic acid. A complete structural characterization of such isomers is impossible by MS alone, given the large number of theoretically possible positional isomers, the limited availability of authentic standards, and the complexity of wine samples that forbids easy routes for peak purifications and nuclear magnetic resonance (NMR) determinations. For example, for five dihydroquercetin isomers detected in the red wines samples, 95 candidate structures were found in the structure database ChemSpider, but only one compound is available to be purchased from Sigma-Aldrich.

Although 264 compounds have been annotated using MS/MS library search, more than 1000 features that are related to true compounds remain unknown. To further characterize the structures or substructures of unknowns, we used MS2Analyzer in combination with online databases such as Phenol-Explorer and METLIN. MS2Analyzer is a software tool using characteristic neutral losses and product ions for small molecule substructure annotation. Because many polyphenols are in glycoside forms, we queried the characteristic neutral losses for pentosides, hexosides, deoxyhexosides, acetylhexosides, and hexuronide in all of the unknown MS/MS spectra with MS2Analyzer. For those MS/MS spectra containing the above neutral losses, their precursors were searched in the Phenol-Explorer and METLIN databases. Potential candidates with matching accurate mass and substructures for 28 features are reported in Table S3. Figure 4 shows an example of MS/MS spectrum, characteristic neutral loss, and potential structure of unknown m/z 521.132 eluting at 1.68 min.

Over the past few years, the data-independent MS/MS acquisition (DIA, specifically SWATH) method has drawn a lot of interest from LC-MS researchers. In contrast to the conventional data-dependent MS/MS acquisition (DDA) method, DIA allows the acquisition of MS/MS spectra for all of the MS peaks simultaneously regardless of their peak intensities. However, DIA is not very suitable for the narrow peaks in our 4 min gradient; longer gradients need to be applied to ensure there are enough data points in each chromatographic peak so MS/MS deconvolution can be performed. To compare DDA and SWATH methods fairly, we extended the gradient time to 12 min and analyzed the six red wines using both methods in positive mode. After data processing, we observed a similar total number of m/z–rt features detected for both methods (1632 for SWATH and 1667 for DDA). However, the SWATH method provided more features with MS/MS spectra (1632 vs 1461) and hence more compound annotations (207 vs 178 structurally annotated peaks). In summary, the SWATH method expands the coverage of the untargeted LC-MS profiling experiments, but may need to sacrifice the chromatography performance to some extent.

MicroLC-MS/MS Analysis of Six Red Wines: Statistics and Visualization.

Using relative quantitation and PCA clustering of all annotated compounds in positive ionization mode (Figure 5), clear differences were observed in the metabolite profiles of the six red wines. Loadings for PC1 and PC2 are available in Table S4 in the Supporting Information. Specifically, Syrah and Cabernet Sauvignon were separated very well from the four other wines along PC1, indicating that they have the overall most similar metabolite profiles. For example, triethyl citrate and syringic acid were found to be much more abundant in Cabernet Sauvignon and Syrah than in the other four wines, shown in Figure 6. Similarly, the six wines were separated on PC2, but not clearly clustered into two groups as on PC1. Other metabolites also showed very large variance among the tested wines. For example, *trans*-resveratrol was up to 6-fold more abundant in Pinot noir and Merlot than in the other four wines, whereas the novel isoresveratrol showed a similar pattern but was less abundant in Merlot. By contrast, caffeic acid and one of its isomer showed very different distribution patterns in the six red wines (Figure 6). To improve the accuracy of relative quantitation, a set of internal standards could be developed to normalize the peak intensities. Considering the complexity of compounds measured and their various physical and chemical

properties, a single internal standard was not adequate to address the difference in the extraction and ionization processes.

One of the potential applications of our untargeted metabolic data was to link the taste of red wines to their chemical components. As a proof of concept, we used the subjective tasting scores from a panel of 14 untrained adults (Table S5) and our metabolic data to build an orthogonal signal correction partial least-squares (O-PLS) model. Q^2 and root mean squared error of prediction (RMSEP) for LV2 were 0.990 and 0.158, respectively. Features that were positively or negatively associated with the tasting scores could be selected, as shown in Figure S1 and Table S6 in the Supporting Information. The sample size in this sensory study may not be sufficient to reveal the true association between the red wine components and overall sensory experience; a larger scale study with more red wines and panelists would be needed for that purpose.

To give an overview of the molecular space and reveal the difference in the metabolite profiles between the red wines with higher and lower tasting scores, a structural and spectral similarity network was built for compounds annotated in positive mode, and the fold differences of peak intensities between Syrah and Pinot noir were mapped onto the network (Figure 7). In this network, chemical structures with Tanimoto similarity score higher than 0.9 were clustered, while isomers were connected based on MS/MS spectra similarity. According to the network, all isomers of dihydroquercetin, resveratrol, and procyanidin B were less abundant in Syrah than in Pinot noir. On the other hand, 2,4(3H,5H)-furandione and syringic acid isomers were more abundant in Syrah. Interestingly, some isomers showed opposite distribution patterns, e.g. caffeic acid was lower in Syrah while two iso-caffeic aids were more abundant in this wine in comparison to the other test wines (Figure 6).

In summary, the optimized microLC-MS/MS screening workflow combined a novel LC method with chemometrics strategies from untargeted metabolomics and was applied to the analysis of red wines. It has multiple advantages compared to other published LC-MS method in the red wine studies, shown in Table 3. First of all, our method requires only 5.5 min, including column wash and re-equilibration, allowing a cost-effective high-throughput operation in large trials. Second, with a flow rate of 0.05 mL/min, each sample needs just 0.275 mL of mobile phase; thus, it takes only 27.5 mL of mobile phase to run 100 samples, which saves costs for both the purchase and discarding of organic solvents. Last, with untargeted data acquisition and chemometrics method, we are able to structurally annotate >200 compounds using comprehensive MS/MS libraries in a single run. Compared to other methods focusing on a few target known compounds, metabolomics is clearly more comprehensive and will build much more robust methods for sensory tasting and quality control outputs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

REFERENCES

(1). Ribereau-Gayon P; Glories Y; Maujean A; Dubourdieu D Handbook of Enology: The Chemistry of Wine Stabilization and Treatment; Wiley: Hoboken, NJ, USA, 2006; Vol. 2.

(2). Rothwell JA; Perez-Jimenez J; Neveu V; Medina-Remon A; M'Hiri N; García-Lobato P; Manach C; Knox C; Eisner R; Wishart DS Phenol-Explorer 3.0: a major update of the Phenol-Explorer database to incorporate data on the effects of food processing on polyphenol content. Database 2013, 2013, bat070. [PubMed: 24103452]

(3). Aznar M; López R; Cacho J; Ferreira V Prediction of aged red wine aroma properties from aroma chemical composition. Partial least squares regression models. J. Agric. Food Chem 2003, 51, 2700–2707. [PubMed: 12696960]

(4). Scalbert A; Johnson IT; Saltmarsh M Polyphenols: antioxidants and beyond. Am. J. Clin. Nutr 2005, 81, 215S–217S.

(5). Smoliga JM; Baur JA; Hausenblas HA Resveratrol and health – a comprehensive review of human clinical trials. Mol. Nutr. Food Res 2011, 55, 1129–1141. [PubMed: 21688389]

(6). Cliff MA; King MC; Schlosser J Anthocyanin, phenolic composition, colour measurement and sensory analysis of BC commercial red wines. Food Res. Int 2007, 40, 92–100.

(7). Kerem Z; Bravdo B-a.; Shoseyov, O.; Tugendhaft, Y. Rapid liquid chromatography–ultraviolet determination of organic acids and phenolic compounds in red wine and must. J. Chromatogr. A 2004, 1052, 211–215. [PubMed: 15527140]

(8). Jaitz L; Siegl K; Eder R; Rak G; Abranko L; Koellensperger G; Hann S LC-MS/MS analysis of phenols for classification of red wine according to geographic origin, grape variety and vintage. Food Chem. 2010, 122, 366–372.

(9). Mirnaghi FS; Mousavi F; Rocha SM; Pawliszyn J Automated determination of phenolic compounds in wine, berry, and grape samples using 96-blade solid phase microextraction system coupled with liquid chromatography–tandem mass spectrometry. J. Chromatogr. A 2013, 1276, 12–19. [PubMed: 23332787]

(10). Greco G; Grosse S; Letzel T Serial coupling of reversed-phase and zwitterionic hydrophilic interaction LC/MS for the analysis of polar and nonpolar phenols in wine. J. Sep. Sci 2013, 36, 1379–1388. [PubMed: 23505207]

(11). Ashokkumar T; Suneetha A; Reddy BV; Appaji SCV Review article on comparative study of new trends in HPLC. Int. J. Adv. Pharm. Anal 2012, 2, 53–55.

(12). Vissers JP Recent developments in microcolumn liquid chromatography. J. Chromatogr. A 1999, 856, 117–143. [PubMed: 10526786]

(13). Wang Q; Feng J; Han H; Zhu P; Wu H; Marina ML; Crommen J; Jiang Z Enantioseparation of N-derivatized amino acids by micro-liquid chromatography using carbamoylated quinidine functionalized monolithic stationary phase. J. Chromatogr. A 2014, 1363, 207–215. [PubMed: 24999069]

(14). Silvestro L; Tarcomnicu I; Dulea C; Attili NRB; Ciuca V; Peru D; Savu SR Confirmation of diosmetin 3-*O*-glucuronide as major metabolite of diosmin in humans, using micro-liquid-chromatography–mass spectrometry and ion mobility mass spectrometry. Anal. Bioanal. Chem 2013, 405, 8295–8310. [PubMed: 23949323]

(15). Grondin C; Zhao W; Fakhoury M; Jacqz-Aigrain E Determination of ciprofloxacin in plasma by micro-liquid chromatography-mass spectrometry: an adapted method for neonates. Biomed. Chromatogr 2011, 25, 827–832. [PubMed: 20872911]

(16). Gritti F; Guiochon G On the extra-column band-broadening contributions of modern, very high pressure liquid chromatographs using 2.1 mm ID columns packed with sub-2 μm particles. J. Chromatogr. A 2010, 1217, 7677–7689. [PubMed: 21044782]

(17). Ma Y; Chassy A; Miyazaki S; Motokawa M; Morisato K; Uzu H; Ohira M; Furuno M; Nakanishi K; Minakuchi H; Mriziq K; Farkas T; Fiehn O; Tanaka N Efficiency of short, small-diameter columns for reversed-phase liquid chromatography under practical operating conditions. J. Chromatogr. A 2015, 1383, 47–57. [PubMed: 25648581]

(18). Nakane K; Shirai S; Saito Y; Moriwake Y; Ueta I; Inoue M; Jinno K High-temperature separations on a polymer-coated fibrous stationary phase in microcolumn liquid chromatography. Anal. Sci 2011, 27, 811. [PubMed: 21828918]

(19). An R; Weng Q; Li J Silica-particle-supported zwitterionic polymer monolith for microcolumn liquid chromatography. J. Sep. Sci 2014, 37, 2633–2640. [PubMed: 25044794]

(20). Tsugawa H; Cajka T; Kind T; Ma Y; Higgins B; Ikeda K; Kanazawa M; VanderGheynst J; Fiehn O; Arita M MS-DIAL: data-independent MS/MS deconvolution for comprehensive metabolome analysis. Nat. Methods 2015, 12, 523. [PubMed: 25938372]

(21). Schmit TM; Rickard BJ; Taber J Consumer valuation of environmentally friendly production practices in wines, considering asymmetric information and sensory effects. J. Agric. Econ 2013, 64, 483–504.

(22). Horai H; Arita M; Kanaya S; Nihei Y; Ikeda T; Suwa K; Ojima Y; Tanaka K; Tanaka S; Aoshima K MassBank: a public repository for sharing mass spectral data for life sciences. J. Mass Spectrom 2010, 45, 703–714. [PubMed: 20623627]

(23). Sawada Y; Nakabayashi R; Yamada Y; Suzuki M; Sato M; Sakata A; Akiyama K; Sakurai T; Matsuda F; Aoki T RIKEN tandem mass spectral database (ReSpect) for phytochemicals: a plant-specific MS/MS-based data resource and database. Phytochemistry 2012, 82, 38–45. [PubMed: 22867903]

(24). Smith CA; O'Maille G; Want EJ; Qin C; Trauger SA; Brandon TR; Custodio DE; Abagyan R; Siuzdak G METLIN: a metabolite mass spectral database. Ther. Drug Monit 2005, 27, 747–751. [PubMed: 16404815]

(25). Ma Y; Kind T; Yang D; Leon C; Fiehn O MS2Analyzer – a software for small molecule substructure annotations from accurate mass MS/MS spectra. Anal. Chem 2014, 86, 10724–10731. [PubMed: 25263576]

(26). Xia J; Mandal R; Sinelnikov IV; Broadhurst D; Wishart DS MetaboAnalyst 2.0 — a comprehensive server for metabolomic data analysis. Nucleic Acids Res. 2012, 40, W127–W133. [PubMed: 22553367]

(27). Grapov D; Wanichthanarak K; Fiehn O MetaMapR: pathway independent metabolomic network analysis incorporating unknowns. Bioinformatics 2015, 31, 2757–2760. [PubMed: 25847005]

(28). Smoot ME; Ono K; Ruscheinski J; Wang P-L; Ideker T Cytoscape 2.8: new features for data integration and network visualization. Bioinformatics 2011, 27, 431–432. [PubMed: 21149340]

(29). Porgalı E; Büyüktuncel E Determination of phenolic composition and antioxidant capacity of native red wines by high performance liquid chromatography and spectrophotometric methods. Food Res. Int 2012, 45, 145–154.

(30). Stein SE; Scott DR Optimization and testing of mass spectral library search algorithms for compound identification. J. Am. Soc. Mass Spectrom 1994, 5, 859–866. [PubMed: 24222034]

(31). Pati S; Crupi P; Benucci I; Antonacci D; Di Luccia A; Esti M HPLC-DAD–MS/MS characterization of phenolic compounds in white wine stored without added sulfite. Food Res. Int 2014, 66, 207–215.

(32). Cui Y; Li Q; Liu Z; Geng L; Zhao X; Chen X; Bi K Simultaneous determination of 20 components in red wine by LC-MS: application to variations of red wine components in decanting. J. Sep. Sci 2012, 35, 2884–2891. [PubMed: 23065941]
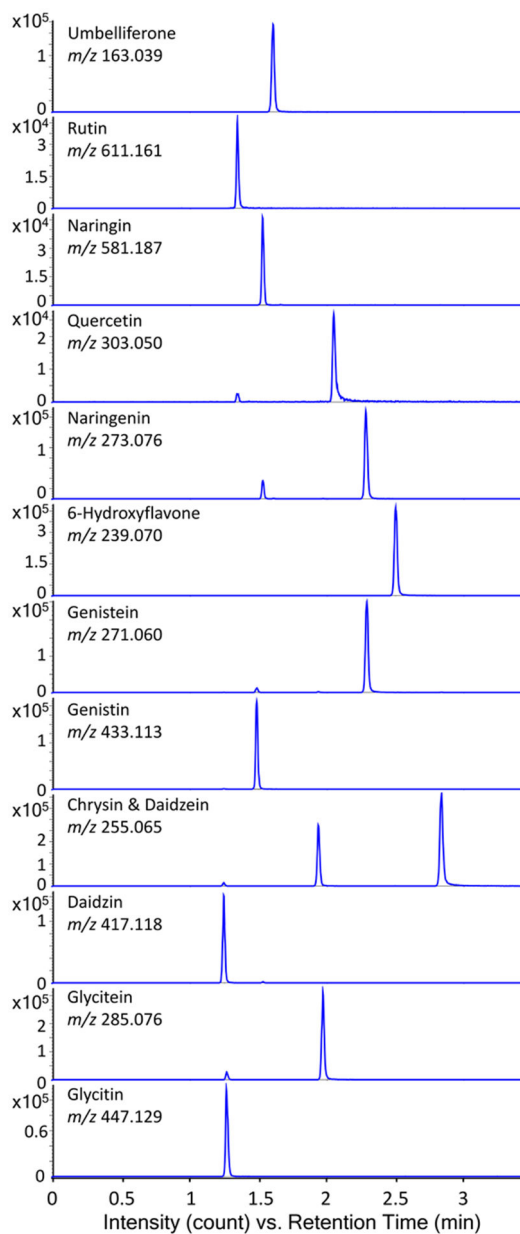
Figure 1.
Extracted ion chromatograms of 13 polyphenol reference standards using the 1 mm i.d. Kinetex C18 column and a 4 min acetonitrile/water gradient at 0.05 mL/min flow rate. Sensitivity and peak capacity calculations were performed on the more abundant peaks if minor impurities were detectable as well in the authentic standards chromatograms.
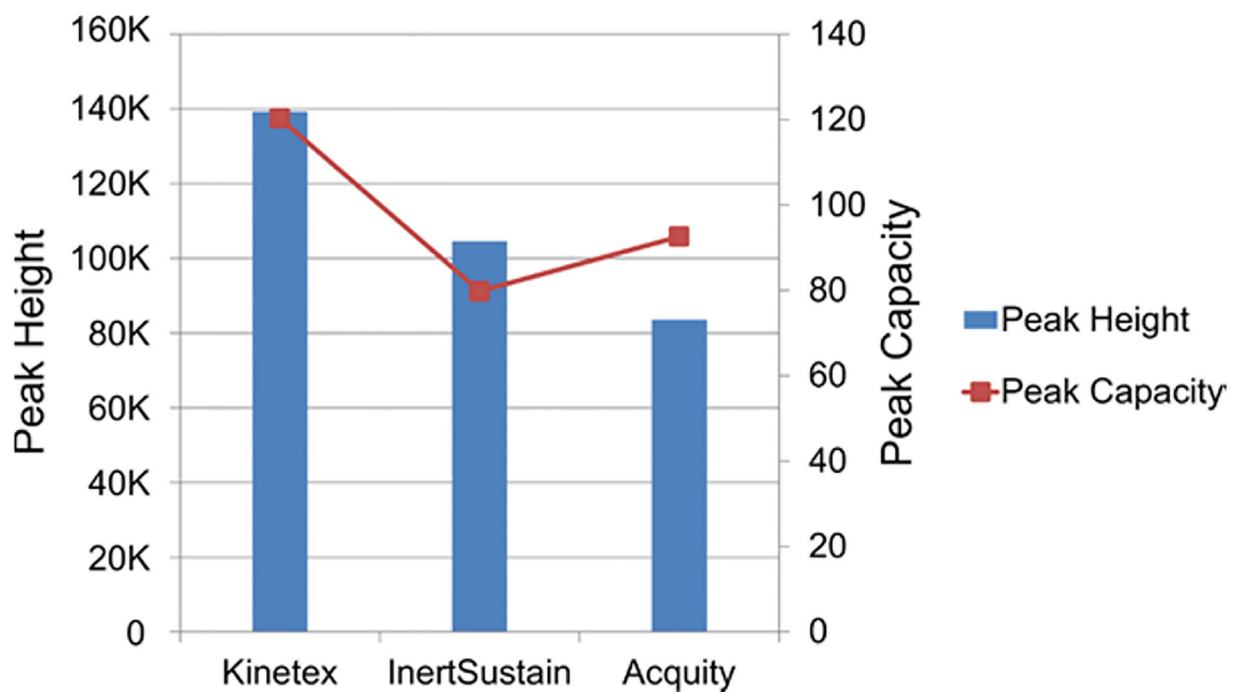
Figure 2.
Comparison of three 1 mm i.d. C18 columns with respect to average peak intensities and peak capacities for 13 polyphenol reference standards using a 4 min acetonitrile/water gradient at 0.05 mL/min flow rate.
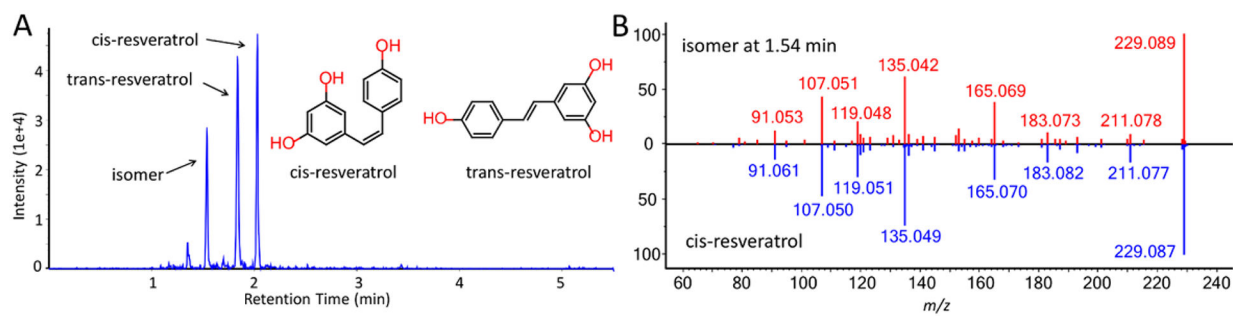
Figure 3.
Example of compound annotation using MS/MS matching: annotation of a novel resveratrol isomer showing (A) the extracted ion chromatogram at *m/z* 229.085 ([M + H]⁺) at 1.54 min and (B) matching the accurate mass MS/MS spectra of the new isomer against *cis*-resveratrol authentic standards.
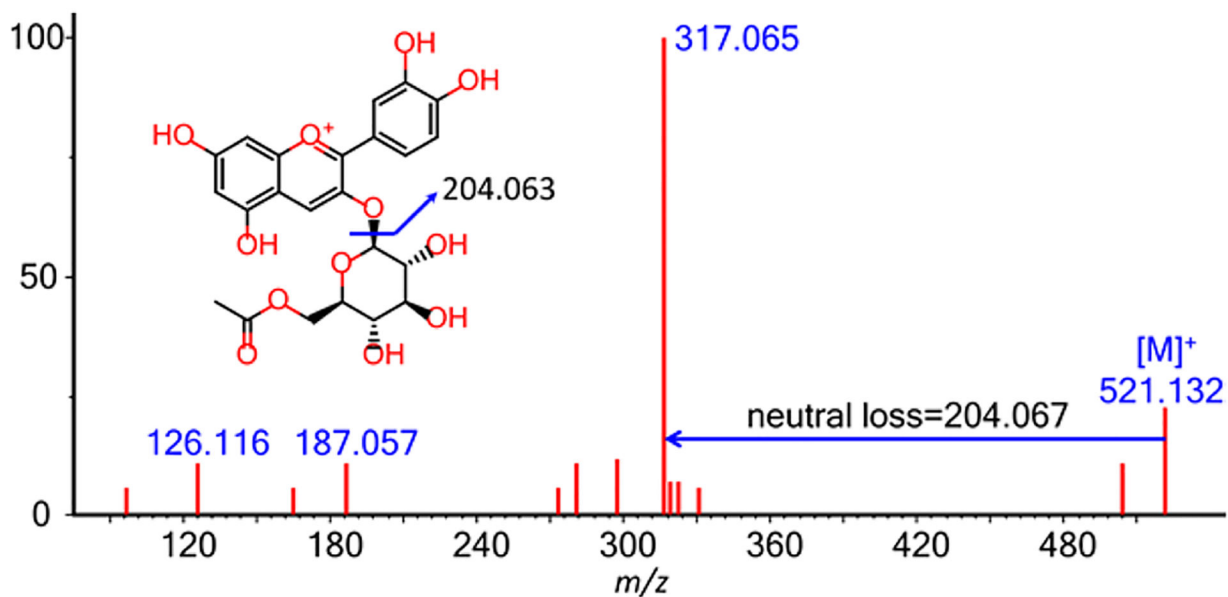
Figure 4.
Example of substructure annotation for unknown compounds using MS2Analyzer. An unknown MS/MS spectrum with precursor m/z 521.132 at 1.68 min in positive mode was annotated as acetylhexoside for the characteristic neutral loss of 204.067. One potential structure candidate, petunidin 3-O-(6″-acetyl-glucoside), was extracted from Phenol-Explorer using accurate mass match.
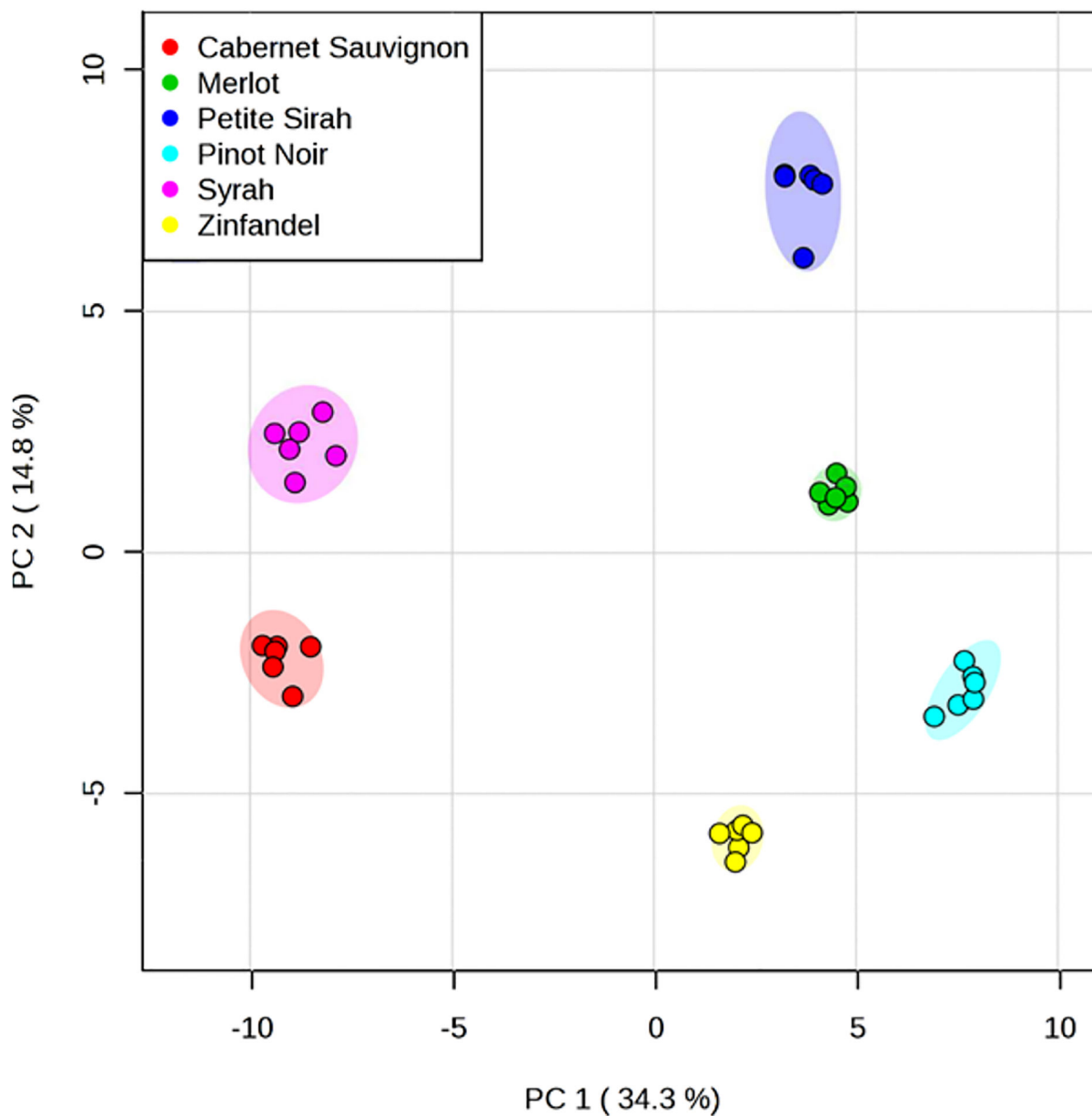
Figure 5.
Principal component analysis (PCA) score plot of the six tested red wines. Total quantitative variances of 177 annotated peaks in positive ionization mode were clustered to reveal the difference and relative similarities of metabolite profiles of different red wines. 95% confidence regions (Hotelling's T2 eclipse) are displayed for each class.
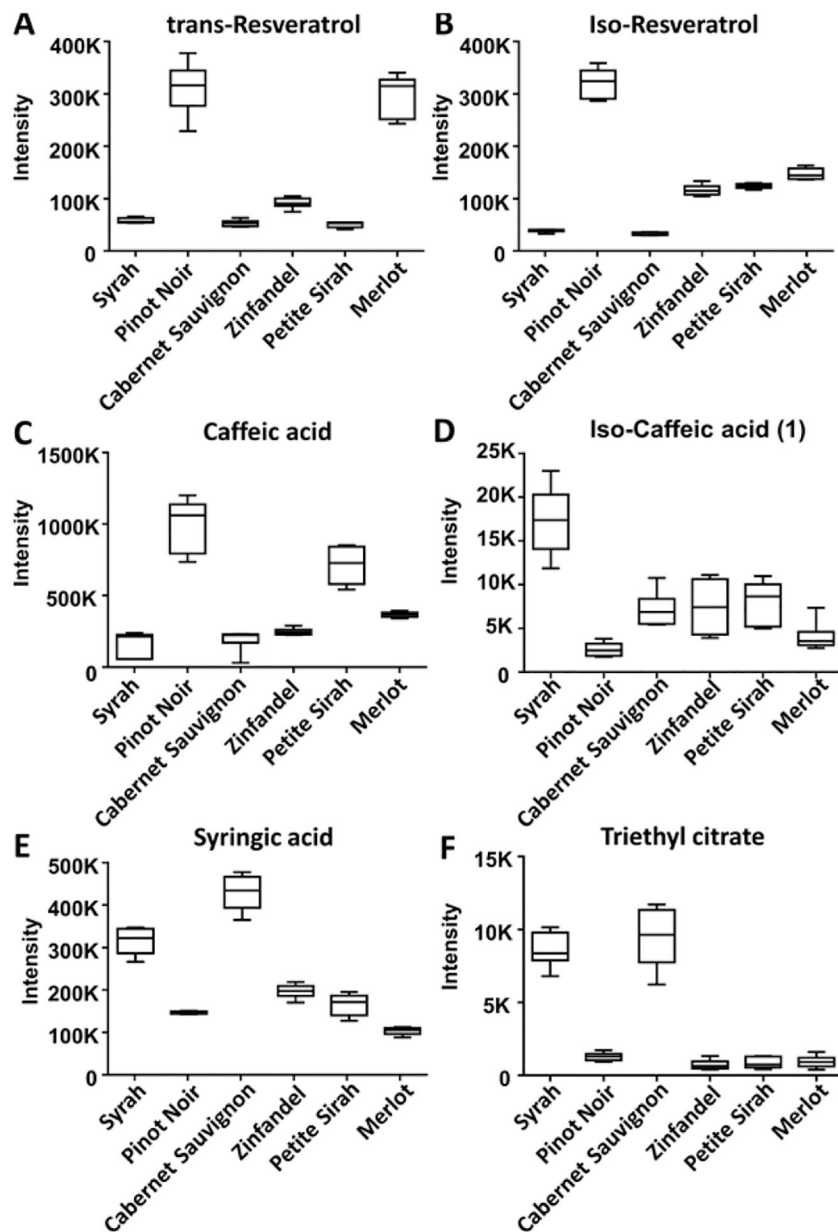
Figure 6.
Peak intensities of selected compounds contributing to the difference of the metabolite profiles of the six red wines: (A) *trans*-resveratrol; (B) isoresveratrol; (C) caffeic acid, negatively correlated to the tasting score; (D) isocaffeic acid, positively correlated to the tasting score; (E) syringic acid; (F) triethyl citrate, present in only two of the red wines.
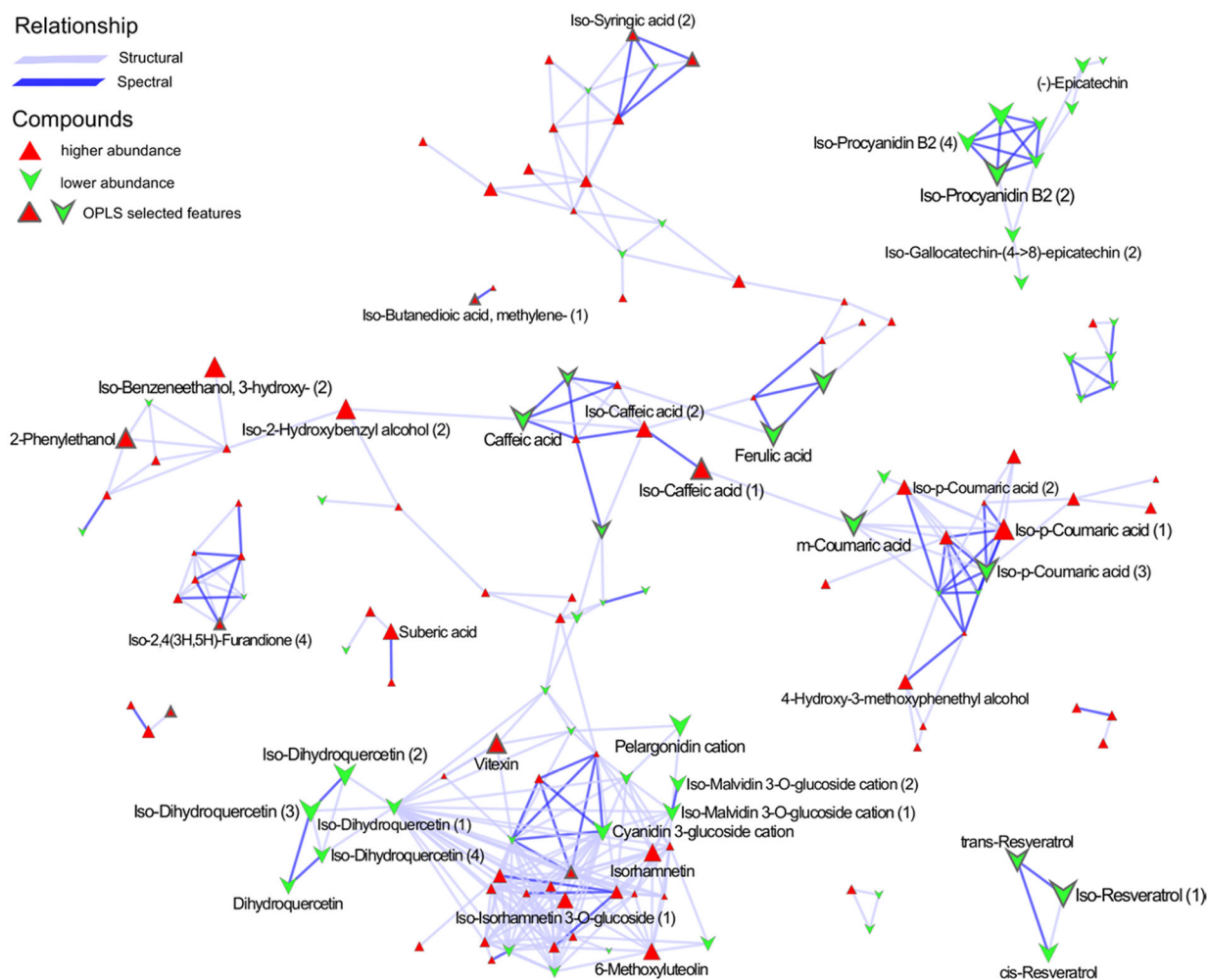
Figure 7.
Chemical structural and mass spectral similarity networks (Tanimoto > 0.9, spectra similarity > 0.4) for compounds annotated in positive ionization mode. Peak intensity fold change of Syrah (highest tasting score) versus Pinot noir (lowest tasting score) is mapped onto the network. OPLS selected features are marked with borders.

Table 1.

Number of Peaks Detected in One Testing of Red Wine Sample Using a Kinetex C18 1.0 mm i.d. Column under Various LC Conditions[a]

flow rate (mL/min)	gradient time				
	4 min	6 min	8 min	12 min	24 min
0.025	571		472		164
0.050	754		572	471	
0.075	687		575		
0.100	664	621	551		

[a]The gradient was 10–90% mobile phase B (acetonitrile with 0.1% acetic acid) at different flow rates and gradient times.

Table 2.

Example Compounds Annotated and Confirmed by Authentic Reference Standards in the Six Red Wines[a]

name	rt (min)	m/z	error (mDa)
gallic acid	0.70	169.014	−0.1
(+)-catechin	1.03	291.086	−0.1
(−)-epicatechin	1.14	291.087	−0.2
caffeic acid	1.18	181.049	0.1
syringic acid	1.19	199.060	0.2
p-coumaric acid	1.43	165.054	0.5
ferulic acid	1.49	195.065	0.7
N-acetyl-L-phenylalanine	1.49	208.096	0.6
dihydroquercetin	1.52	305.066	−0.1
DL-indole-3-lactic acid	1.57	206.081	0.6
isofraxidin	1.62	223.060	0.6
myricetin	1.68	319.045	0.0
3-(2-hydroxyethyl)indole	1.79	162.091	0.4
trans-resveratrol	1.84	229.086	0.2
quercetin	1.98	303.050	−0.2
cis-resveratrol	2.03	229.086	0.3
phloretin	2.18	275.091	0.2
naringenin	2.22	273.076	0.2

[a]All compounds were detected as $[M + H]^+$ except for gallic acid ($[M - H]^-$).

Table 3.

Comparison of the MicroLC-MS Method to Recent Alternative Methods

study	LC time (min)	flow rate (mL/min)	mobile phase 100 samples (mL)	compounds annotated
this study	5.5	0.05	27.5	200+
Pati et al. (2014)[31]	40	0.2	800	26
Cui et al. (2012)[32]	40	0.8	3200	20
Greco et al. (2012)[10]	27	0.4	1080	13
Mirnaghi et al. (2012)[9]	20	0.2	400	8
Jaitz et al. (2010)[8]	13	0.4	520	11