

Video Article

The Terroir Concept Interpreted through Grape Berry Metabolomics and Transcriptomics

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Abstract

Terroir refers to the combination of environmental factors that affect the characteristics of crops such as grapevine (*Vitis vinifera*) according to particular habitats and management practices. This article shows how certain terroir signatures can be detected in the berry metabolome and transcriptome of the grapevine cultivar Corvina using multivariate statistical analysis. The method first requires an appropriate sampling plan. In this case study, a specific clone of the Corvina cultivar was selected to minimize genetic differences, and samples were collected from seven vineyards representing three different macro-zones during three different growing seasons. An untargeted LC-MS metabolomics approach is recommended due to its high sensitivity, accompanied by efficient data processing using MZmine software and a metabolite identification strategy based on fragmentation tree analysis. Comprehensive transcriptome analysis can be achieved using microarrays containing probes covering ~99% of all predicted grapevine genes, allowing the simultaneous analysis of all differentially expressed genes in the context of different terroirs. Finally, multivariate data analysis based on projection methods can be used to overcome the strong vintage-specific effect, allowing the metabolomics and transcriptomics data to be integrated and analyzed in detail to identify informative correlations.

Video Link

The video component of this article can be found at <https://www.jove.com/video/54410/>

Introduction

Large-scale data analysis based on the genomes, transcriptomes, proteomes and metabolomes of plants provides unprecedented insight into the behavior of complex systems, such as the terroir characteristics of wine which reflect the interactions between grapevine plants and their environment. Because the terroir of a wine can be distinct even when identical grapevine clones are grown in different vineyards, genomics analysis is of little use because the clonal genomes are identical. Instead it is necessary to look at correlations between gene expression and the metabolic properties of the berries, which determine the quality traits of wine. The analysis of gene expression at the level of the transcriptome benefits from the similar chemical properties of all transcripts, which facilitates quantitative analysis by exploiting universal characteristics such as hybridization to immobilized probes on microarrays. In contrast, universal analytical methods in proteomics and metabolomics are more challenging because of the huge physical and chemical diversity of individual proteins and metabolites. In the case of metabolomics this diversity is even more extreme because individual metabolites differ vastly in size, polarity, abundance and volatility, so no single extraction process or analytical method offers a holistic approach.

Among the analytical platforms suitable for non-volatile metabolites, those based on high performance liquid chromatography coupled to mass spectrometry (HPLC-MS) are much more sensitive than alternatives such as HPLC with ultraviolet or diode array detectors (HPLC-UV, HPLC-DAD) or nuclear magnetic resonance (NMR) spectroscopy, but quantitative analysis by HPLC-MS can be influenced by phenomena such as the matrix effect and ion suppression/enhancement¹⁻³. The investigation of such effects during the analysis of Corvina grape berries by HPLC-MS using an electrospray ionization source (HPLC-ESI-MS), showed that sugars and other molecules with the lowest retention times were strongly underreported, probably also reflecting the large number of molecules in this zone, and that the abundance of other molecules could be underestimated, overestimated or unaffected by the matrix effect, but the data normalization for the matrix effect seemed to have limited impact on the overall results^{4,5}. The method described herein is optimized for the analysis of medium-polarity metabolites that accumulate at high levels in grape berries during ripening, and which are significantly impacted by the terroir. They include anthocyanins, flavonols, flavan-3-ols, procyanidins, other flavonoids, resveratrol, stilbenes, hydroxycinnamic acids and hydroxybenzoic acids, which together determine the color, taste and health-related properties of wines. Other metabolites, such as sugars and aliphatic organic acids, are ignored because quantitation

by HPLC-MS is unreliable due to the matrix effect and ion suppression phenomena⁵. Within the polarity range selected by this method, the approach is untargeted in that it aims to detect as many different metabolites as possible⁶.

Transcriptomics methods that allow thousands of grapevine transcripts to be monitored simultaneously are facilitated by the availability of the complete grapevine genome sequence^{7,8}. Early transcriptomics methods based on high-throughput cDNA sequencing have evolved with the advent of next-generation sequencing into a collection of procedures collectively described as RNA-Seq technology. This approach is rapidly becoming the method of choice for transcriptomics studies. However, a large body of literature based on microarray, which allow thousands of transcripts to be quantified in parallel by hybridization, has accumulated for grapevine. Indeed, before RNA-Seq became a mainstream technology, many dedicated commercial microarray platforms had been developed allowing grapevine transcriptome to be inspected in great detail. Among the vast variety of platforms, only two allowed genome-wide transcriptome analysis⁹. The most evolved array allowed the hybridization of up to 12 independent samples on a single device, thus reducing the costs of each experiment. The 12 sub-arrays each comprised 135,000 60-mer probes representing 29,549 grapevine transcripts. This device has been used in a large number of studies¹⁰⁻²⁴. These two platforms have now been discontinued but a new custom microarray has recently been designed and represents a more recent development as it contains an even greater number of probes representing additional newly discovered grapevine genes²⁵.

The large-scale datasets produced by transcriptomics and metabolomics analysis require suitable statistical methods for data analysis, including multivariate techniques to determine correlations between different forms of data. The most widely used multivariate techniques are those based on projection, and these can be unsupervised, such as principal component analysis (PCA), or supervised, such as bidirectional orthogonal projection to latent structures discriminant analysis (O2PLS-DA)²⁶. The protocol presented in this article utilizes PCA for exploratory data analysis and O2PLS-DA to identify differences between groups of samples.

Protocol

1. Select Appropriate Materials and Construct a Sampling Plan

1. Begin the experiment by developing an appropriate sampling plan. There is no generic and universal approach so evaluate each plan on a case-by-case basis. Ensure that the sampling plan states the sampling places, times and the precise sampling procedure. See **Figure 1** for the sampling plan used in this case study.

NOTE: In this case study, grape berries from a single clone (*Vitis vinifera* cv. Corvina, clone 48) were collected from seven commercial vineyards in three different macro-zones in the province of Verona (Lake Garda, Valpolicella and Soave). The principal features of each vineyard are summarized in **Table 1**. Berries were collected during three growing seasons (2006, 2007, and 2008) at three time points, corresponding to veraison (the onset of ripening), mid-ripening and ripe berries.

1. For each of the accessions (vineyard/year/ripening stage), harvest 30 clusters from different positions along two vine rows, with randomized heights and locations on the plant.
2. Select three berries randomly from each cluster, avoiding those with visible damage and/or signs of infection.
3. Repeat steps 1.1.1 and 1.1.2 to obtain three independent pools.
4. Deseed the berries and freeze the pericarp immediately in liquid nitrogen.
5. Crush 10 frozen berries from each pool with an automatic mill grinder, and divide each powdered sample into two equal parts, one for transcriptomics analysis and one for metabolomics analysis.
6. Store the powders at -80 °C.

	AM	BA	BM	CS	FA	MN	PM
Macro-zone	Soave	Lake Garda	Valpolicella	Lake Garda	Valpolicella	Valpolicella	Soave
Height (m)	250	120	450	100	130	250	130
Rootstock	41B	S04	K5BB	420A	420A	K5BB	41B
Row direction	E-W	N-S	E-W	E-W	E-W	N-S	N-S
Training system	Overhead System (Pergola)	Overhead System (Pergola)	Vertical Shoot Positioning (Guyot)	Overhead System (Pergola)	Overhead System (Pergola)	Vertical Shoot Positioning (Guyot)	Vertical Shoot Positioning (Guyot)
Soil type	Silty clay	Loam	Clay	Loam	Clay Loam	Silt loam	Clay loam
Planting layout (m)	3.20 x 1.00	4.50 x 0.80	4.00 x 1.25	3.50 x 1.20	3.50 x 0.75	2.80 x 1.00	1.80 x 0.80
Total lime %	3.9	19.3	18.3	14.4	31	5.9	27.9
Active lime %	0.5	2.6	9.4	6.3	11.3	3.1	8.3
Sand %	15	47	66	42	29	13	36
Loam %	43	36	21	37	39	67	36
Clay %	42	17	13	21	32	20	28
Soil pH	8.3	7.9	7.8	8.2	8.2	7.8	7.9
Organic substance (%)	2.9	2.5	2.2	1.2	2.9	1.6	2.5
Exchangeable phosphorus (mg/kg)	26	73	73	68	48	47	64
Exchangeable potassium (mg/kg)	190	376	620	230	168	154	126
Exchangeable magnesium (mg/kg)	272	468	848	623	294	293	183
Exchangeable calcium (mg/kg)	6500	5380	7358	6346	4652	10055	2878
Berry Reducing Sugars 2006	211.25 ± 1.20	176.20 ± 0.42	187.40 ± 0.00	203.70 ± 1.13	212.55 ± 0.64	195.20 ± 0.00	211.65 ± 0.64
Berry Reducing Sugars 2007	190.00 ± 1.27	165.25 ± 0.49	153.00 ± 0.42	203.60 ± 0.71	210.90 ± 0.71	192.25 ± 0.64	188.70 ± 1.84
Berry Reducing Sugars 2008	191.35 ± 0.64	178.90 ± 0.57	170.05 ± 0.49	205.15 ± 1.48	188.70 ± 0.57	169.35 ± 0.49	108.05 ± 1.06
Berry pH 2006	3.01 ± 0.01	2.96 ± 0.01	2.84 ± 0.00	2.9 ± 0.00	2.98 ± 0.00	3.02 ± 0.00	3.06 ± 0.01
Berry pH 2007	2.97 ± 0.00	3.00 ± 0.00	2.74 ± 0.00	3.07 ± 0.01	2.98 ± 0.00	2.87 ± 0.01	3.09 ± 0.00
Berry pH 2008	2.83 ± 0.00	3.04 ± 0.01	2.71 ± 0.00	2.98 ± 0.01	2.98 ± 0.00	2.82 ± 0.00	3.11 ± 0.00
Harvesting Date	2006	2007	2008				
Veraison	8-Aug	18-Jul	12-Aug				
Mid Ripening	4-Sep	8-Aug	2-Sep				
Ripe	18-Sep	29-Aug	23-Sep				

Table 1: Principal features of each vineyard and sample collection dates. m = meters, E-W = East-West, N-S = North-South.

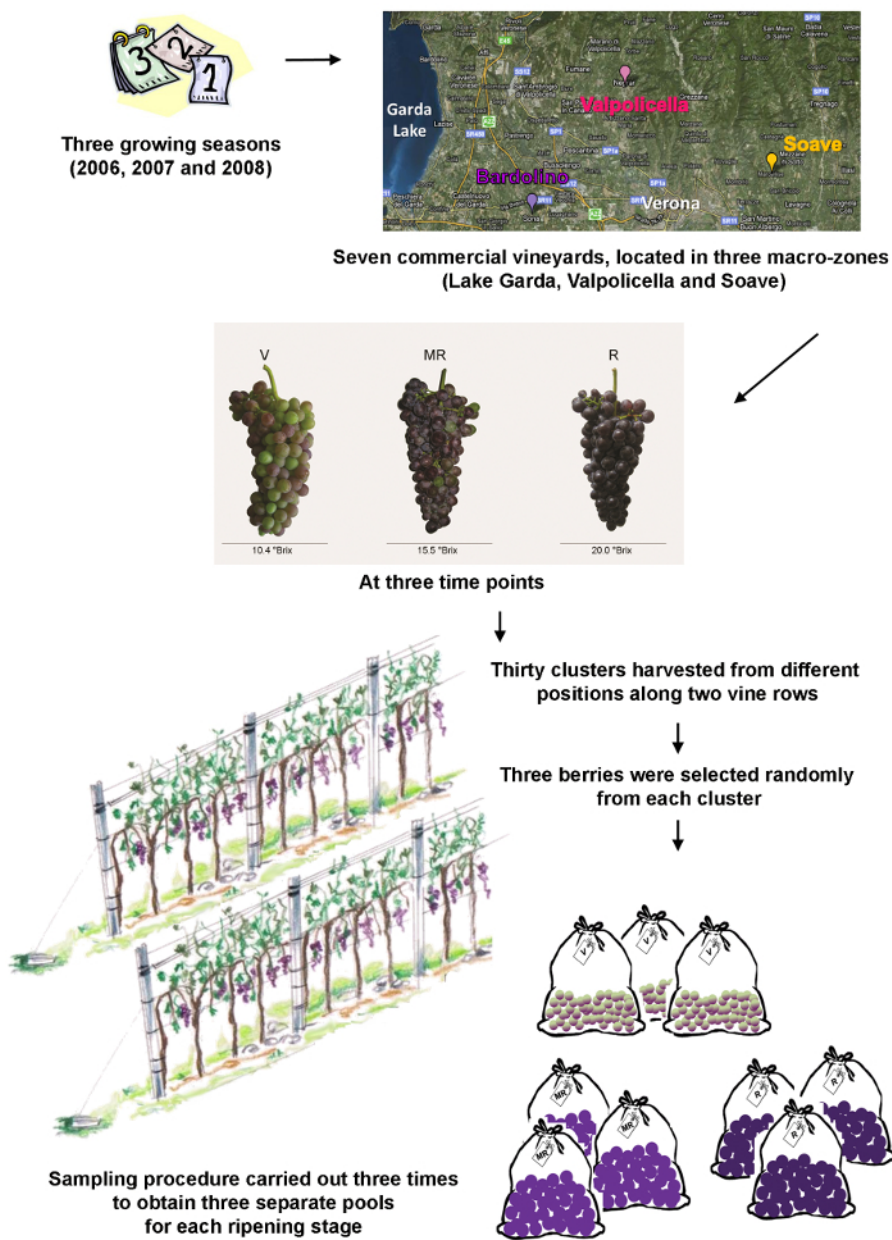


Figure 1: Schematic representation of the sampling procedure. The three wine production macro-zones are located in the surroundings of the city of Verona, Veneto region, Italy. The three time points are veraison (V) representing the onset of ripening in viticulture, mid-ripening (MR) and ripe berries (R). [Please click here to view a larger version of this figure.](#)

2. Prepare Berry Powder Extracts, Analyze the Metabolites and Process the Data

1. Prepare the Berry Powder Samples for Analysis.
 1. Prepare the metabolic extracts of the berry samples at room temperature in three volumes (w/v) of methanol acidified with 0.1% (v/v) formic acid in an ultrasonic bath at 40 kHz for 15 min. Use LC-MS-grade water and formic acid, and HPLC-grade methanol.
 2. Centrifuge the extracts at 16,000 x g for 10 min at 4 °C.
 3. Dilute the supernatant from step 2.1.2 in two volumes (v/v) of deionized water and pass through a 0.2-µm filter.
2. Analyze the Berry Extracts by HPLC-ESI-MS.
 1. Set up the HPLC-ESI-MS system according to the supplier's recommendations.
NOTE: In this case study, the setup comprised an HPLC system equipped with an autosampler, connected in-line with an ion trap mass spectrometer and electrospray ionization source.
 2. Connect the HPLC system with a C18 guard column (7.5 x 2.1 mm²) and a Reversed Phase C18 column (150 x 2.1 mm², particle size 3 µm).

3. Prepare the solvents used as the mobile phase. Use 5% (v/v) acetonitrile, 0.5% (v/v) formic acid in water as Solvent A and 100% acetonitrile as solvent B. Use LC-MS-grade acetonitrile, water and formic acid.
4. Run the HPLC separation with a linear gradient of solvents A and B at a constant flow rate of 0.2 ml min⁻¹ using a sample injection volume of 30 µl.
 1. Initially equilibrate the column with 100% solvent A. Following sample injection, establish a gradient from 0% to 10% solvent B in 5 min, from 10% to 20% solvent B in 20 min, from 20% to 25% solvent B in 5 min, and from 25% to 70% solvent B in 15 min.
 2. Analyze each sample in duplicate. Randomize sample analysis to avoid instrument-driven effects. Allow 20 min for re-equilibration with 100% solvent A between each analysis.
5. Acquire mass spectra in alternate negative and positive ionization modes. For the results described in this case study, set the parameters as in **Table 2**. The precise machine setting depends on the specific platform.
NOTE: Alternatively, use other suitable methods according to the specific platform.
 1. In all cases, as with any separation platform, make sure to use a suitable re-equilibration time, in order to have retention time reproducibility. When the number of samples is high (above ten samples), analyze them in batches of 9-10 samples, with chromatographic column cleaning programs (slow gradients between the two elution solvents) between each batch. To have enough retention time reproducibility, ensure that the first chromatographic analysis of each batch is a blank analysis (*i.e.*, analysis of the solvent).
6. Also acquire mass spectra in fragmented negative and positive ion modes setting the fragmentation options (two ion precursors, MS³). Annotate the metabolites by fragmentation tree analysis (MS/MS and MS³) according to manufacturer's instructions.
NOTE: This is particularly suitable for plant metabolites because they are often glycosylated, therefore the first fragmentation (MS/MS) tends to remove the sugar moieties leaving the free aglycone ion, and subsequent fragmentation (MS³) helps to identify the aglycone against an authentic standards library.
7. Acquire MS/MS and MS³ spectra in the *m/z* range 50-1,500 with a fragmentation amplitude of 1 V. Alternatively, use other suitable methods according to the specific platform.
8. For each *m/z* signal, compare the MS/MS and MS³ fragmentation patterns and retention times to the authentic standards library according to manufacturer's instructions.
NOTE: Many type of platforms include software facilities to build this kind of library through the analysis of authentic standards. This should identify many of the signals but not all signals will be annotated.
9. For the remaining anonymous signals, compare the MS/MS and MS³ fragmentation patterns with those published in the literature, searching for *m/z* values (*i.e.*, digit "m/z 353" to look for publications in which molecules with such *m/z* are mentioned) with any of the common search engine freely available, or use online databases such as MassBank (www.massbank.jp/en/database.html) and the Human Metabolome Database (www.hmdb.ca/search/spectra?type=ms_search).

Mass spectrometer components	Function	Parameters
Electrospray Ionization Source	Nebulizing Gas	50 psi, 350 °C
	Drying gas	10 L min ⁻¹
Ion trap and detector	Scan	Full scan mode, 13,000 <i>m/z</i> per second, range 50-1,500 <i>m/z</i>
	Target mass	400 <i>m/z</i>
	Collision Gas	Helium
	Vacuum pressure	1.4 x 10 ⁻⁵ mbar
	Capillary source	+4,000 V
	End plate offset	-500 V
	Skimmer	-40 V
	Cap exit	-121 V
	Oct 1 DC	-12 V
	Oct 2 DC	-1.7 V
	Lens 1	+5 V
	Lens 2	+60 V
	ICC for positive ionization mode	20.000
ICC for negative ionization mode	7,000	

Table 2: Principal parameter set for acquiring mass spectra.

Operation	Selection	Function	Parameters	Values
Peak Detection	Mass detection	Centroid	Noise level	3,500
	Chromatogram builder	Highest data point	min time span	0.15
			min height	4,000
			m/z tolerance	0.3
Peak Detection	Peak deconvolution	Local minimum search	Chromatographic threshold	70
			Search minimum in RT range (min)	0:50
			Minimum relative height	15%
			Minimum absolute height	4,000
			Min ratio of peak top/edge	2
			Duration range (min)	0-10
Isotopes	Isotopic peaks grouper	-	m/z tolerance	1.2
			RT tolerance	0:50
			monotonic shape	No
			Maximum charge	3
			Representative isotope	No
Alignment	Join aligner	-	m/z tolerance	1.2
			Weight for m/z	10
			Retention time tolerance	0:50
			Weight for RT	5
			Require same charge state	No
			Require same ID	No
Gap filling	Peak finder	-	Intensity tolerance	20%
			m/z tolerance	0.9
			Retention time tolerance	0:40
			RT correction	No
Filtering	Duplicate Peak filter	-	m/z tolerance	1.2
			RT tolerance	0:30
			Require same identification	No

Table 3: Mzmine workflow with specific values to process negative LC-MS grape berry data files.

3. Process the LC-MS Data.

1. Access or download a data processing software package that can extract relevant information from many raw chromatograms and build a data matrix in which each detected metabolite is quantified in each sample.
NOTE: The protocol steps below are tailored for the open-source software MZmine v2.14 (<http://mzmine.sourceforge.net>). It is an open-source software for mass-spectrometry data processing, with the main focus on LC-MS data.²⁷
2. Transform the LC-MS chromatogram data into netCDF format using the software provided by the equipment manufacturer. For the results described here, use the Bruker Daltonics Esquire v5.2 and Data Analysis v3.2 softwares and perform steps according to the manufacturer's instructions.
NOTE: Other converters may be used (various free-ware converters are available).
3. Import the .cdf files into the software.
4. Implement the peak detection, alignment, gap filling and peak filtering procedures with the parameters reported in **Table 3**.
 1. As first step, select an imported .cdf file, then go to Visualization → TIC/XIC Visualizer. Put the cursor upon the base of the smallest peak in the chromatogram and take note about the minimum intensity signal of the base ion. Then go to Raw Data Methods → Peak Detection.
 2. Select Mass Detection and fill the signal level to detect individual ions for each scan and create an ion list.
NOTE: Check the algorithm prior to performing the mass detection - it depends on the mass spectrometer. In our case, we select Centroid.
 3. Select Chromatogram Builder and fill with the signal level to connect data points from the ion list and build a chromatogram for each mass value. See the parameters specified in the mass spectrometer's manual to adjust the Min Time Span and m/z tolerance.

4. Next, go to Peak List Methods → Peak Detection → Chromatogram Deconvolution. Select the appropriate algorithm (in this case Local Minimum Search) to allow the separation of each chromatogram into individual peaks.
 5. Manually inspect the chromatograms to find out the appropriate values for the following parameters. Set the Chromatographic Threshold to 30% to remove the noise and Search Minimum in RT Range to 2 (min) to identify the presence of minimum locals to discriminate two peaks.
 6. Set Minimum Relative Height at 2.0. Manually inspect the chromatogram to identify the minimum absolute height of signal that corresponds to a peak and not to the background; set this value as Absolute Height (for instance, 10,000 in the presented experiment).
 7. Set Min Ratio of Peak Top/Edge to 1.1 to state the minimum ratio between the top and the lowest data point intensities of a peak to be recognized as a true peak.
 8. Manually inspect the chromatograms to see which is the minimum duration of the various peaks in the used chromatographic conditions (for instance, between 0.2 and 2 min, depending on the compound, in the presented experiments), and consequently use these values as Peak Duration Range (min) to set the range of acceptable peak length as duration.
 9. Then go to Peak List Methods → Isotopes → Isotopic Peaks Grouper to group isotopes in one peak, usually in the most intense. Note: The parameters depend on the resolution of the mass spectrometer and the reproducibility of the retention times.
 10. Finally, go to Peak List Methods → Alignment → Join Aligner to align peaks depending on their *m/z* and retention time by using a match score.
5. After aligning the peaks, fill any data gaps by clicking on Peak List Methods → Gap Filling → Peak Finder. Finally, filter duplicate data points by clicking on Peak List Methods → Filtering → Duplicate Peak Filter.
 6. Export the resulting dataset as a .csv file.
 7. Manually change the .csv extension to a .txt extension. If no file extensions are visible, change the computer settings to reveal file extensions. Go to File Explorer Options → View → Advanced Settings and uncheck the box 'Hide extensions for known file types'.
 8. Import the .txt files into a spreadsheet. This produces a data matrix in which all the detected metabolites, recognized by an identification number, *m/z* value and retention time, are quantified in all samples in terms of their peak area values.

3. Prepare Berry Powder Extracts for Transcriptome Analysis and Process the Data

1. Extract Total RNA from the Berry Samples and Determine the RNA Quality.
 1. Extract total RNA from the berry samples.
NOTE: In this case study, a proprietary kit and a modified procedure that ensures the complete removal of molecules that interfere with RNA analysis, such as polysaccharides and polyphenols, were used. The instructions below are specific for this kit¹⁸.
 1. Weigh 400 mg of berry powder for each sample, divide it into two portions and place 200 mg each in two microfuge tubes.
 2. Add 900 μ l of the lysis solution containing β -mercaptoethanol (provided in the kit) to each 200 mg of powder and vortex immediately and vigorously for at least 30 sec. Heat the sample at 56 °C for 5 min shaking at 800 rpm.
 3. Centrifuge the samples at maximum speed in a benchtop microfuge for 10 min to pellet cellular debris.
 4. Pipet 700 μ l of the supernatant into the filtration column provided in the kit (blue retainer ring) seated in a 2 ml collection tube. Close the cap and centrifuge at maximum speed in a benchtop microfuge for 1 min to remove residual debris. Repeat this step twice using the same filtration column but a fresh collection tube, resulting in three tubes each containing ~700 μ l of the clarified lysate.
 5. Pipet 750 μ l of binding solution (supplied in the kit) into each tube of clarified lysate and mix immediately by pipetting up and down at least five times. Transfer 700 μ l of this mixture to the binding column provided in the kit (red retainer ring) seated in a 2 ml collection tube. Close the cap and centrifuge at maximum speed in a benchtop microfuge for 1 min to bind the RNA.
 6. Decant the flow-through fraction, invert the collection tube and tap it briefly on a clean pad of absorbent paper to drain the residual liquid.
 7. Return the column to the collection tube and pipet the remaining mixture into the same column and repeat the centrifugation and decanting steps. Repeat until the entire mixture has been filtered into the same red binding column.
 8. Now follow the remaining instructions in the kit and elute the RNA in 50 μ l elution buffer (supplied in the kit) and store it at -80 °C ready for the quality control steps.
 2. Determine the quantity and purity of the RNA using a spectrophotometer. Record the absorbance ratios which reveal the degree of contamination with proteins ($A_{260/280}$) and polyphenols/polysaccharides ($A_{260/230}$).
NOTE: RNA suitable for microarray hybridization should score at least 1.8 for both ratios.
 3. Determine the integrity of the RNA.
NOTE: Various systems can be used. A digital acquirer that performs a capillary electrophoretic run in combination with a fluorescent dye was used in this case study. RNA suitable for microarray hybridization should have a RNA Integrity Number (RIN) of at least 8.
2. Prepare Samples and Hybridize the RNA to a Custom Microarray.
 1. Set the starting amount of total RNA for microarray analysis to 200 ng by diluting the RNA solution obtained in the step 3.1.1.8 with deionized RNase-free water. Add the appropriate amount of RNA to a 1.5 ml microcentrifuge tube in a final volume of 1.5 μ l.
 2. Add 2 μ l of diluted spike mix to each RNA sample and follow the manufacturer's instructions to synthesize first strand cDNA, transcribe this into cRNA and label the cRNA with cyanine 3CTP.
 3. Purify the labeled cRNA according to the manufacturer's instructions and elute in 30 μ l RNase-free water.
 4. Determine the yield and specific activity of each cRNA by recording three values on a spectrophotometer: cyanine 3 dye concentration ($\text{pmol } \mu\text{l}^{-1}$), RNA purity ($A_{260/280}$) and cRNA concentration ($\text{ng } \mu\text{l}^{-1}$). Use the formulae found in the manufacturer's instruction manual to calculate the cRNA yield (μg) and the specific activity ($\text{pmol Cy3 per } \mu\text{g cRNA}$).
NOTE: Recommended yields and specific activities differ on the basis of the specific microarray format. In this case study a 4-pack 44K format was chosen, the recommended yield was 1.65 and the specific activity was 9.
 5. Design the custom microarray using suitable software for probe design.

1. For the results described in this case study, prepare a new custom microarray, designed on the 4-pack 44K format by using a web-based application for custom microarray designs and oligonucleotide libraries. Design probes to match 34,651 target transcripts, including 29,971 predicted transcripts from the Pinot noir V1 array, 4,500 new loci identified in the Pinot cultivar by Corvina transcriptome reconstruction and 180 private Corvina genes²⁵.
NOTE: This involved the production of 34,651 specific 60-mer probes, comprising 29,798 Pinot noir predicted transcripts, 4,392 new Pinot loci and 179 Corvina private genes.
6. Prepare the hybridization assembly based on 4-pack format specifications as follows.
 1. Place 1.65 µg of Cy3-labeled cRNA in a final volume of 41.8 µl of deionized RNase-free water. Add 11 µl of 10x Blocking Agent and 2.2 µl 25x Fragmentation Buffer. Incubate at 60°C for 30 min in a thermostatic bath to fragment RNA. Immediately cool on ice for 1min.
 2. Finally, add 55 µl of 2x hybridization buffer, mix well by pipetting and spin for 1 min at 15,500 x g at RT. Promptly place the microcentrifuge tube on ice. Use immediately, do not store it.
7. Load the custom microarray as follows.
 1. Load a gasket slide with the label facing up into the base of a hybridization chamber. Slowly load 100 µl of hybridization sample obtained in step 3.2.6.2 onto each gasket well, dispensing the liquid with the tip of the pipette, avoiding bubbles.
 2. Slowly place the custom microarray facing down, ensuring that the numeric barcode is facing up. Make sure that the sandwich pair is properly aligned. Finally place the cover of the hybridization chamber onto the sandwiched slides and hand-tighten the clamp onto the chamber. Rotate the assembled chamber to assess the mobility of bubbles.
8. Place assembled slide chamber in a rotisserie in a hybridization oven set to 65 °C. Set the rotator to rotate at 10 rpm. Allow hybridization to proceed for 17 hr.
9. Proceed with the microarray slide wash as follows.
 1. First, prepare three slide-staining dishes and fill them with the appropriate washing buffers: 2 dishes with Wash Buffer 1 at RT and 1 dish with pre-warmed (37 °C) Wash Buffer 2.
 2. Disassemble the hybridization chamber and remove the sandwich. With the microarray slide numeric barcode facing up, submerge the sandwich into the first slide-staining dish filled with Wash Buffer 1 at RT and, with the help of clean forceps, separate the gasket from the microarray slide. Quickly transfer the microarray slide into a slide rack and place it in the second slide-staining dish filled with Wash Buffer 1 at RT.
 3. Put the slide-staining dish onto a magnetic stirrer and wash 1 min with moderate stirring. Quickly transfer the slide rack into the third slide-staining dish filled with pre-warmed (37 °C) Wash Buffer 2 and wash 1 min with moderate stirring.
 4. Slowly remove the rack from the slide-staining dish and carefully remove the slide from the rack, avoiding droplets.
NOTE: Do not add Triton X-102 to the washing buffers and omit the acetonitrile washing step.
10. Store the washed chip in the dark at room temperature.
3. Scan the Microarray and Extract the Salient Features.
 1. Place the microarray slide into a suitable scanner and scan each array using the parameter settings recommended in the microarray manufacturer's instructions manual. For the results described here, place each microarray slide into a Slide Holder to facilitate the scanning procedure.
 2. Import the output *.shp* file in an appropriate software that is able to convert a digital signal into numeric fluorescent values. Check the quality control report to ensure that the hybridization procedure was successful.
 1. For the results described here, use the parameter settings recommended in the instruction manual of the feature extraction software and inspect the quality control report to ensure that the parameters listed in **Table 4** are within the normal range given by the manufacturer.

Metric Name	Upper Limit	Lower Limit	Description
AnyColorPrntFeatNonUnif	1.00	NA	Percentage of features that are feature non-uniformity outliers in either channel
DetectionLimit	2.00	0.10	Average plus 1 standard deviation of the spike ins below the linear concentration range
absGE1E1aSlope	1.20	0.90	Absolute of slope of fit for Signal vs. Concentration of E1a probes
MedCVProcSignal	8.00	NA	Median %CV for the Processed Signal
gNegCtrlAveBGSubSig	5.00	-10.00	Average of background subtracted signal of all inlier negative controls (BGSubSignal is calculated by subtracting a value called BGUsed from the feature mean signal)
gNegCtrlAveNetSig	40.00	NA	Average of net signal of all inlier negative controls
gNegCtrlSDevBGSubSig	10.00	NA	Standard deviation of background subtracted signals of all inlier negative controls
gNonCntrlMedCVProcSignal	8.00	NA	Median %CV for the Processed Signal of the non-control probes
gSpatialDetrendRMSFilter	15.00	NA	Residual of background detrending fit

Table 4: Principal parameters to be checked to verify the quality of microarray hybridization.

4. Process the Microarray Data.
 1. Once all the microarray slides have been scanned and the quality control has been assessed positively, prepare a tab-delimited data-matrix by selecting the *gProcessedSignal* values from each single-subarray outcome file, representing the raw fluorescence intensities of each probe.
 2. In a spreadsheet, normalize the data on the 75th percentile within each array (*P* values) and calculate the average of all the *P* values among all different sub-arrays to calculate the *R* ratio.
 3. Then, on the same spreadsheet, normalize each *gProcessedSignal* value to the *R* ratio of its own sub-array.

4. Carry Out the Detailed Statistical Analysis of the Metabolomics and Transcriptomics Data

1. Prepare the Statistical Analysis Software.

NOTE: In this case study, a software able to perform PCA, PLS-DA and O2PLS-DA was used.

 1. Import the metabolomics and transcriptomics data. Go to File → New Regular Project → New Regular Project to import the data matrix obtained with MZmine software. Then click on Edit → Transpose the whole matrix → Home and assign the appropriate Primary and Secondary ID → Finish.
 2. Mean center the data and scale them using the pareto scale. In Home window, go to Edit → M1 and change the appropriate parameters depending the data. For the results described here, change the Scale from Unit Variance to Par.
 3. Scale the transcriptomics data using the Unit Variance setting.
2. Carry Out the Multivariate Statistical Analysis.
 1. Implement the PCA as shown in **Figure 2**. In this case study, PCA reveals the main differences between samples, reflecting the different ripening stages and growing seasons.
 1. In the Workset window, select PCA-X as model type. Press Autofit. Pay attention to the R2X(cum) and Q2(cum) values as they give an idea about the quality of the model.

NOTE: Generally, the higher the values the better the model, but models with very high R2X(cum) might over-fit the data. As empirical rule, we stop adding Principal Components when the Q2(cum) value starts to decrease.
 2. Then, select Scores → Scatter to see the plot that shows the possible grouping of the samples.
 3. Inspect the PCA Score Plot. If a good model is obtained (Q2cum >0.5), use the same classes of samples to build a O2PLS-DA model (step 4.2.2).
 2. Build two O2PLS-DA models using the samples classified by macro-zone and validate the models using a permutation test with 200 permutations.
 1. Assign the classes by going to Home window → New As → M1 → Observations. Set the desired classes. Then, change the Model Type from PCA-X to O2PLS-DA. Press Autofit. Ensure that the number of components of the PLS-DA model is the same as that of the O2PLS-DA.

2. To validate the O2PLS-DA model, go to Analyze CV-ANOVA and see the right p-value. Moreover, click on New As in the Home window → M2 and change the Model Type from O2PLS-DA to PLS-DA. Press Autofit.
 3. Go to Analyze → Permutations and perform 200 permutations (de-select Recalculate Permutations option).
Note: The final output shows a window in which the R2 value should generally hit the Y-axis in values under 0,4, while the Q2 should hit the negative part of the Y-axis. If R2 and/or Q2 values are incorrect, reduce the number of components both in PLS-DA and O2PLS-DA models.
 4. Proceed to select M2 in Project Window and Click on Scores → Scatter to see the plot and the position of the sample classes. To observe what metabolites characterize one or more specific classes, go to Plot/List → Scatter.
 5. Change the Select Data Type to Observations and Loadings → Add Series and modify the Item in X-Axis and Series into pq(corr) and Pred Comp into 1 and either 2 or further components when present, respectively.
 6. With the mouse's right button pressed on the plot, go to Property → Color and select By Terms to distinguish the plot symbols among molecules and classes. Go to Layout → Format Plot → Axis and/or Styles to modify the plot with the desired characteristics.
3. Based on the results of the metabolomics O2PLS-DA analysis, present differences in the relative levels of specific metabolites or classes of metabolites as histograms.
 4. Based on the results of the transcriptomics O2PLS-DA analysis, retrieve and assign differentially modulated genes to Gene Ontology (GO) classifications²⁸.
 5. Identify relationships between metabolite levels and gene expression manually.

Representative Results

The case study described in this article yielded a final data matrix comprising 552 signals (m/z features) including molecular ions plus their isotopes, adducts and some fragments, relatively quantified among 189 samples (7 vineyards x 3 ripening stages x 3 growing seasons x 3 biological replicates). The total number for data points was therefore 104,328. Fragmentation tree analysis resulted in the annotation of 282 m/z features, corresponding to metabolites plus adducts, isotopes and fragments. Exploratory analysis of the whole data matrix by PCA showed that samples clustered according to the ripening stage (veraison, mid-ripening and ripe) along the first and second principal components (t1-t2, **Figure 2A**), and according to the growing season along the third principal component (t1-t3, **Figure 2B**).

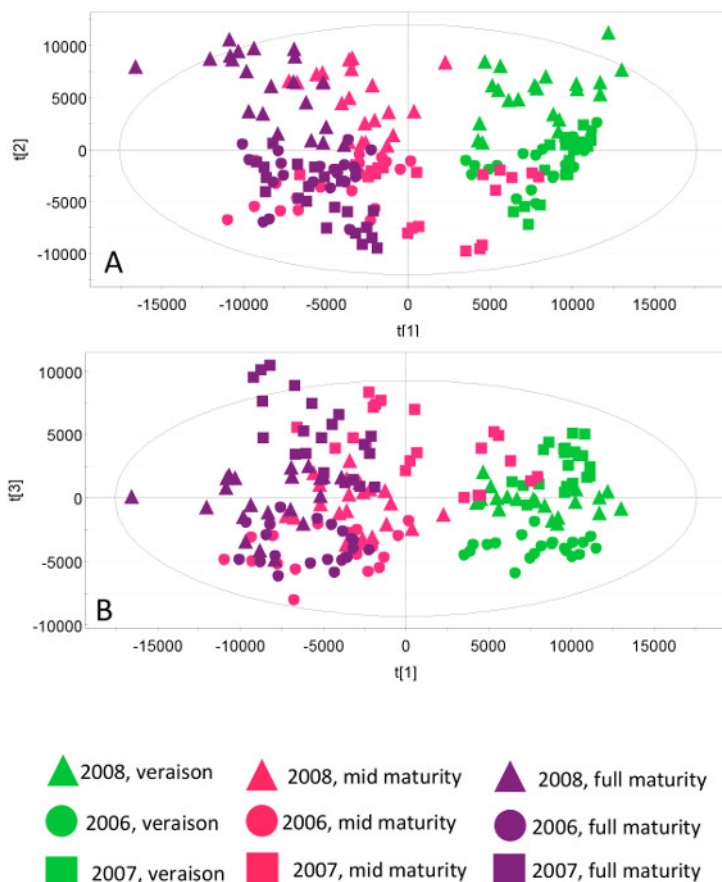


Figure 2: PCA of the whole metabolomics data matrix. Unsupervised PCA-X score scatter plots showing the clustering of grapevine samples collected during three different growing seasons (2006, 2007, and 2008) and ripening stages (veraison, mid-ripening and ripe berries). In the first plot (A) the samples clustered according to the ripening stages, whereas in the second (B) they clustered according to the growing season. The colors highlight the ripening stages as green (veraison), pink (mid-ripening) and purple (ripe berries). The symbols represent the growing seasons: circles (2006), squares (2007) and triangles (2008). Component $t1$: Q2: 0.34; R2X: 0.331; Q2cum: 0.304; Component $t2$: Q2: 0.185; R2X: 0.155; Q2cum: 0.433; Component $t3$: Q2: 0.145; R2X: 0.0924; Q2cum: 0.515. [Please click here to view a larger version of this figure.](#)

The unsupervised PCA was unable to reveal specific terroir features, which are hidden under the prevalent vintage effects, so a supervised O2PLS-DA approach was applied to two separate datasets, *i.e.*, berries at veraison and fully ripe berries. This allows the exploration of metabolic differences representing the three macro-zones (Lake Garda, Valpolicella and Soave) in two key stages of ripening, to identify specific terroir signatures representing these zones at these ripening stages.

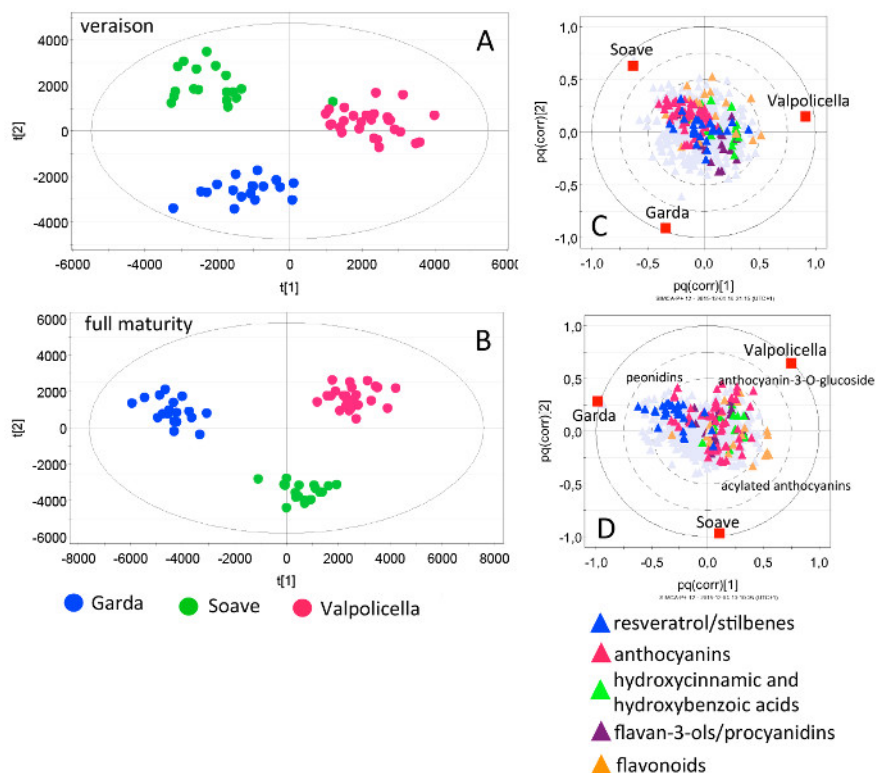


Figure 3: O2PLS-DA of veraison and ripe grape berries. O2PLS-DA score scatter plots showing the clustering of grapevine samples collected in the Lake Garda (blue), Soave (green) and Valpolicella (pink) macro-zones at veraison (A) and when the berries were ripe (B). The metabolites responsible for the clustering observed in (A) and (B) are visible in correlation loading plots (C) and (D) respectively. Groups of metabolites, represented as triangles, are blue for resveratrol and stilbenes, pink for anthocyanins, green for hydroxycinnamic and hydroxybenzoic acids, purple for flavan-3-ols/procyanidins, and yellow for other flavonoids. A: component P1: Q2: 0.278; R2X: 0.0529; Q2cum: 0.278; component P2: Q2: 0.33; R2X: 0.0394; Q2cum: 0.608. B: component P1: Q2: 0.353; R2X: 0.0639; Q2cum: 0.353; component P2: Q2: 0.188; R2X: 0.0374; Q2cum: 0.541. [Please click here to view a larger version of this figure.](#)

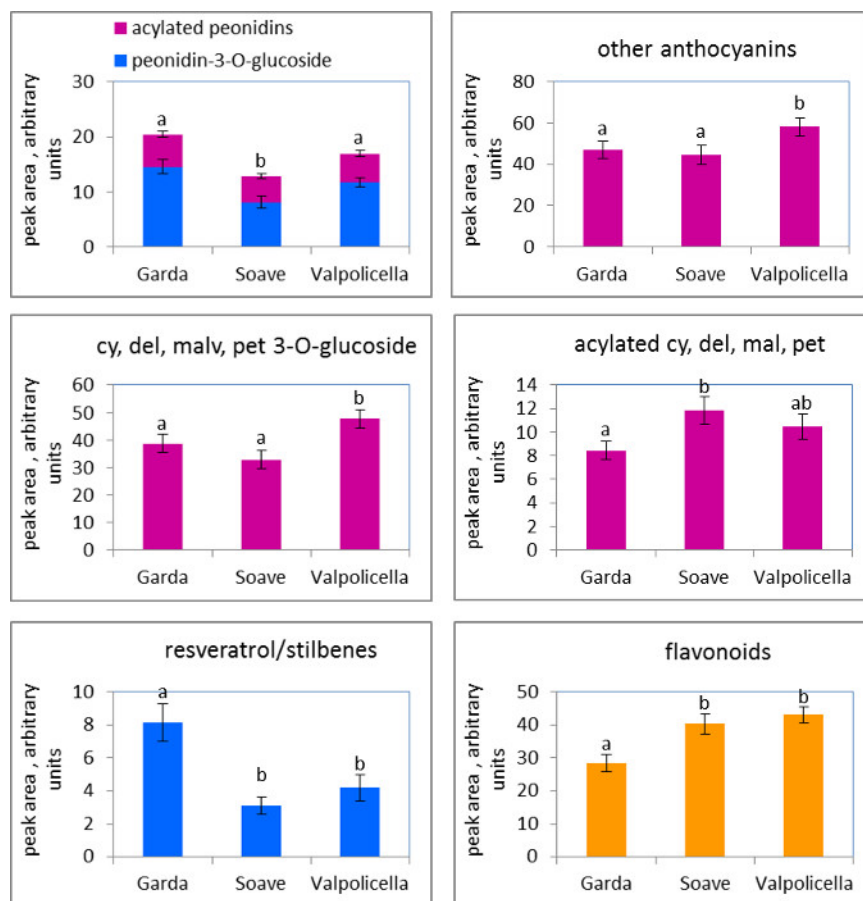


Figure 4: Distribution of metabolic markers among the three macro-zones: Lake Garda, Soave, and Valpolicella. Metabolite levels reflect the mean \pm standard error ($n = 18$ for Garda and Soave, 27 for Valpolicella macro-zone) of different glycosylated and acylated anthocyanins, resveratrol/stilbenes and flavonoids in ripe Corvina berries in the three different growing seasons (2006, 2007, and 2008). Different letters represent groups that were significantly different as determined by a t-test ($p < 0.05$). [Please click here to view a larger version of this figure.](#)

The specific signatures of the seven individual vineyards were identified using a specialized statistical approach¹⁰. Two O2PLS-DA models were built, one exploring the differences between the berries grown in the three macro-zones at veraison, and the other exploring the differences in the three macro-zones among mature berries. The models were cross-validated using a permutation test (200 permutations) showing that the model describing the mature berries across the three macro-zones was valid, whereas only the Lake Garda samples were distinct from the other samples at veraison (not shown).

The score plots of the two models, which demonstrate how the samples from the three macro-zones at veraison and maturity are separated according to the distribution of metabolites, are shown in **Figures 3A** and **3B**. The loading plots of these model expressed as $pq(\text{corr})$, *i.e.*, the correlation between p (the class of samples) and q (the metabolites), are shown in **Figures 3C** and **3D**. In the loading plots, the red squares represent the class of samples (macro-zones) and the colored triangles represent the individual metabolites. The distance between the metabolites and the samples reflects their relationships. The metabolites are color-coded according to their chemical class.

Given the low statistical validity of the veraison model as shown by permutation test, it is likely to suffer from overfitting. Therefore, the following observations refer solely to the mature berry samples, whose model was statistically valid. In the loading plot shown in **Figure 3D**, the stilbenes are shifted towards the Lake Garda macro-zone, whereas the flavonoids are shifted towards the Soave and Valpolicella macro-zones. A closer look at individual metabolites reveals the asymmetric distribution of anthocyanins, with peonidin and its derivatives more prevalent in the Lake Garda macro-zone and other anthocyanins more prevalent in the Valpolicella macro-zone (**Figure 4**). Simple anthocyanin-3-O-glucosides are more prevalent in the Valpolicella macro-zone whereas acylated and coumarated derivatives are more prevalent in the Soave macro-zone.

The transcriptomics component of this case study was originally out using a transcriptomics platform that is no longer supported. As a consequence, we set up an alternative procedure, with the still available platform; the new platform contains more probes than the old one, including 249 more Pinot noir predicted transcripts, 4392 newly identified Pinot loci and 179 Corvina private genes²⁵.

Discussion

This article describes the metabolomics, transcriptomics and statistical analysis protocols used to interpret the grape berry terroir concept. Metabolomics analysis by HPLC-ESI-MS is sensitive enough to detect large numbers of metabolites simultaneously, but relative quantitation is affected by the matrix effect and ion suppression/enhancement. However, a similar approach has already been used to describe the ripening and post-harvest withering of Corvina berries, and the correction of matrix effects had a limited impact on the results⁵. Furthermore, a recent large-

scale multi-instrument inter-laboratory study to determine the comparative robustness of NMR and LC-MS for untargeted metabolomics using the same samples revealed that the different instruments and technologies yielded consistent results⁶. The case study described herein involved the collection and analysis of samples at three ripening stages from seven vineyards in three macro-zones over three growing seasons. Herein, the terroir signature was investigated at the macro-zone level (Lake Garda and Soave, each represented by two vineyards, and Valpolicella, represented by three vineyards) using PCA and O2PLS-DA multivariate statistics. The differences among individual vineyards are more subtle and require more sophisticated and sensitive statistical approaches¹⁰. Various steps of the proposed protocols are critical and have to be considered in order to successfully answer to the biological questions about terroir influences on grape quality.

First of all, the appropriate sampling plan is critical: the choice of one specific clone to minimize the genetic differences and the multiple-year duration of the sampling allows to highlight the real, sound differences between the various terroirs. On the other side, the proposed research was performed within a terroir that is particularly suitable for viticulture, and this could minimize the terroir-differences on grape quality. Thus, it would be very interesting to compare the same cultivar grown in a less optimal zone but, obviously, these vineyards could simply be not available.

From the technical point of view, a highly reproducible metabolite separation through HPLC is critical to obtain good data matrix, since the higher the differences in retention time in the various analyses, the higher the number of mistakes in peak alignment in the final dataset. Thus, it is of critical importance to set an appropriate re-equilibration time, to divide the samples into batches of 9-10 samples with cycles of chromatographic column cleaning between them and to use blank samples as the first sample of each batch. LC-MS is affected by a certain degree of instability, which may result in differences between samples that are instrument-driven. This problem can be partially solved by the randomization of sample analysis, as shown in this paper. An improvement of the method is the use of appropriate quality control samples in each batch, that can inform on the platform state. An appropriate standard compound mix (*i.e.*, standard compounds with *m/z* values and retention times spanning over the whole *m/z* and retention time range) and a new control sample obtained by mixing equal parts of powder from all the samples can inform about platform effects and also eventually be used for *a-posteriori* data normalization.

Another important point in order to compare metabolomics and transcriptomics results was to use exactly the same material (*i.e.*, the same crushed samples) for both the analyses. In the data analysis, it is critical to use an appropriate statistics method, such as OPLS-DA, which allows easy interpretation of the differences in terms of gene expression and metabolite accumulation with respect to methods such as PCA. One of the major limitations of this kind of approach is the absence of strong methods for transcriptomics and metabolomics data integration. The development of suitable methods for different types of data correlation is necessary in order to build a strong and reliable correlation between transcriptomics and metabolomics data.

The techniques described in this article clearly revealed the ripening and vintage effects on the berry metabolome, but specific chemical signatures representing the three macro-zones were also identified in ripe berries underneath the prevalent vintage effect. Interestingly, the analysis of veraison berries did not yield a statistically valid model, indicating that the terroir concept is predominantly manifested by metabolites that accumulate during ripening (*e.g.*, anthocyanins and stilbenes) than those already present in the immature berries.

Disclosures

The authors have nothing to disclose.

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