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Untargeted Metabolomics Discriminates Grapes and Wines from Two Syrah Vineyards Located in the Same Wine Region

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Abstract: The influence of terroir in determining wine sensory properties is supported by the specific grape microbiome and metabolome, which provide distinct regional wine characteristics. In this work, the metabolic composition of grapes, must and wine of the Syrah grape variety cultivated on two sites in the same region was investigated. Concomitantly, a sensorial analysis of the produced wines was performed. Ultra-high-resolution liquid chromatography coupled with tandem mass spectrometry (UHPLC-Q-ToF-MS/MS) was applied to identify grape and wine metabolites. Untargeted metabolomics was used to identify putative biomarkers for terroir differentiation. More than 40 compounds were identified, including 28 phenolic compounds and 15 organic acids. The intensity evolution of the analyzed chemical compounds showed similar behavior during the fermentation process in both terroirs. However, the metabolic analysis of the grape, must and wine samples enabled the identification of an anthocyanin, chrysanthemin, as a putative biomarker of terroir 1. The overall sensorial quality of the wines was also evaluated, and according to the hitherto reported results, the wines from site 1 scored better than the wines from site 2. The results highlight the potential of metabolomics to assess grape and wine quality, as well as terroir association.

Keywords: metabolomics; grapes; wine; terroir

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1. Introduction

Wine characteristics are the result of multiple factors, including the terroir and cultivar, as well as viticultural and vinification practices. The quality of wines is often associated with the geographical region of production. Specific growing conditions determine the final fruit composition, which contributes to the specific chemical and sensorial characteristics of wine, conferring distinct regional characteristics onto the produced wines. Several works have been published on the interaction between different environmental conditions on the grape metabolome [1–5]. Anesi and co-workers [3] showed a terroir-specific response of the metabolome and transcriptome in grape berries from a single clone of the Corvina variety, cultivated in seven different vineyards over several vintages.

The metabolites present in the grapes are determined by the grape variety, soil, edapho-climatic conditions and management practices [6], which then influence the entire process of wine making. In addition to grape metabolites, the grape microbiome and winery technology have also been correlated with the chemical composition of wine and contribute to specific wine characteristics [7].

Metabolomics involves the use of powerful technologies to identify the broad spectrum of metabolites that are present in different samples, namely in grape and wine samples. Over the past decade, many studies have been carried out to investigate the metabolic profile of grapes and wines, as reviewed by Pinu [8]. Techniques such as gas chromatography coupled with mass spectrometry (GC-MS) [9–11] and nuclear magnetic resonance (NMR) [12,13] are the approaches most frequently used to determine the variety of grape and wine based on the metabolic composition.

Ultra-high-performance liquid chromatography coupled with time-of-flight high-resolution tandem mass spectrometry (UHPLC/Q-ToF-MS/MS) provides detailed information on sample composition due to its high sensitivity. This technique has been used to understand the molecules involved in wine flavor and taste [14] and can help to distinguish between several cultivars [15]. The identification of compounds, most of the time, draws on databases, but not all the compounds can be found in these databases, mainly when untargeted metabolomics is performed. Therefore, the use of MS2 is appropriate and helps in the identification process. This was the case of the present work [16].

The main objective of the present work was to understand how terroir influences the grape and wine metabolite profiles, as well as the sensory parameters, in the elemental wines produced. The dynamics of the fermentation process and the impact on the must and wine quality were evaluated by metabolic analysis. Comparative organoleptic analysis was performed on the elemental wines produced. The study focused on comparative data of the Syrah grape variety cultivated in two vineyards in the same Demarcated Wine Region of Lisbon (Portugal), and distinct metabolic profiles could be assigned. Thus, local markers could distinguish wines produced in different vineyards within the same region.

2. Materials and Methods

2.1. Grape, Must and Wine Samples

Syrah grapes were collected from two different vineyards located at site 1 (S1) and site 2 (S2). The maturation was controlled through physicochemical analysis up to harvest. Grapes were randomly collected from the entire vineyard field at optimal maturation and transported to the INIAV experimental winery. From each site, four biological replicate samples for the grape analysis were prepared by collecting 50 berries each from different bunches. The samples were immediately frozen and stored at −80 °C until the time of use.

The grapes processed for microvinifications were crushed, destemmed and supplemented with 20–30 mg/L of SO_2 (potassium metabisulfite). Microvinifications involving two biological replicates each of 50–60 L volume were performed under the same conditions in stainless steel deposits. After homogenization, *Saccharomyces cerevisiae* yeast starter was added (30 g/hL) following rehydration, according to the manufacturer's instructions. The fermentation progress was monitored through daily measurements of the density and temperature after homogenization. Must samples were collected in duplicate during fermentation at 3 different timepoints: initial fermentation after must homogenization, mid-fermentation and end-fermentation. The must samples were immediately frozen at −80 °C.

After fermentation, the clarified wines were stored in 20 L glass vessels until bottling. Wine samples were collected at the moment of bottling, approximately 5 months after clarification. Table 1 summarizes the sample description and designation.

Table 1. Description, number of replicates and designation of the samples.

Sample Designation	Number of Samples *per* Site	Description	Local
Grape 1 (S1.1)	4	50 crushed grape berries	Site 1
Grape 2 (S2.1)	4		Site 2
Initial 1 (S1.2)	2	50 mL of must at initial fermentation	Site 1
Initial 2 (S2.2)	2		Site 2

Table 1. *Cont.*

Sample Designation	Number of Samples *per* Site	Description	Local
Middle 1 (S1.3) Middle 2 (S2.3)	2 2	50 mL of must at mid-fermentation	Site 1 Site 2
End 1 (S1.4) End 2 (S2.4)	2 2	50 mL of must at end-fermentation	Site 1 Site 2
Wine 1 (S1.5) Wine 2 (S.2.5)	2 2	50 mL wine collected upon bottling	Site 1 Site 2

2.2. Reagents

Acetonitrile (ACN), isopropanol (iPrOH), acetic acid, formic acid and sodium hydroxide (NaOH) were obtained from Fisher Chemical (Porto Salvo, Portugal). All the chemicals were analytical grade. Ultra-pure water (MilliQ H_2O) was obtained through a water purification system (Merck Millipore, Burlington, MA, USA).

2.3. Metabolic Profiling

The chromatographic analyses were carried out with an Elute autosampler for ultra-high-performance liquid chromatography (UHPLC; Bruker, Bremen, Germany) using an Intensity Solo 2 C18 reverse-phase column (100 × 2.1 mm, 2.0 µm; Bruker, Bremen, Germany). The grape samples were crushed after unfreezing, and the liquid obtained was used for the analysis, as described below. All the samples, including the grape juice, the fermentation must and the wine, were first diluted in MilliQ H_2O in proportions of 1:2. A volume of 5 µL of each sample was injected (auto injector) into the system using a gradient composed of MilliQ H_2O + 0.1% formic acid (solution A) and ACN + 0.1% formic acid (solution B), as follows: 0 min—95% A; 1.5 min—95% A; 25% A—13.5 min; 0% A—18.5 min; 0% A—21.5 min; 95% A—23.5 min; 95% A—30 min. The flow rate was set to 0.250 mL/min, and the column was kept at 35°C.

For the mass spectrometry, an Impact II quadrupole time-of-flight (QTOF; Bruker, Bremen, Germany) spectrometer was used, and the data were acquired through the DataAnalysis® 4.4 software. The method consisted of MS/MS scans in negative ionization modes. Signals in the m/z 100–5000 range were recorded. The capillary voltage was set to 3500 V and 4000 V for the negative and positive ionization modes, respectively. The dry gas was kept at 8.0 L/min and at 200 °C. The collision cell energy was set to 5.0 eV, and a loop of 20 µL was used. The internal calibration solution consisted of 250 mL MilliQ H_2O, 250 mL iPrOH, 750 µL acetic acid, 250 µL formic acid and 0.5 mL 1N NaOH solution. The compounds were identified by considering the molecular formulas suggested by the DataAnalysis program from Bruker, while the chemical structure was searched in Pubchem, MetLin, and HMDB and checked using MS2 fragmentation through the program MassFrag from Bruker. The validation of the detection parameters was assessed by running the pure compounds for chrysanthemin and salicylic acid (both from Sigma-Aldrich, Barcelona, Spain) under the same conditions as those used for the samples under analysis. Data directly obtained through UHPLC/Q-ToF-MS/MS were used for the comparison of the samples.

2.4. Sensory Analysis

The sensory analysis was carried out by the INIAV-Dois Portos expert panel using nine highly trained and independent wine tasters in a standardized tasting room with individual white booths. Standard winetasting glasses (ISO 3591:1977) were filled with 30 mL of the wine, which was served between 16 and 18 °C. Three visual, ten flavor and seven taste characteristics were evaluated using a scale between 0 (absence) and 9 (highest intensity), and the overall quality was also assessed (0–20).

2.5. Statistical Analysis

Statistical analysis was conducted using the Metaboscape® software from Bruker (Bremen, Germany) that performs principal component analysis (PCA), and volcano tests were used for the identification of differences between the samples.

One-way ANOVA with alpha = 0.01 (99% confidence level) and 0.05 (95% confidence level) was performed to identify statistical differences between the compounds present in the grapes and wine from both terroirs. The calculations were performed using Excel Statics software.

One-way ANOVA with alpha = 0.05 (significance level) and alpha = 0.95 (confidence limits) was performed to assess the sensory results of each replicate wine in order to evaluate the effect of the site factor. Bartlett's, Cochran's and Hartley's tests were used in order to test the homogeneity of the variances [17]. When the effect of the site factor was detected, the calculation of the least significant difference (LSD), using Fisher's LSD test with alpha = 0.05, was applied for the comparison of the different averages [18]. All the calculations were carried out using the software Statistica 7.0 (Stat Soft Inc., Tulsa, OK, USA).

3. Results and Discussion

The sample collection and fermentation trials took place during the 2018 vintage. The field experiment was set up in vineyards of *V. vinifera* cv. Syrah, located at two different sites (S1 and S2) of the Demarcated Win Region of Lisbon. Based on the Harmonized World Soil Database (FAO), both vineyards belong to the reference soil group of "Cambisols". The S1 area is characterized as calcaric cambisols, while S2 is in an area of chromic cambisols.

The maturation was controlled through physicochemical analysis. The results of the main characteristics analyzed at harvest are presented in Table 2.

Table 2. Main characteristics of the grape musts at the date of harvest at the two sites under study.

Site	Date of Harvest	Brix	Sugar ($g \cdot L^{-1}$)	Mass Density (20 °C)	pH	Total Acidity ($g \cdot L^{-1}$ tart ac.)
S1	27 September 2018	21.9	213.6	1.090	3.38	4.6
S2	4 October 2018	21.4	207.8	1.088	3.54	5.4

3.1. Compound Identification by UHPLC-MS/MS

Samples from grapes and wine originating from site 1 and site 2, with 4 replicates of each, were analyzed by UHPLC-MS/MS, and the results were assessed through principal component analysis (PCA, Metaboscape) in order to identify similarities and differences. Four comparisons were performed: grapes versus wine from terroir 1 (S1.1 vs. S1.5), Figure 1a, and from terroir 2 (S2.1 vs. S2.5), Figure 1b; grapes from terroir 1 versus grapes from terroir 2 (S1.1. vs. S2.1.), Figure 1c; and wine from terroir 1 versus wine from terroir 2 (S1.5 vs. S2.5), Figure 1d. Four replicates of each sample were analyzed, each one being indicated by «R» (Figure 1).

PCA analysis allowed us to show that all four replicates were grouped together in the case of both the grape and wine samples from both terroirs, highlighting their distinctness. In each terroir, the separation of the samples was achieved by comparing grapes and wine (Figure 1a,b). Likewise, when comparing the grape samples (Figure 1c) or wine samples (Figure 1d), separate groups of each terroir could be observed.

As all the replicates were grouped together, one of them in each condition was used for compound identification. The identification was then attempted regardless of the sample origin, and the proposed compounds and their retention time (Rt), molecular formula, experimental m/z and its associated error, together with the fragmentation, MS/MS, which was used to confirm the suggestion, are indicated in Table 3. A total of 56 compounds were putatively identified, including sugars, amino acids, organic acids and phenylpropanoids.

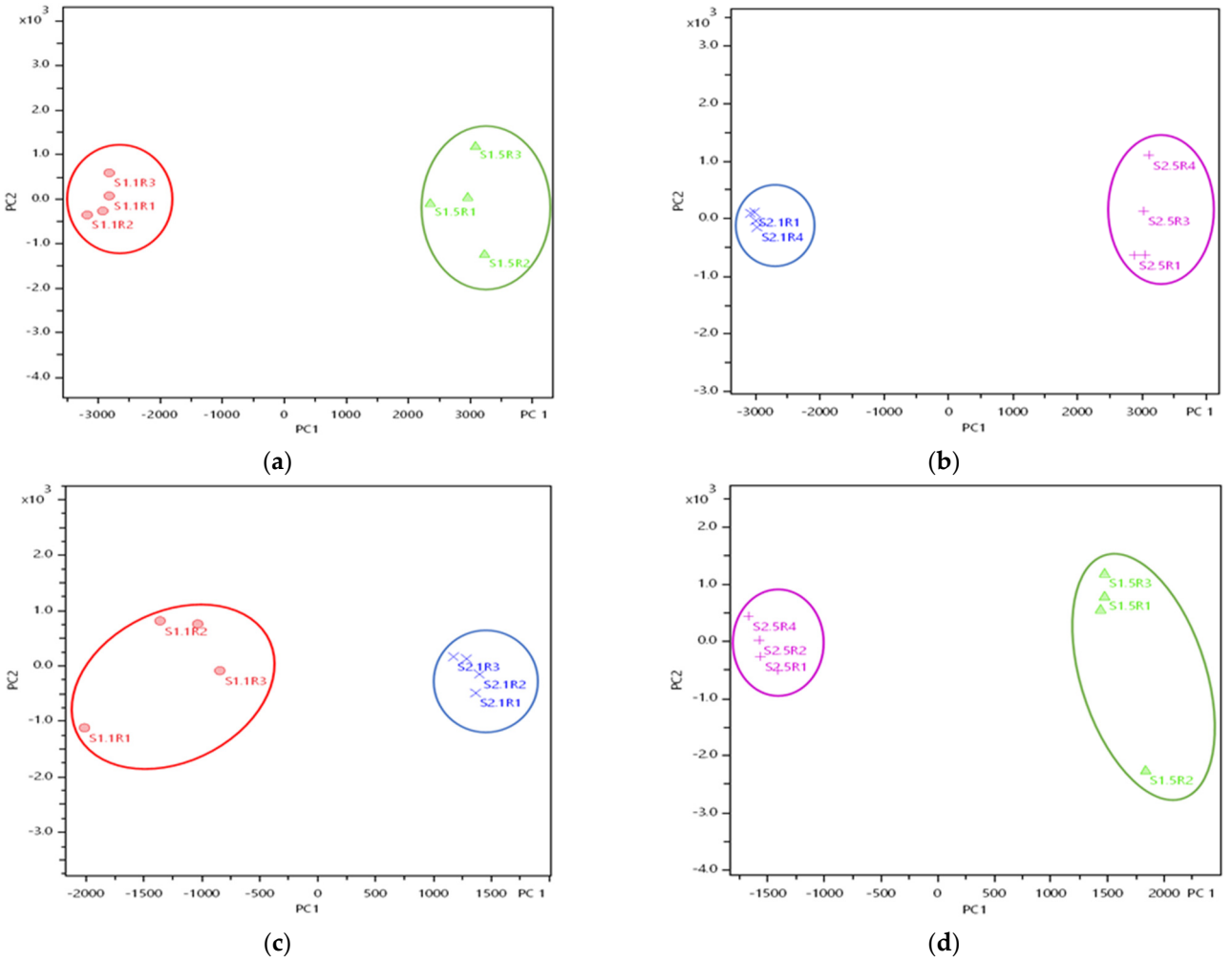


Figure 1. Comparison of (**a**) grapes and wine from site 1 (S1.1 vs. S1.5): PC1(90.8%), PC2(93.8%), R^2(cum) = 0.961, Q^2(cum) = 0.796; (**b**) grapes and wine from site 2 (S2.1 vs. S2.5): PC1(93%), PC2(95.3%), R^2(cum) = 0.848, Q^2(cum) = 0.796; (**c**) grapes from site 1 and site 2 (S1.1 vs. S2.1): PC1(60.1%), PC2(75.4%), R^2(cum) = 0.879, Q^2(cum) = 0.569; and (**d**) wine from site 1 and site 2 (S1.5 vs. S2.5): PC1(71.2%), PC2(77.4%), R^2(cum) = 0.98, Q^2(cum) = 0.671. Principal component analysis (PCA). Where the replicates (R) are similar, the label cannot be discriminated in the figures.

Table 3. Putative compounds identified using UHPLC-MS/MS in the negative mode. Retention time (RT), molecular formula and mass spectrometric data of the molecular ions and observed fragments of compounds present in all the samples.

RT (min)	Formula	$[M\text{-}H]^-_{experimental}$	Error (ppm)	Main MS/MS	Proposed Compound
1.0	$C_6H_{12}O_6$	179.0561	0.0	89.0243 (7.1%); 71.0136 (65.8%); 59.0139 (100%)	α-D-glucose
1.0	$C_7H_{14}O_8$	225.0617	−0.4	113.0243 (6.4%); 89.0245 (21.8%); 71.0138 (65%); 59.0140 (100%)	glucoheptonic acid
1.1	$C_4H_6O_6$	149.0089	1.6	87.0086 (15.9%); 72.9931 (100%); 59.0141 (24.5%)	tartaric acid
1.2	$C_4H_6O_5$	133.0140	2.0	71.0137 (100%); 59.0146 (6.3%)	malic acid
1.2	$C_4H_4O_4$	115.0038	−1.0	71.0149 (100%)	fumaric acid

Table 3. *Cont.*

RT (min)	Formula	$[M-H]^-_{experimental}$	Error (ppm)	Main MS/MS	Proposed Compound
1.3	$C_6H_{10}O_7$	193.0354	−0.1	111.0089 (23.7%); 103.0040 (27.8%); 87.0087 (47.1%); 75.0087 (29.1%); 59.0139 (100%)	2-keto-D-glucuronic acid
1.5	$C_5H_6O_5$	145.0143	−0.3	101.0243 (23.2%); 85.0297 (6.9%); 57.0349 (100%); 55.0195 (23.4%)	ketoglutaric acid
1.8	$C_6H_8O_7$	191.0195	1.1	111.0087 (64.9%); 87.0083 (100%); 67.0182 (12%); 57.0344 (19.4%)	citric acid
1.8	$C_5H_8O_5$	147.0298	0.7	129.0197 (4.1%); 103.0413 (4.6%); 101.0248 (29.1%); 85.0295 (23.4%); 57.0348 (100%)	(S)-2-hydroxyglutarate
1.8	$C_5H_8O_5$	147.0297	1.2	115.0028 (10.5%); 103.0398 (9.6%); 71.0136 (100%)	citramalic acid
1.9	$C_5H_{10}O_4$	133.0504	1.7	87.0073 (10.8%); 71.0137 (100%)	2,3-dihydroxy-3-methylbutanoic acid
1.9	$C_{20}H_{32}N_6O_{12}S_2$	611.1437	1.7	611.1418 (13.9%); 306.0754 (100%); 272.0894 (27.1%); 143.0460 (17.2%); 128.0349 (12.2%)	glutathione, oxidized
1.9	$C_6H_6O_6$	173.0091	0.5	129.0206 (18.2%); 111.0086 (13.9%); 85.0292 (100%); 67.0189 (5.9%)	aconitic acid
2.0	$C_4H_6O_4$	117.0193	0.5	73.0293 (100%); 55.0207 (9.7%)	succinic acid
2.1	$C_6H_{10}O_5$	161.0453	1.4	59.0138 (31.9%); 57.0349 (100%)	3-hydroxy-3-methyl-glutaric acid
2.4	$C_7H_6O_5$	169.0141	0.8	125.0241 (100%); 97.0292 (33%); 79.0190 (21.1%); 69.0345 (51.6%)	gallic acid
2.7	$C_6H_{10}O_6$	177.0404	0.5	87.0078 (7.8%); 72.9934 (100%); 59.0141 (77.2%); 44.9997 (8.3%)	4-ethoxy-2,3-dihydroxy-4-keto-butyric acid
3.5	$C_{13}H_{16}O_{10}$	331.0669	0.5	169.0137 (100%); 125.0244 (34.9%)	glucogallic acid
3.9	$C_6H_{10}O_5$	161.0456	−0.3	71.0141 (87.6%); 59.0146 (8.3%); 45.0351 (17.9%);	2-dehydro-3-deoxy-L-rhamnotic acid
3.9	$C_{15}H_{14}O_7$	305.0667	−0.1	237.0752 (10.4%); 219.0637 (16.4%); 167.0344 (35.7%); 139.0399 (32%); 125.0239 (100%); 109.0288 (14.1%)	epigallocatechin
4.1	$C_{14}H_{20}O_8$	315.1085	0.2	153.0557 (100%); 123.0451 (58%)	vanilloloside
4.1	$C_7H_6O_4$	153.0194	−0.6	109.0304 (100%); 91.0185 (17.1%); 53.0408 (9.7%)	protocatechuic acid
4.2	$C_8H_{10}O_3$	153.0553	2.5	123.0449 (100%); 121.0287 (15.2%); 109.0313 (13.1%); 95.0496 (23.5%); 93.0353 (16.9%); 81.0339 (22.1%); 71.0137 (12.6%)	hydroxytyrosol
4.3	$C_9H_8O_4$	179.0349	0.5	135.0455 (100%); 117.0379 (16.7%); 107.0496 (12%); 89.0395 (3.5%); 65.0029 (9.9%)	caffeic acid
4.4	$C_{13}H_{12}O_9$	311.0409	0.0	179.0348 (74.6%); 149.0089 (41%); 135.0451 (100%); 87.0085 (27.5%)	caftaric acid

Table 3. *Cont.*

RT (min)	Formula	$[M-H]^-_{experimental}$	Error (ppm)	Main MS/MS	Proposed Compound
4.6	$C_{22}H_{24}N_4O_4$	407.1714	2.6	203.0818 (100%); 159.0918 (4.3%); 142.0661 (5.9%); 116.0503 (22.8%)	Phe Trp Gly (in any order)
4.7	$C_{11}H_{12}N_2O_2$	203.0825	0.4	142.0667 (26.6%); 116.0505 (100%); 74.0249 (10.3%)	tryptophan
4.7	$C_{30}H_{26}O_{12}$	577.1343	1.6	451.1024 (8.9%); 425.0866 (18.3%); 407.0768 (54.5%); 289.0714 (100%); 161.0244 (13.3%); 137.0234 (8.9%); 125.0243 (35.8%); 123.0448 (10.5%);	procyanidin B2
5.0	$C_7H_{12}O_5$	175.0609	1.6	113.0618 (18.4%); 101.0604 (8.4%); 85.0663 (24.1%); 69.0345 (14.9%)	3-isopropylmalic acid
5.0	$C_{13}H_{12}O_8$	295.0453	2.1	163.0391 (41.3%); 149.0086 (84.1%); 119.0501 (100%); 87.0087 (16.8%)	*cis*-coutaric acid
5.1	$C_7H_{12}O_5$	175.0610	0.9	131.0708 (2.9%); 115.0401 (81.3%); 113.0607 (44.8%); 85.0656 (100%); 59.0143 (22.7%)	2-isopropylmalic acid
5.1	$C_9H_8O_3$	163.0401	0.1	119.0504 (100%); 117.0349 (9.4%); 93.0341 (9.7%)	2-hydroxycinnamic acid
5.2	$C_{15}H_{18}O_8$	325.0923	1.7	163.0400 (69.9%); 119.0501 (100%); 93.0349 (8.3%)	melilotoside
5.4	$C_{23}H_{24}O_{12}$	491.1198	−0.6	343.0832 (7.9%); 331.0809 (17.3%); 329.0654 (100%); 313.0362 (13%)	malvidin-3-O-glucoside
5.5	$C_{19}H_{28}O_{12}$	447.1505	0.8	401.1455 (91.3%); 269.1033 (100%); 161.0452 (36.2%); 101.0244 (14.7%); 89.0242 (10.1%)	4-methoxyphenyl 4-O-(b-D-galactopyranosyl)-b-D-glucopyranoside
5.6	$C_{15}H_{14}O_6$	289.0719	−0.6	245.0820 (43.2%); 221.0829 (36.6%); 203.0723 (65.1%); 179.0357 (33.2%); 165.0207 (17.4%); 151.0395 (84.4%); 149.0237 (38.2%); 137.0244 (52.1%); 123.0461 (94.7%); 109.0296 (100%);	catechin
6.0	$C_{14}H_{18}O_9$	329.0878	0.8	167.0348 (100%); 123.0449 (17.2%); 81.0343 (10.3%)	vanillic acid glucoside
6.1	$C_{21}H_{20}O_{13}$	479.0824	1.5	479.0823 (31.2%); 317.0283 (26.4%); 287.0197 (11.6%);	myricetin 3-glucoside
6.1	$C_6H_{12}O_3$	131.0712	0.9	113.0621 (6.2%); 85.0666 (37.7%);	hydroxyhexanoic acid
6.5	$C_{21}H_{18}O_{13}$	477.0672	0.5	301.0356 (100%); 151.0029 (4.1%); 109.0296 (2.3%)	quercetin 3-O-glucuronide
6.5	$C_{21}H_{20}O_{12}$	463.0880	0.4	463.0870 (30.9%); 301.0335 (63.4%); 271.0246 (25.8%); 255.0290 (9%); 243.0295 (16.5%)	quercetin 3-O-glucoside
6.6	$C_{22}H_{22}O_{13}$	493.0989	−0.2	493.0969 (23%); 463.0856 (12.3%); 331.0432 (47.6%); 330.0374 (100%); 315.0143 (10.4%); 287.0189 (15.8%); 203.0343 (22.6%)	laricitrin 3-O-glucoside
6.8	$C_{15}H_{12}O_7$	303.0511	−0.2	285.0360 (4.3%); 275.0552 (5.1%); 229.0133 (31.1%); 217.0493 (10.2%); 153.0181 (36%); 152.0115 (20.8%); 151.0401 (25.6%); 151.0026 (19.5%); 125.0245 (100%);	taxifolin

Table 3. *Cont.*

RT (min)	Formula	$[M\text{-}H]^-_{experimental}$	Error (ppm)	Main MS/MS	Proposed Compound
6.9	$C_{21}H_{20}O_{11}$	447.0938	−1.1	447.0928 (44.1%); 285.0385 (44%); 255.0301 (29.9%); 227.0351 (57.8%); 183.0446 (15%)	chrysanthemin
7.0	$C_{23}H_{24}O_{13}$	507.1139	1.1	507.1144 (100%); 387.0689 (2.3%); 345.0597 (25.1%); 329.0280 (4.3%); 301.0347 (5.1%); 273.0404 (6%); 234.0507 (1.2%); 151.0032 (4.2%)	syringetin-3-O-glucoside
7.0	$C_{22}H_{22}O_{12}$	477.1026	2.7	477.1024 (100%); 315.0487 (22.5%); 271.0237 (26.6%); 243.0291 (32.5%); 201.0184 (10.5%); 199.0399 (14.2%)	isorhamnetin 3-glucoside
7.3	$C_{20}H_{22}O_8$	389.1241	0.1	227.0716 (100%); 185.0604 (3.0%); 143.0504 (6.5%)	piceid
7.5	$C_{15}H_{10}O_8$	317.0302	0.2	317.0299 (13.4%); 178.9983 (84.8%); 165.0193 (11.2%); 151.0037 (100%); 137.0244 (83%); 109.0303 (13.9%); 107.0139 (11.1%)	myricetin
7.9	$C_7H_6O_3$	137.0241	2.5	93.0339 (100%); 65.0403 (11.3%)	salicylic acid
8.5	$C_{15}H_{10}O_7$	301.0354	0.0	301.0337 (11.9%); 151.0040 (100%); 149.0250 (10.7%); 121.0304 (28.1%); 107.0140 (14.3%);	quercetin
8.7	$C_{14}H_{12}O_3$	227.0713	0.3	227.0716 (17.2%); 185.0595 (27.0%); 183.0794 (13.0%); 182.0736 (31.8%); 157.0660 (10.4%); 143.0496 (100%);	*cis*-resveratrol
8.9	$C_{11}H_{12}O_4$	207.0664	−0.5	207.0666 (10.3%); 179.0359 (12.3%); 161.0244 (51.1%); 135.0452 (100%); 133.0295 (71.2%)	ferulic acid
9.3	$C_{15}H_{12}O_5$	271.0616	−1.4	271.0600 (8.1%); 227.1283 (22.9%); 187.0405 (13.5%); 177.0177 (6.8%); 169.0144 (10.7%); 165.1283 (13.3%); 151.0048 (40.4%); 145.0302 (5.1%); 119.0501 (100%)	naringenin
9.4	$C_{15}H_{10}O_6$	285.0403	0.7	285.0399 (100%)	kaempferol
9.5	$C_{16}H_{12}O_7$	315.0508	0.8	315.0516 (13.1%); 151.0035 (15.1%)	isorhamnetin
10.1	$C_{11}H_{12}O_3$	191.0714	−0.2	163.0408 (2.1%); 145.0300 (33.0%); 119.0509 (33.1%); 117.0348 (100%)	ethyl coumarate

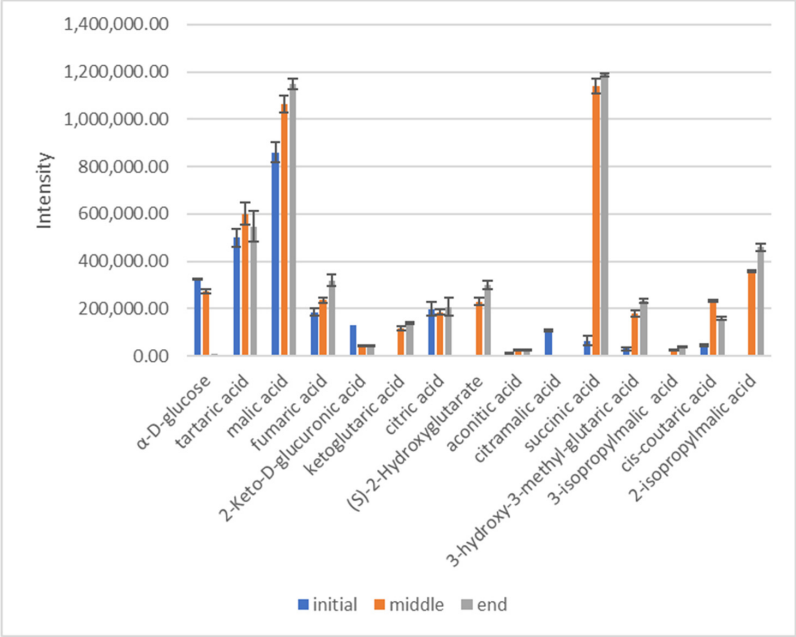
For this identification, the procedure described in Section 2 was followed. That is, the molecular mass was used in the DataAnalysis program to propose several chemical formulas, each with an error of estimation and with two or three proposals, according to the lowest error, and the databases were used to obtain chemical structures. These structures were drawn by employing MassFrag, and the MS^2 indicated in the chromatograms were used to verify each chemical structure proposed. Salicylic acid was applied as a standard to control the methodology in use, and chrysanthemin was also used as a standard to verify the hypothetical biomarker that is proposed in this work.

3.2. Metabolic Profile Evolution during Fermentation

In order to follow the evolution of each compound during fermentation, samples were collected at the initial (after must homogenization—S1.2 and S.2.2), middle (mid-fermentation—S1.3 and S2.3) and end stages of fermentation (end-fermentation—S1.4 and S2.4).

After analyzing all the replicates, the intensities of each compound were averaged, and the data were displayed according to the chemical type, glucose, organic acids, phenolic acids and their derivatives and the phenolic compounds (Figures 2 and 3). Figure 2 shows the data obtained during fermentation for the grapes from site 1, while Figure 3 contains the data obtained during fermentation for the grapes from site 2.

(**a**)



(**b**)

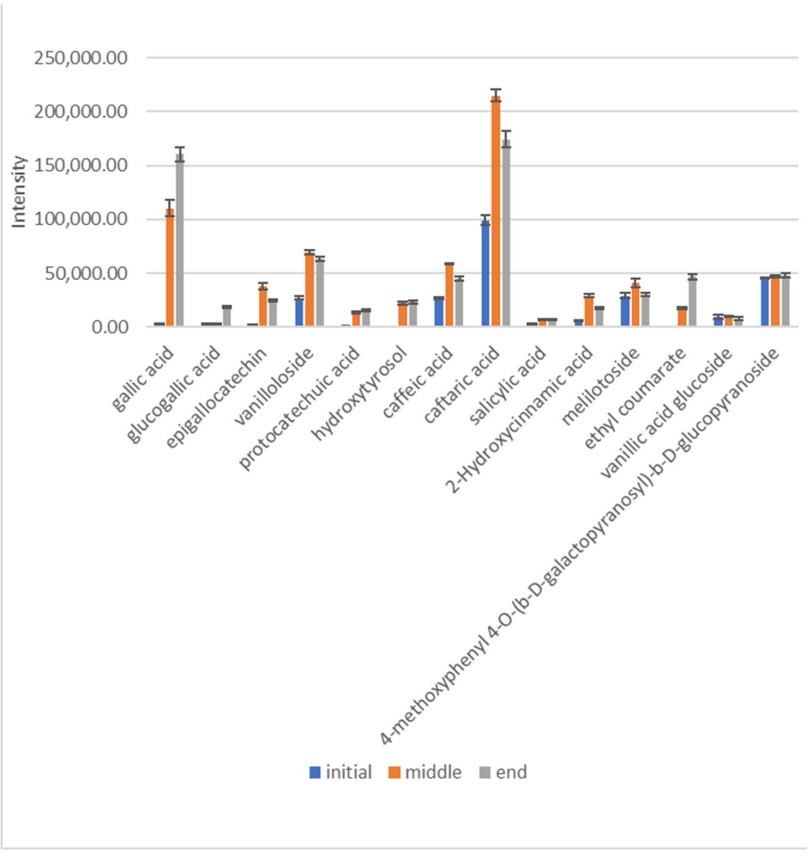


Figure 2. *Cont.*

(**c**)

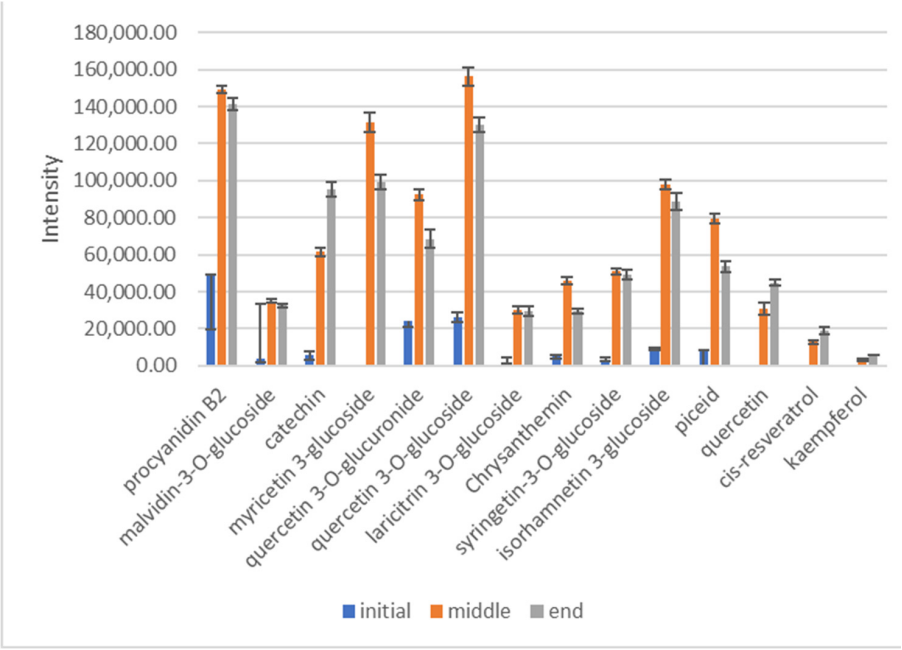


Figure 2. Evolution of the compounds present in the must during the fermentation process for the grapes from site 1: (**a**) glucose and organic acids; (**b**) phenolic acids and their derivatives; (**c**) phenolic compounds. The compound appearing as 4-methoxyphenyl 4-O-(b-D-corresponds to 4-methoxyphenyl 4-O-(b-D-galactopyranosyl)-b-D-glucopyranoside.

(**a**)

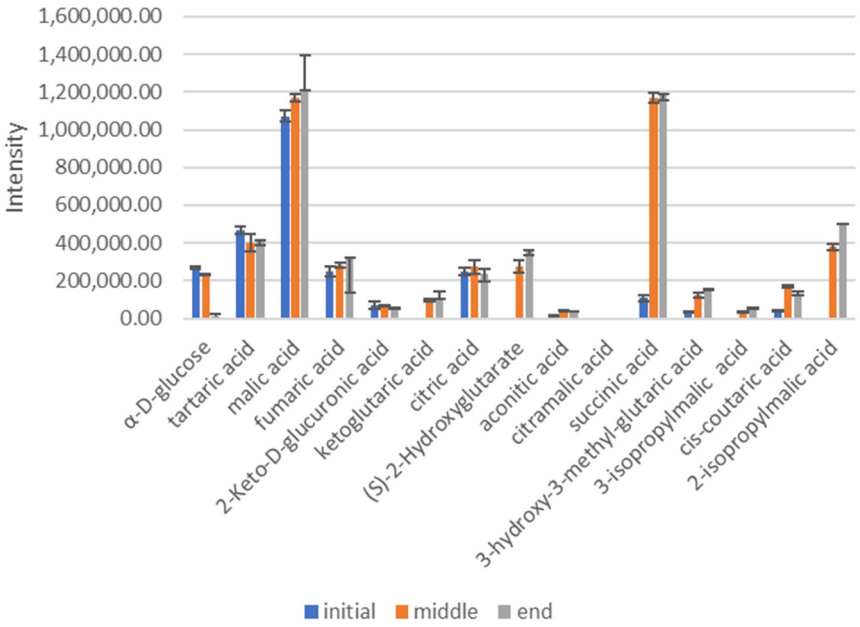
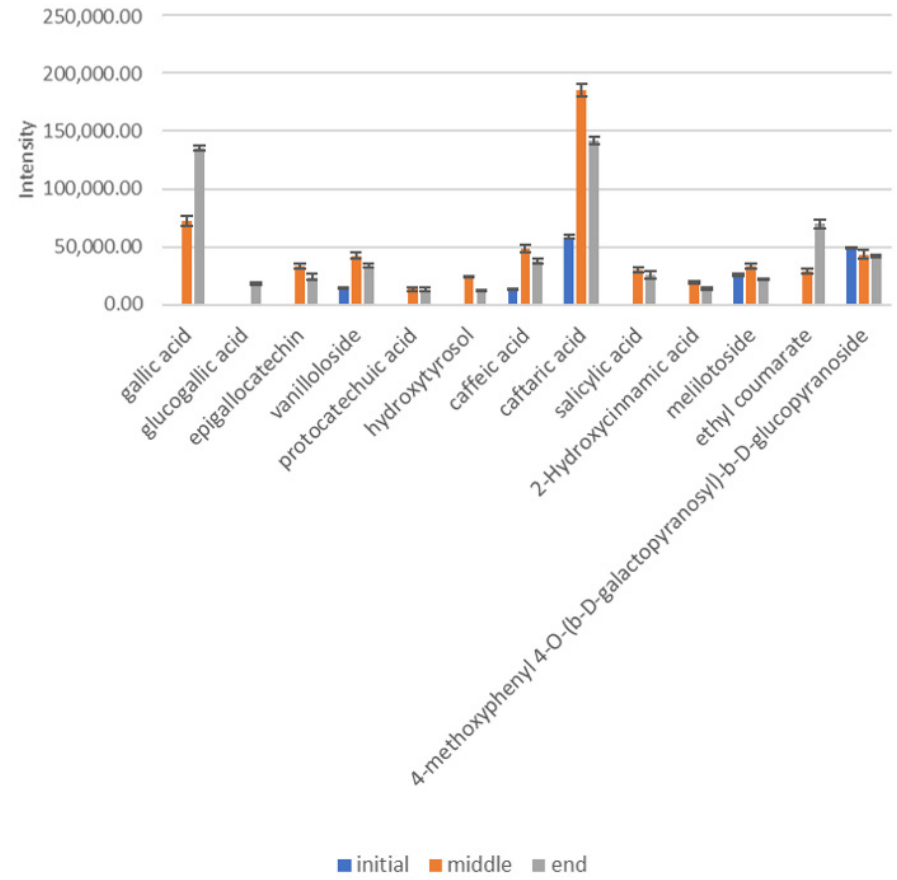


Figure 3. *Cont.*

(**b**)



(**c**)

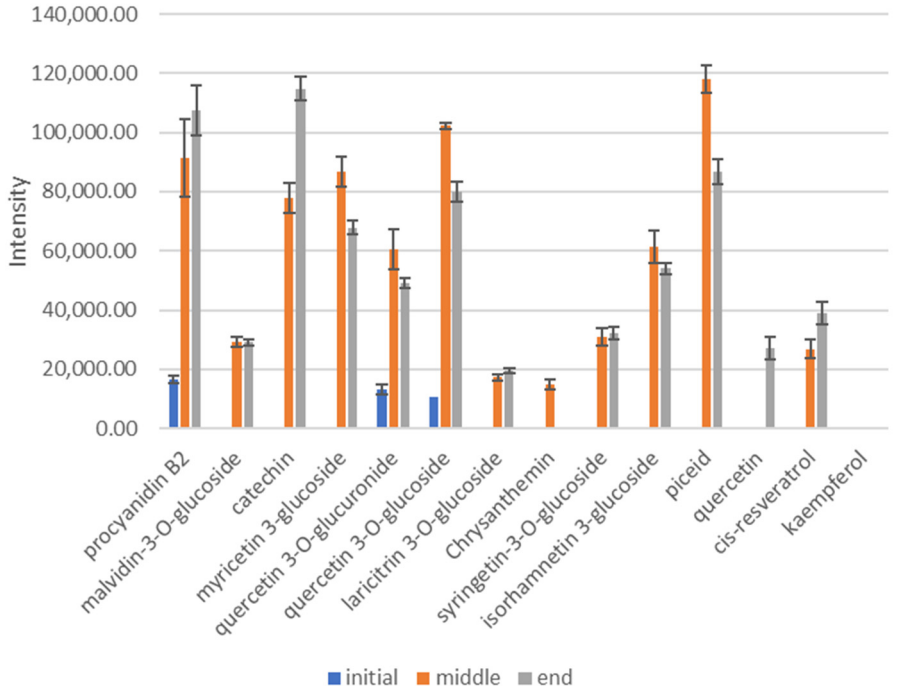


Figure 3. Evolution of the compounds present in the must during the fermentation process for the grapes from terroir 2: (**a**) glucose and organic acids; (**b**) phenolic acids and their derivatives; (**c**) phenolic compounds. The compound appearing as 4-methoxyphenyl 4-O-(b-D- corresponds to 4-methoxyphenyl 4-O-(b-D-galactopyranosyl)-b-D-glucopyranoside.

As expected, some compounds decreased in their intensity to values that are not measurable while fermentation is ongoing, as in the case of glucose or citramalic acid, indicating that they are metabolized during the fermentation process (Figures 2a and 3a). On the contrary, compounds such as ketoglutaric, succinic acid and 2-isopropylmalic acid are formed during fermentation (Figures 2a and 3a).

When focusing on site 1, regarding the phenolic acids and their derivatives (Figure 2b), it is worth noting that compounds such as gallic acid, epigallocatechin, hydroxytyrosol and ethyl coumarate, although showing very low intensities in the must at beginning of fermentation, increased in their intensity throughout fermentation. Phenolic acids such as caffeic and caftaric seem to increase in their intensity up to the middle of fermentation, followed by a slight decrease towards the end of fermentation, while maintaining a higher level when compared to the beginning of fermentation (Figure 2b). Caffeic acid is an important compound that seems to contribute to color stability and protection against oxidation [19].

It is interesting to note that most phenolic compounds are formed during the fermentation process, as shown in Figure 2c. Compounds such as myricetin 3-glucoside, quercetin, *cis*-resveratrol and kaempferol, although not detected at the beginning of fermentation, showed measurable values by the end of fermentation. In particular, resveratrol is a polyphenol found mainly in grape seeds, as well in the peel of red grape berries, which showed an increase in the extraction amount during the winemaking process [19]. The presence of this compound is interesting, as it is known mainly for its antioxidant, anti-inflammatory and antitumoral properties [20].

The same comparison was carried out for site 2, as shown in Figure 3. Similar results were obtained for the evolution of glucose and organic acids (Figure 3a), as well as the phenolic acids and their derivatives compounds (Figure 3b), except for citramallic, which was not detected during the fermentation of the Syrah grapes from this site.

The phenolic compound chrysanthemin (Figure 3c) was only detected at mid-fermentation in the must samples from site 2, although it was detected at all stages of must fermentation for the grapes of site 1 (Figure 2c). Although appearing in mid-fermentation at site 2, it was at a concentration level inferior to 50% that of site 1. Kaempferol was not detected in the terroir 2 fermentation samples. The flavonols myricetin, quercetin, laricitrin, syringetin, isorhamnetin and kaempferol play important roles in the color stabilization of young red wines, as well as in the sensory perception of astringency and bitterness [21,22]. Except for chrysanthemin and kaempferol, the differences encountered between the terroirs seemed to be related to the level of intensity, as the patterns of presence and evolution during fermentation were quite similar. This is not surprising, since the same cultivar was used.

3.3. Metabolic Profile Comparison between the Terroirs for the Grape and Wine Samples

The metabolic profile of each sample was determined, and the intensity of each compound was evaluated and compared between grapes and wines from each terroir. The data are displayed according to the chemical type, glucose and organic acids (Figure 4a), phenolic acids and their derivatives (Figure 4b) and the phenolic compounds (Figure 4c), and the differences encountered between samples are marked with an asterisk (* for alpha = 0.05= or ** for alpha = 0.01). The comparisons include the grape samples from each terroir, wines from each terroir and grapes and wine from the same terroir.

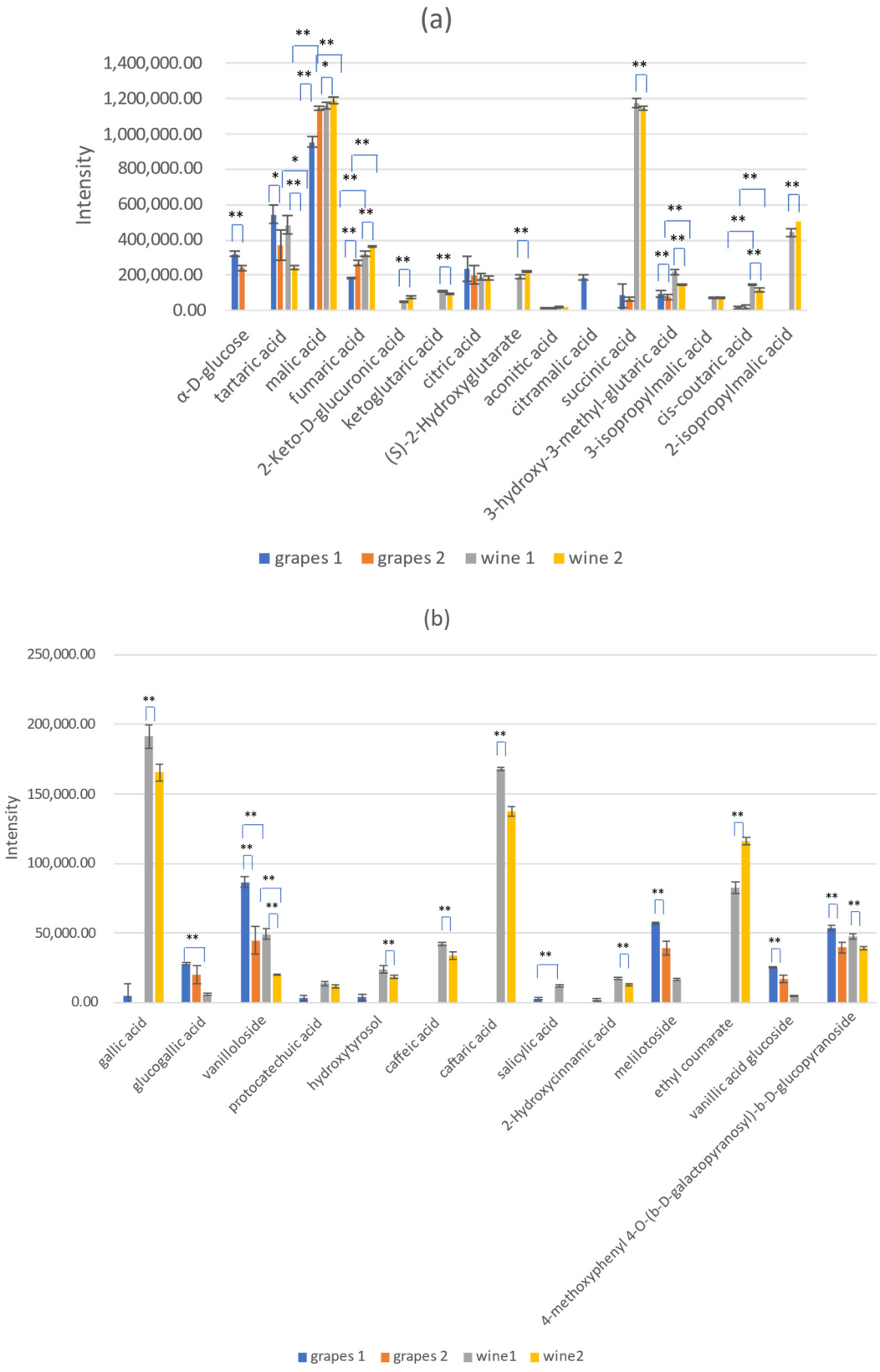


Figure 4. *Cont.*

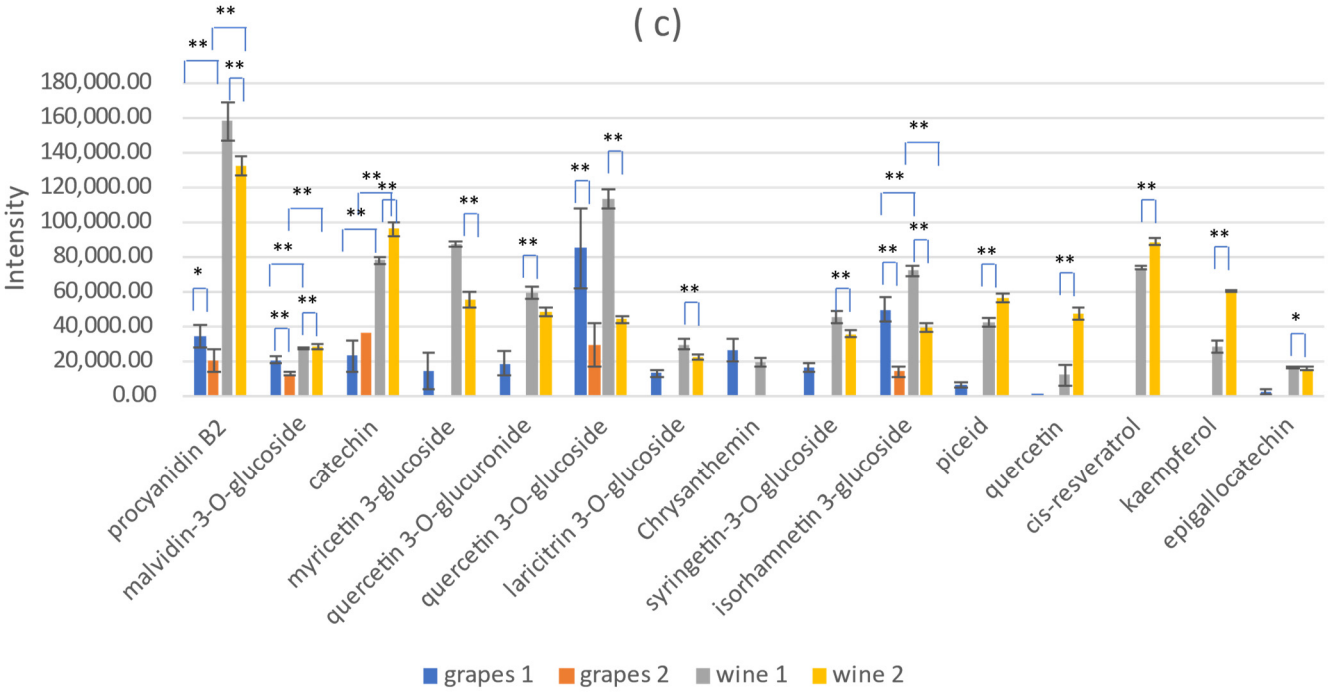


Figure 4. Quantification of compounds present in the samples from site 1 (grapes 1 and wine 1) and site 2 (grapes 2 and wine 2): (**a**) glucose and organic acids; (**b**) phenolic acids and their derivatives; (**c**) phenolic compounds (*; **—differences at the 95% and 99% significance levels, respectively). Compound appearing as 4-methoxyphenyl 4-O-(b- . . .) corresponds to 4-methoxyphenyl 4-O-(b-D-galactopyranosyl)-b-D-glucopyranoside.

As displayed in Figure 4a, the differences encountered between the grapes from site 1 and site 2 are significantly different (99% confidence level) for most the organic acids and glucose. When the organic acids and glucose from the wines were analyzed, there were significant differences encountered between the sites for all the compounds, except for citric acid in both the grapes and wine and 3-isopropylmalic acid in the wines, among which no differences could be found.

Most of the phenolic acids and glucosides (Figure 4b), as well as the phenolic compounds (Figure 4c), formed during fermentation showed significant differences between the wines, with most at a significance level of 99% with *p*-values less than 0.001, and the majority were higher for the samples from site 1. Gallic and caftaric acids were the most abundant phenolic acids. In fact, gallic acid is considered the most important phenolic acid in red wine and stands out as the precursor of all hydrolysable tannins [22]. Various parameters such as the soil, climate, maturity and viticultural practices can influence the grape anthocyanin composition, evidencing a terroir relationship [23,24]. Relative amounts of most anthocyanins showed higher levels in the wine than in the corresponding grapes, however this relationship was not always observed [25,26].

Polyphenols are compounds with a great influence on sensory properties such as appearance, color, astringency, bitterness, and flavor [27,28]. Compounds with a great impact in the sensorial analysis, such as quercetin, myricetin, catechin and malvidin-3-O-glucoside, showed clear differences between the wines, which certainly contributed to the sensorial analysis performed by a trained panel, as discussed later. The most frequently studied bioactive compound, resveratrol, although not detected in the grapes using the method presented herein, was found in the wines, as during winemaking, there is an increase in the extraction of phenolic compounds present mainly in grape skin and seeds. It is evidenced that although this compound was present in both wines, the wines showed significant differences in intensity.

3.4. Search for Biomarkers of Terroir Distinctiveness

The analysis of Figure 4 reveals the hypothesis of some existing biomarkers that allow one to differentiate the wines from the two terroirs. Wine samples collected at the moment of bottling, approximately 5 months after clarification, were analyzed.

Regarding the comparison between the wine samples from both terroirs, it was possible to observe that chrysanthemin was only present in terroir 1. This compound is marked in green with its name shown in Figure 5. The other compounds were discarded, as they had log2 of fold change values that were very low. The location was also found to be one of the factors that influenced the chrysanthemin (cyanidine-3-O-glucoside) concentration in the fruits and wine of the grape variety Pinot Noir [26].

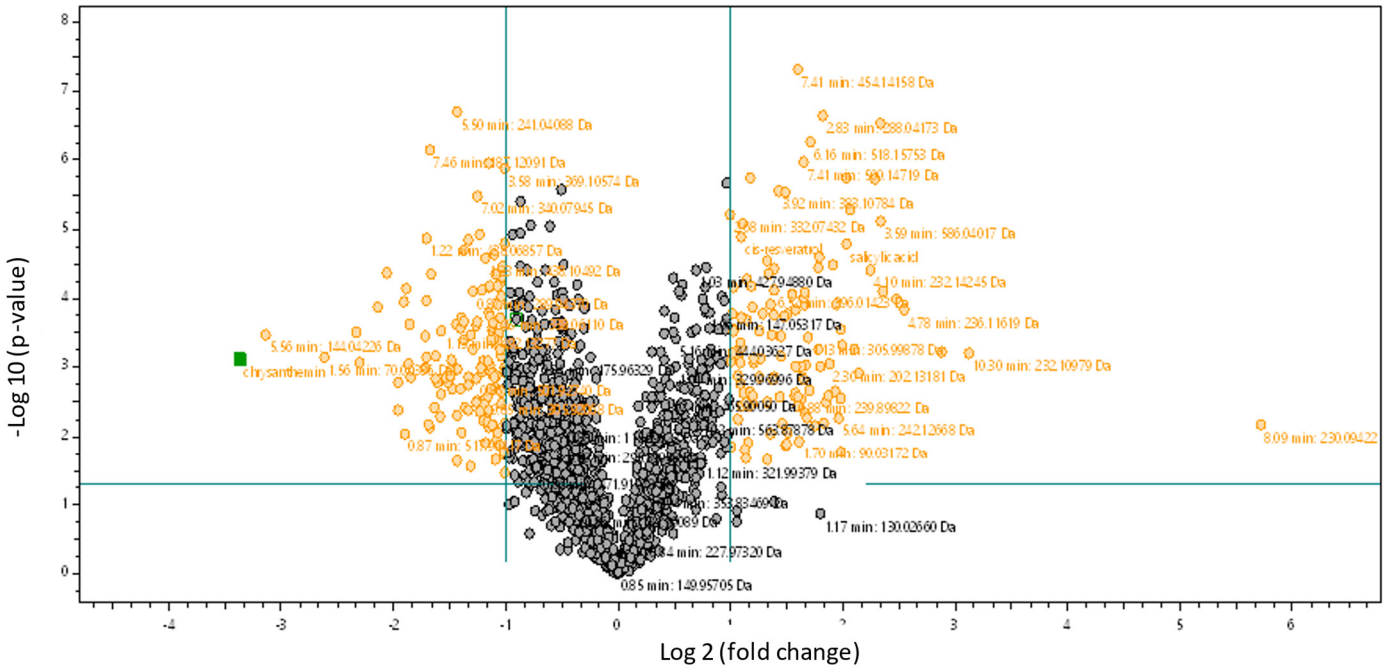


Figure 5. Volcano plot of the comparison of the wine samples from both terroirs. The plot represents the −log10 *p*-value on the y-axis vs. the log2 fold change on the x-axis (when $p < 0.05$, the metabolites were considered statistically different). A negative or positive log2 fold change indicates the higher intensity of the metabolites in the wine 1 and wine 2 samples, respectively. Compounds marked in green correspond to the one that was present only in terroir 1, with both the relevant fold change and $p < 0.05$.

3.5. Evaluation of Wine Sensory Properties

The wines obtained from the grapes collected in the two terroirs were evaluated by the INIAV trained panel in one session using the descriptors listed in Table 4.

Regarding the visual characteristics, an identical classification of the clarity/brightness was obtained for the wines from both sites. The wines from site 1 exhibited a higher aroma of red fruits/berries, nutty and dried fruits and jelly/jam, which contributed to the significantly higher aroma positive intensity obtained for these wines. Wines from site 2 presented a lower classification of color intensity and color quality with highly significant differences, respectively.

Concerning the flavor attributes, the wines from site 1 were clearly classified with higher rates for most of the attributes evaluated. The higher polyphenol (Figure 4) content of this wine may contribute to the higher rates of astringency, body and complexity obtained, as supported by the literature [21,22]. It should be mentioned that some tasters noted the presence of a sulfide/reduced aroma in the wines from site 2. The overall quality of the wines was also evaluated, and according to the hitherto reported results, the wines from site 1 scored better than the wines from site 2.

Table 4. ANOVA results used to evaluate the site effect on each sensory descriptor of the wines obtained. Values are the average of the replicates (average ± std. dev.).

	S1	S2	Effect
Clarity/brightness	8.4 ± 0.08	8.4 ± 0.08	ns
Color intensity	8.1 ± 0.08 b	7.4 ± 0.16 a	**
Color quality	8.3 ± 0.00 b	7.6 ± 0.08 a	***
Red fruits/berries	5.2 ± 0.08 b	1.8 ± 1.26 a	***
Nutty and dried fruits	2.7 ± 0.47 b	1.5 ± 0.39 a	***
Jelly/jam	2.7 ± 0.24 b	1.2 ± 0.24 a	***
Dried vegetable	1.4 ± 0.63	2.0 ± 0.63	ns
Spices	1.4 ± 0.31	1.1 ± 0.16	ns
Chocolate/coffee	0.2 ± 0.16 a	0.4 ± 0.00 b	*
Smoke	1.0 ± 0.16	1.3 ± 0.08	ns
Wood	1.2 ± 0.16	1.0 ± 0.47	ns
Vanilla	0.1 ± 0.00	0.3 ± 0.16	ns
Positive intensity	6.8 ± 0.16 b	3.3 ± 0.86 a	***
Acid	4.4 ± 0.24 b	3.8 ± 0.08 a	*
Sweet	2.6 ± 0.08 b	1.7 ± 0.08 a	***
Bitter	2.6 ± 0.00	2.2 ± 0.00	ns
Astringency	4.2 ± 0.00 b	3.2 ± 0.47 a	***
Body	5.8 ± 0.16 b	4.1 ± 0.24 a	***
Complexity	6.2 ± 0.08 b	3.6 ± 0.31 a	***
Harmonious persistence	6.6 ± 0.08 b	4.1 ± 0.63 a	***
Overall quality (0 a 20)	13.9 ± 0.20 b	9.6 ± 1.26 a	***

Average values in the same line followed by different letters indicate significant differences, according to Fisher's LSD test ($\alpha = 0.05$). The level of significance is indicated: ns—not significant; * significant ($\alpha < 0.05$); ** very significant ($\alpha < 0.005$); *** highly significant ($\alpha < 0.001$).

4. Conclusions

A thorough metabolic profile of the Syrah grape variety was obtained, enabling the putative identification of 56 compounds. The comparative metabolic profiles of this grapevine variety, cultivated in two vineyards, showed differences, especially in the compound intensity. A higher polyphenol content was observed for site 1, which was corroborated by the sensory results, corresponding to a higher wine quality. The metabolomic analysis enabled the identification of the biomarker chrysanthemin for terroir differentiation. Although observed in the same Demarcated Wine Region of Lisbon (Portugal), this marker seems to distinguish the wines produced on the different sites and may eventually be related to a better wine quality. This is of utmost importance, considering that Syrah is a variety cultivated worldwide, with a high commercial value.

Studying an increasing number of grape varieties and sites (vineyards) in future work will offer new insights into the identification of biomarkers that could support the concept of terroir. The identification of regional-specific metabolites that could be attributed to terroir specificity will offer new opportunities for wine marketing and authenticity and improve consumers' wine knowledge.

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