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Performance comparison of electrospray ionization and atmospheric pressure chemical ionization in untargeted and targeted liquid chromatography/mass spectrometry based metabolomics analysis of grapeberry metabolites

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RATIONALE: Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are both used to generate ions for the analysis of metabolites by liquid chromatography/mass spectrometry (LC/MS). We compared the performance of these methods for the analysis of Corvina grapevine berry methanolic extracts, which are complex mixtures of diverse metabolites.

METHODS: Corvina berries representing three ripening stages (veraison, early-ripening and full-ripening) were collected during two growing seasons, powdered and extracted with methanol. Untargeted metabolomic analysis was carried out by LC/ESI-MS and LC/APCI-MS. Processed data files were assembled into a data matrix for multivariate statistical analysis. The limits of detection (LODs), limits of quantification (LOQs), linear ranges, and matrix effects were investigated for strongly polar metabolites such as sucrose and tartaric acid and for moderately polar metabolites such as caftaric acid, epicatechin and quercetin 3-*O*-glucoside.

RESULTS: Multivariate statistical analysis of the 608 features revealed that APCI was particularly suitable for the ionization of strongly polar metabolites such as sugars and organic acids, whereas ESI was more suitable for moderately polar metabolites such as flavanols, flavones and both glycosylated and acylated anthocyanins. APCI generated more fragment ions whereas ESI generated more adducts. ESI achieved lower LODs and LOQs for sucrose and tartaric acid but featured narrower linear ranges and greater matrix effects.

CONCLUSIONS: ESI and APCI are not complementary ion sources. Indeed, ESI can be exploited to analyze moderately polar metabolites, whereas APCI can be used to investigate weakly polar/non-polar metabolites and, as demonstrated by our results, also strongly polar metabolites. ESI and APCI can be used in parallel, exploiting their strengths to cover the plant metabolome more broadly than either method alone. Copyright © 2016 John Wiley & Sons, Ltd.

Untargeted metabolomics based on liquid chromatography/mass spectrometry (LC/MS) aims to screen a large proportion of the non-volatile metabolites in a given biological system. The most common atmospheric pressure ionization methods in this context are electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI).[1] The combination of these two methods for untargeted metabolomics allows the analysis of a broad range of metabolites with diverse chemical and physical properties. ESI is particularly efficient for strongly and moderately polar metabolites with a large molecular mass, whereas APCI is mainly used for non-polar and weakly polar metabolites.[2] Both ESI and APCI are soft ionization methods, i.e. they generate intact molecular ions or few fragments during the first round of ionization. These features make them ideal in research fields such as drug development, disease monitoring, and the compositional analysis of foods, beverages and herbal products.

Wine is a source of health-promoting secondary metabolites, especially antioxidant polyphenols such as resveratrol and anthocyanins.[3] The molecular composition of wine is strictly related to the fermentation process and the metabolic profile of the grapevine berries, which depends on the developmental and/or ripening stage[4] and the pedoclimatic conditions.[5–7] Berries undergo a developmental phase characterized by rapid cell division and growth, during which malate and other organic acids accumulate rapidly in the vacuoles.[4,8,9] This is followed by a ripening phase involving sugar accumulation, softening, and changes in color, followed by an increase in pH and the accumulation of polyphenols and flavor compounds.[4,8]

Corvina is an Italian grapevine cultivar which is widely used for the production of Bardolino and Valpolicella wines. It is also combined with Corvinone and Rondinella berries to produce premium wines such as Amarone and Recioto after

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post-harvest dehydration.[10–12] We have previously described the dynamic nature of the Corvina berry metabolome during development, ripening and withering, especially the changes occurring at veraison, the onset of ripening.[12]

Here we compare the performance of ESI and APCI for the untargeted analysis of non-volatile metabolites at different stages of Corvina berry ripening. We focus particularly on the strongly polar metabolites, which were efficiently ionized by APCI with a lower susceptibility to the matrix effects that severely affect ESI.

EXPERIMENTAL

Plant material and sampling

Eight-year-old grapevine plants (*Vitis vinifera* cv. Corvina, clone 48) grafted onto 41B rootstock (*Vitis vinifera* cv. Chasselas x *Vitis berlandieri*) were cultivated in a calcareous clay soil, oriented east-west, and the training system was the classical Pergola Trentina Semplice. The vineyard was located in Negrar near Verona, at 250 meters above sea level. Berries were sampled during the 2007 and 2008 growing seasons at three developmental time points, corresponding to V for veraison (18 July 2007 and 12 August 2008), R1 for early ripening (8 August 2007 and 2 September 2008), and R2 for late ripening (29 August 2007 and 23 September 2008). For each sample, three biological replicates were collected at each time point. A biological replicate was a pool of 30 deseeded berries from five clusters collected from five different plants. The pools of deseeded berries were frozen in liquid nitrogen, powdered with a pestle and mortar and stored at –80°C.

Metabolite extraction

Thirty deseeded berries were homogenized and metabolite extracts were prepared as previously described.[12] Briefly, 300-mg aliquots of powdered samples were extracted with 900 µL (three volumes p/v) of ice-cold methanol containing 0.1% formic acid, sonicated at 40 kHz for 15 min in an ultrasonic bath (Falc Instruments, Bergamo, Italy) at room temperature, centrifuged for 10 min at 16,000 *g* at 4°C, and passed through 0.2-µm filters before storing the extracts at –20°C.

Metabolite detection and quantification

High-performance liquid chromatography/mass spectrometry (HPLC/MS) was carried out using a Gold 127 HPLC system (Beckman Coulter, Fullerton, CA, USA) equipped with a C18 guard column (7.5 × 2.1 mm) and an analytical Alltima HP C18 column (150 × 2.1 mm, particle size 3 µm; Alltech Associates Inc., Derfield, IL, USA). The methanolic extracts were diluted 1:2 and 30 µL of each sample was injected. Each sample was analyzed in duplicate. Two solvents were used: 5% (*v*/v) formic acid, 5% (*v*/v) acetonitrile in water (solvent A), and 100% methanol (solvent B). A solvent gradient was established from 0 to 10% B in 5 min, from 10 to 20% B in 20 min, from 20 to 25% B in 5 min, from 25 to 70% B in 15 min and from 70 to 0% B in 1 min, followed by 31 min equilibration between samples. The flow rate was set to 200 µL·min^{-1}. The HPLC system was coupled on-line with a Esquire 6000 ion trap mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany) with an ESI or APCI ion source in negative ion mode.

MS data were recorded up to 60 min and collected using Esquire Control v5.2 and processed using Data Analysis v3.2 (Bruker Daltonics). Negative ion mass spectra were recorded by selecting a target of 400 *m/z* and a range of 50–1500 *m/z*. MS/MS and MS^3 spectra were also recorded in negative ion mode at a fragmentation amplitude at 1 V. Nitrogen was used as the nebulizing gas and drying gas for both ion sources. The ESI parameters were 50 psi at 350°C for the nebulizing gas and 10 L·min^{-1} for the drying gas. The APCI parameters were 50 psi at 350°C for the nebulizing gas and 5 L·min^{-1} for the drying gas, with a vaporizer temperature of 450°C. Helium was used as the collision gas. Additional ESI parameters: capillary source, 4000 V; end plate offset, –500 V; skimmer, –40 V; cap exit, –121 V; Oct 1 DC, –12 V; Oct 2 DC, –1.70 V; lens 1, 5 V; lens 2, 60 V. Additional APCI parameters: capillary source, 2000 V; end plate offset, –500 V; skimmer, –40 V; capillary exit, –121 V; Oct 1 DC, –12 V; Oct 2 DC, –1.70 V; lens 1, 5 V; lens 2, 60 V. MS-based quantitation was expressed as the peak area in arbitrary units (AU).

The HPLC/APCI and ESI-MS methods for the analysis of sucrose, tartaric acid, caftaric acid, epicatechin and quercetin-3-*O*-glucoside were validated using serial dilutions of standard compounds. From preliminary results, sucrose and tartaric acid standards with concentration ranges of 0.0001 µg·mL^{-1} to 10 mg·mL^{-1} and caftaric acid, epicatechin and quercetin 3-*O*-glucoside with concentration ranges of 0.005 µg·m L^{-1} to 0.1 mg·m L^{-1} were analyzed in duplicate to determine the lower limit of detection (LOD), lower limit of quantification (LOQ) and the linear range. The LOD and LOQ were assessed at signal-to-noise (S/N) ratios of 3 and 10, respectively. The column was injected with 30 µL of each sample and elution was carried out using the previously described gradient of solvent B with the same ESI and APCI parameters as specified above.

LC/MS data extraction, alignment and analysis

Raw LC/MS data files were converted from their native .d format into net.cdf files before they were processed using MZmine v2.10[13] to obtain a data matrix suitable for multivariate analysis. The metabolomics data were processed using SIMCA v13.0 (Umetrix AB, Umea, Sweden). Pareto scaling, centering and logarithmic transformations were immediately applied to the entire dataset. The data matrix, comprising 36 samples (observations) and 608 signals (variables), was used for unsupervised principal component analysis (PCA) to define sample clusters. Missing values were considered as 'missing' (not as zero). Supervised statistical analysis was then performed by assigning the new clusters as Y classes in partial least-squares discriminant analysis (PLS-DA) and orthogonal partial least-squares discriminant analysis (OPLS-DA). The PLS-DA models were validated with a permutation test (400 permutations) to avoid overfitting, and each corresponding OPLS-DA model was cross validated by analysis of variance (ANOVA) with a significance threshold of $p < 0.01$.

For OPLS-DA, we used the pq(corr) parameter, which states the correlation between p (based on the X component of the model, the metabolites) and q (based on the Y component of

the model, the class), to determine which metabolites contributed most to the class separation. Only metabolites satisfying pq(corr) >0.9 and <−0.9 were considered as significant contributors to the differences between groups of samples. To confirm such differences, univariate statistical analysis was performed by using the t-test (p-values <0.01).

Matrix effect evaluation

The matrix effects for sucrose, tartaric acid, caftaric acid, epicatechin and quercetin-3-*O*-glucoside were evaluated using serial dilutions of a grape methanolic extract.[12,14,15] Specifically, a methanolic extract of R2 berries collected in 2007 was diluted several times (1:2, 1:5, 1:10, 1:25, 1:50, 1:100, 1:250, 1:500, 1:750, 1:1000, 1:5000 and 1:10000) for the analysis of sucrose and tartaric acid, whereas a methanolic extract of R3 berries collected in 2007 was diluted (1:2, 1:3, 1:5, 1:8, 1:10, 1:12, 1:15, 1:20, 1:25, 1:50, 1:75 and 1:100) for the analysis of caftaric acid, epicatechin and quercetin-3-*O*-glucoside. Each sample was analyzed in duplicate by LC/APCI-MS and LC/ESI-MS. The peak areas were determined and normalized for the dilution factor, and normalized peak areas were plotted against dilution factors. By considering the highest dilution point at which the curves levelled off, it was possible to obtain a correction factor for the matrix effect based on the ratio of the normalized areas of the most diluted sample and the usual working dilution.

Annotation of metabolites

The m/z values, retention times and fragmentation patterns (MS/MS and MS^3) were used to determine the identity of each metabolite based on our in-house library of commercial standards, and on metabolite fragmentation patterns reported in the literature or the MassBank online database[16] when commercial standards were not available. Neutral losses of 132, 146 and 162 Da were considered to indicate losses of pentose, deoxyhexose and hexose sugars, respectively. Accordingly, the following annotations were used for aglycones and other portions of certain detected compounds: caffeic acid, m/z = 179, MS/MS = 135; coumaric acid, m/z = 163, MS/MS = 119; sinapic acid, m/z = 223, MS/MS = 149, 164, 179 and 207; ferulic acid m/z = 193, MS/MS = 134, 149 and 178.

RESULTS

ESI and APCI were compared for the untargeted metabolomic analysis of Corvina berries collected at three stages (V, R1 and R2) during two growing seasons (2007 and 2008). Homogenized berry samples were extracted with methanol and analyzed by LC/ESI-MS and LC/APCI-MS in parallel. A representative LC/APCI-MS chromatogram is shown in Fig. 1, revealing three major elution zones (A, B and C) depending on the gradient of the least polar solvent.

Strongly polar metabolites eluted in zone A, including sugars and organic acids. The most abundant metabolites based on our putative annotations were caffeoyl tartaric acid (caftaric acid) and coumaroyl tartaric acid (coutaric acid). Moderately polar compounds eluted in zone B, including five glycosylated anthocyanins and quercetin-3-*O*-glucoside. Finally, the least polar molecules eluted in zone C, including resveratrol hexoside, kaempferol hexoside and coumarated anthocyanins. A comprehensive list of 122 metabolites based on our putative annotations is provided in Supplementary Table S1 (see Supporting Information). The main metabolites of grape samples, their structures and fragmentation patterns are shown in Fig. 2.

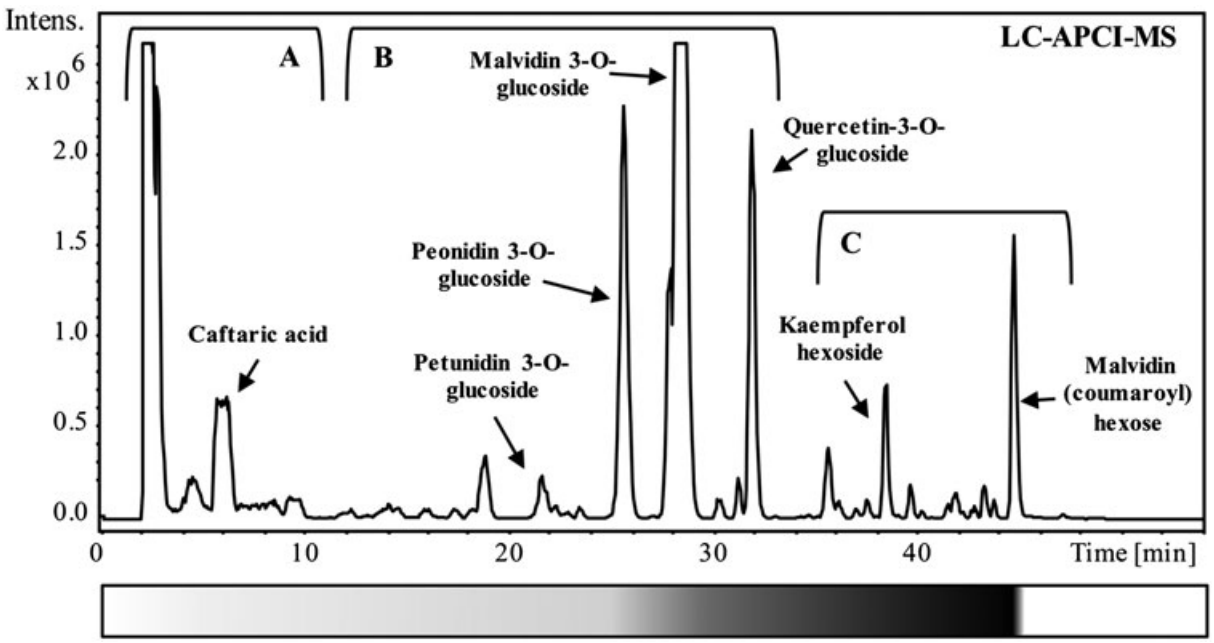

Figure 1. LC/APCI-MS base peak chromatogram of a methanolic extract of R2/2007 Corvina berries. Three different elution zones are shown, representing strongly polar (A), moderately polar (B) and weakly polar (C) compounds. The lower bar shows the elution gradient starting from 0% solvent B (white) to 70% solvent B (black).

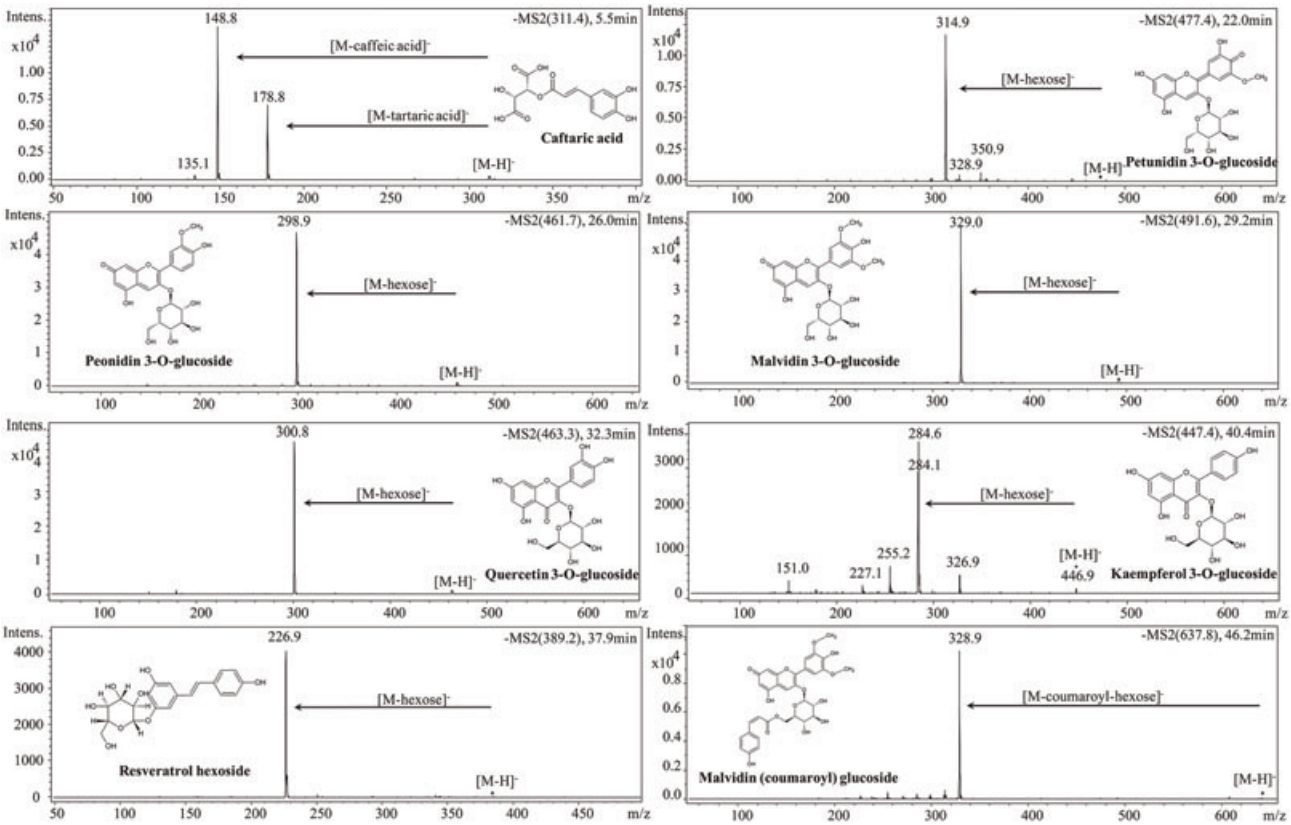

Figure 2. LC/ESI-MS fragmentation patterns and structures of the main grape secondary metabolites.

Multivariate data analysis – PCA-X

The chromatographic .d files were processed using MZmine v2.10, yielding a data matrix comprising 36 samples (observations) and 608 *m/z* features (variables) suitable for multivariate data analysis. Among the features, 15 were detected only by ESI (4 were putatively identified as adducts of malvidin and peonidin 3-*O* glucosides, dihydroquercetin deoxyhexoside and quercetin glucoronide), whereas six unidentified features were detected only with APCI; the other *m/z* features were detected with both the ionization sources. All the detected features were included in the data matrix for the comparison. Initially, SIMCA v13.0 was used to perform unsupervised PCA, revealing a clear separation between the samples analyzed by LC/ESI-MS and LC/APCI-MS along the first principal component (PC1), explaining 62.9% of the variation (Fig. 3). No clustering between the R1 and R2 samples was observed along the second principal component (PC2), whereas the V samples clustered away from the others, indicating the V metabolome is distinct from the ripening stages.

Multivariate data analysis – OPLS-DA

OPLS-DA was initially applied to the entire dataset using the clusters identified by PCA (ESI vs APCI) as classes. OPLS-DA models are easier to interpret than PCA results because they separate non-correlated systematic variations in X (orthogonal information) from the correlated information between X (the metabolite peak areas) and Y (the assigned sample classes). The S-loading plot in Fig. 4(A) shows that many molecules strongly correlate (pq(corr) >0.9) with either ESI (including flavones, acylated and glycosylated anthocyanins, and some flavanols) or APCI (pq(corr)<−0.9; including sugars and organic acids). Interestingly, the same plot revealed that adducts were mainly detected by ESI. The correlations and the t-test results between particular molecules and ionization methods are listed in Supplementary Table S2 (see Supporting Information).

The correlation between APCI and strongly polar metabolites such as sugars and organic acids was also observed by plotting the pq(corr) of the loadings against the

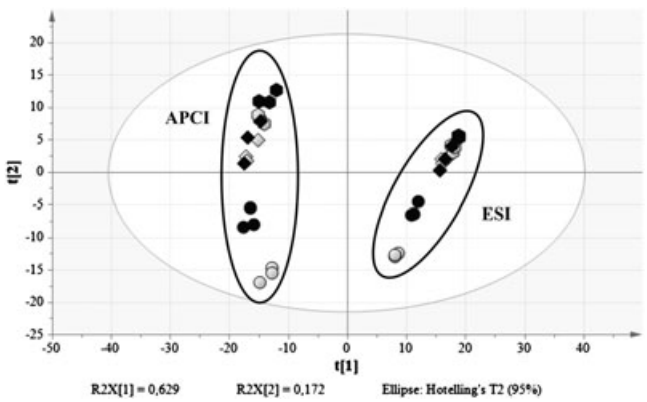

Figure 3. Unsupervised PCA-X score scatter plot. The two circles represent the samples analyzed using the APCI and ESI methods. Black and gray indicate samples collected in the 2006 and 2007 seasons, respectively. Circles, diamonds and hexagons represent the V, R1 and R2 stages, respectively.

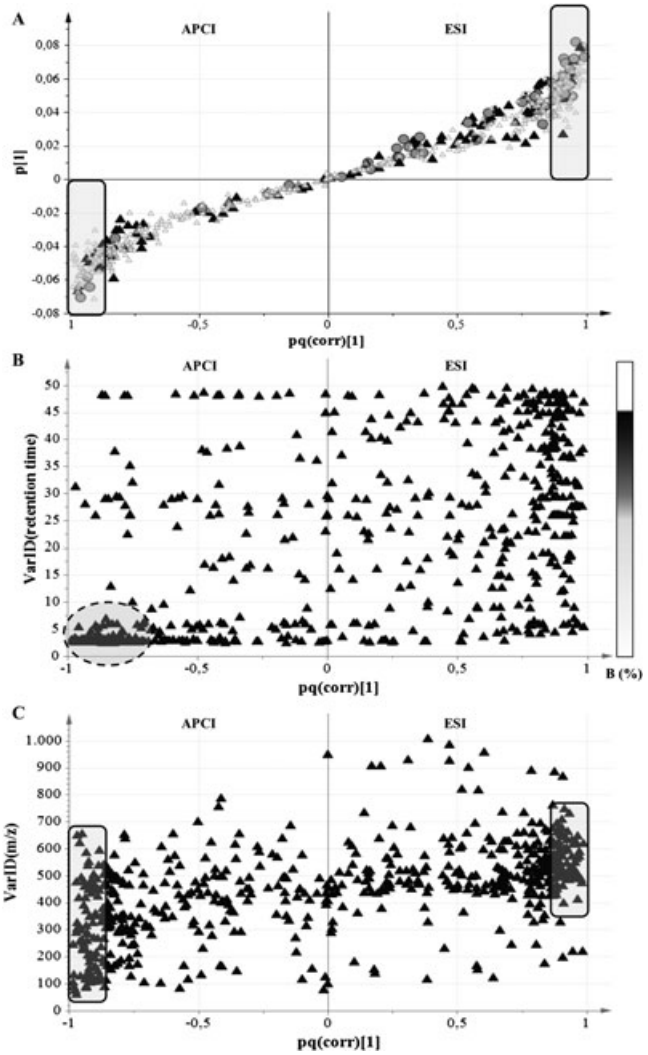

Figure 4. OPLS-DA correlation loading plots. (A) The entire dataset was split into two classes: samples ionized with APCI (class 1) or ESI (class 2). Black triangles indicate annotated molecules, dark gray circles indicate adducts, and small gray triangles represent unidentified molecules. (B) OPLS-DA correlation loading plot highlighting the retention time axis. Each triangle is a molecule. As indicated by the circle, many strongly polar metabolites correlated closely with APCI at the beginning of elution. (C) OPLS-DA correlation loading plot highlighting the *m/z* axis. Each triangle is a molecule. Molecules with pq(corr) values greater than ±0.9 are enclosed in boxes.

retention time (Fig. 4(B)). Indeed, metabolites with lower retention times, theoretically corresponding to strongly hydrophilic molecules that bind weakly or not at all to the reversed-phase C18 column, were ionized more efficiently by APCI, whereas ESI seemed to ionize molecules regardless of their polarity, but was more efficient with moderately and weakly polar metabolites.

Finally, when the loadings were plotted against the *m/z* values, rt/mz features with low *m/z* values were better correlated with APCI than ESI (Fig. 4(C)). These features included sugars and organic acids, as well as multiple fragments, suggesting that APCI is a harder ionization method resulting in a higher frequency of fragmentation directly in the ion chamber.

Comparison of APCI and ESI using representative standard compounds

ESI is strongly recommended as an ion source for the analysis of polar compounds.[2,17] We therefore compared the performance of LC/APCI-MS and LC/ESI-MS for the analysis of two of the most abundant polar compounds in berries, i.e. sucrose and tartaric acid. Moreover, caftaric acid, epicatechin and quercetin-3-*O*-glucoside, which are particularly abundant in grape berries, were analyzed as well. We compared the linear range, LOD and LOQ for each method using standard sucrose, tartaric acid, caftaric acid, epicatechin and quercetin 3-*O*-glucoside solutions, as shown in Table 1.

Different concentration ranges were chosen after preliminary experiments with standard compounds. The LODs and LOQs were lower for ESI than APCI with all reference compounds, showing that ESI can allow the detection and quantification of these molecules at levels down to ~10 ng. However, the linear range for sucrose and tartaric acid was wider for APCI than ESI, indicating that APCI may be better for the analysis of these molecules in untargeted metabolomics experiments, especially in organs such as fruits, where the concentration of sugars and aliphatic organic acids is higher than many other metabolites.

We also investigated the matrix effect for sucrose and tartaric acid by analyzing diluted R2 grape extracts (Table 1). When APCI was used as the ion source, the matrix effect reduced the sucrose and tartaric acid signals by 89% and 37%, respectively. However, when ESI was used as the ion source, the matrix effect reduced the sucrose and tartaric acid signals by 98% and 90%, respectively. Similar observations were noted for caftaric acid, epicatechin and quercetin-3-*O*-glucoside in a diluted R3 grape extract ionized with ESI, showing that signals were lowered by 36%, 48% and 48%, respectively (Table 1). On the contrary, APCI was not affected by matrix effect for caftaric acid and epicatechin at the dilution used in the comparison experiment (1:3). The matrix effect for quercetin-3-*O*-glucoside was not possible to assess with APCI since the signal was sharply lost after a few dilution points, thus preventing us calculating the correction factor.

Measurement of specific metabolite levels in berry samples

Various rt/mz features were assigned putative annotations based on reference spectra, and also their MS/MS and MS^3 fragmentation patterns, as sugar molecular ions, fragments or adducts, including one dihexose, four dihexose derivatives and three dihexose adducts with malic acid, arginine and glycine, respectively. The relative abundance of sugars (sum of peak areas) at the three ripening stages as determined by LC/ESI-MS and LC/APCI-MS is summarized in Fig. 5. The APCI source revealed a clear profile of sugar accumulation from V to R1 and R2, but this trend was barely apparent when the ESI source was used. Furthermore, the tartaric acid signal was much stronger when APCI was used instead of ESI, providing a much clearer picture of the dynamic profile of this molecule at different ripening stages and in different vintages (Fig. 5).

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Table 1. The sensitivity and range of LC/ESI-MS and LC/APCI-MS for metabolite analysis

Ion source	Molecules	LOD (S/N = 3) (μg)	LOQ (S/N = 10) (μg)	Linear range (μg)	Equation*	R^2	n	m.e. (%)	c.f.
APCI	Sucrose	0.0075	0.015	0.015–3	y = 1E + 08x − 5E + 06	0.9959	9	89	6.36
	Tartaric acid	0.0075	0.015	0.03–3	y = 9E + 06x + 91022	0.9963	7	37	1.10
	Caftaric acid	0.02	0.04	0.05–1	y = 1E + 08x − 4E + 06	0.9996	4	0	1
	Epicatechin	0.0005	0.0025	0.01–1	y = 1E + 08x − 3E + 06	0.9980	7	0	1
	Quercetin h.	0.005	0.015	0.05–1	y = 1E + 09x − 5E + 07	0.9995	4	-	-
ESI	Sucrose	0.0003	0.0015	0.003–0.3	y = 3E + 07x + 144687	0.9913	9	98	11.39
	Tartaric acid	0.0003	0.0015	0.015–0.225	y = 1E + 07x + 219025	0.9968	6	90	6.51
	Caftaric acid	0.0003	0.0025	0.0025–0.1	y = 1E + 08x + 151835	0.9987	8	36	1.55
	Epicatechin	0.0003	0.00075	0.00075–0.02	y = 2E + 08x − 29942	0.9985	8	48	1.92
	Quercetin h.	5E-06	0.00025	0.00025–0.1	y = 7E + 08x + 750098	0.9978	12	48	1.80

The limit of detection (LOD) and limit of quantification (LOQ) were defined as the minimum acceptable signals, with signal to noise (S/N) ratios of 3 and 10, respectively.
*Least-squares linear regression analysis.
Regression equation: y = ax + b, where x is the amount of compounds in μg and y is the detection response (peak area of the extracted signal).
R^2 is the correlation coefficient determined from *n* points for each calibration curve.
m.e. (%): percentage of the matrix effects; c.f.: correction factor; quercetin h: quercetin 3-*O*-glucoside.

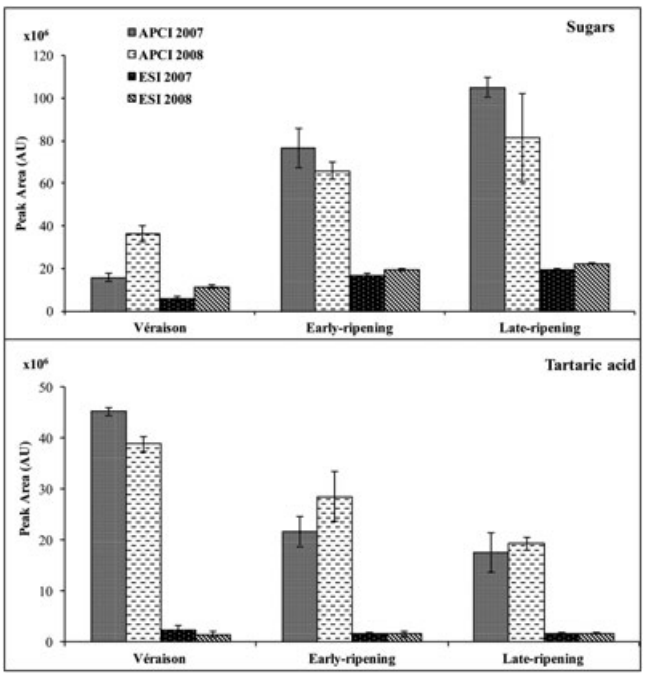

Figure 5. The relative abundance of sugars and tartaric acid in grape berries at different time points and vintages, based on the signals for molecular ionization produced by APCI and ESI. AU: arbitrary units. The error bars represent standard deviations (*n* = 3). Univariate statistical analysis confirmed that each comparison was statistically significant (t-test; *p*-value <0.05).

DISCUSSION

Corvina berries collected at three different ripening stages (V, R1 and R2) during the 2007 and 2008 growing seasons were used to prepare methanolic extracts for analysis by LC/ESI-MS and LC/APCI-MS. The data were processed with MZmine and multivariate statistical analysis was applied to the resulting data matrix in order to compare the performance of the two ion sources. Among the 608 *m/z* features, 15 were detected only by ESI and 6 with APCI. Since the two ion sources are both atmospheric pressure ionization (API) sources and they were used to analyze the same methanolic grape extracts, we did not expect to find many *m/z* features detected only with one of them. Few peak area values were missing in the data matrix (less than 10%), indicating that the chromatographic peak extraction, deconvolution, alignment and gap filling operations were correctly performed by MZmine. In fact, a high abundance of missing values in the data matrix (more than 10%) is undesired because this might affect the multivariate statistical analysis by reducing the representativeness of the samples. Missing values were considered as missing rather than zero because zero is in turn a mathematic value and therefore influencing the final statistical output. PCA revealed clear separation along PC1 for the samples analyzed by the two different methods, and this explained 62.9% of the variation. This suggested that the two ion sources can be distinguished by their ability to better ionize specific types of molecules, probably reflecting the different methods of ionization. A supervised OPLS-DA model was generated in which the two classes were based on the ionization source (APCI as class 1 and ESI as class 2). Surprisingly, the model showed that strongly polar metabolites such as sugars and organic acids correlated more closely with the APCI class than the ESI class, apparently contradicting earlier reports in which APCI rather than ESI is recommended for the ionization of weakly polar/non-polar metabolites,[2] including triterpenes,[18] carotenoids and chlorophylls,[19] long-chain fatty acids,[20] resveratrol[21–23] and triacylglycerols.[24,25] ESI is claimed to be highly sensitive, with good S/N ratios over a wide range of metabolites.[26] However, we found that moderately and weakly polar molecules such as flavanols, flavones, and acylated and glycosylated anthocyanins correlated more closely with the

ESI class, in agreement with previous reports concerning anthocyanins,[27] prodelphinidins and flavanols,[28] stilbenes,[29] and other polyphenols.[14,30] Furthermore, ESI is reported to ionize highly polar metabolites efficiently,[2,17] as well as non-polar molecules.[31] However, our analysis did not yield any data for non-polar metabolites with either method because the extraction protocol favored the recovery of strongly, moderately and weakly polar metabolites. Moreover, recent experiments have shown that APCI is suitable for the analysis of strongly polar metabolites, including endogenous fructose and sorbitol in human nerve tissues,[32] the glucose-d2/glucose ratio in human serum and plasma,[33] and the presence of monosaccharides and disaccharides in matrices such as orange juice, a well-characterized sugar solution, and a solution derived from the enzymatic hydrolysis of sunflower seeds.[34] An APCI source has also been used to investigate the differentiation of underivatized monosaccharides as an alternative to ESI-MS.[35]

APCI is less efficient for the ionization of molecules with a high molecular weight, probably due to the preferential transit of small molecules in a vapor and the strong fragmentation in MS^1, which also makes each product ion more difficult to identify. Although APCI is considered as a soft ionization method,[2,36] the intrinsic ionization mechanism and the nature of the target molecule can increase the degree of fragmentation.[37] ESI is also considered as a soft ionization method due to the production of molecular ions without fragmentation in MS^1. Indeed, we observed a strong correlation between the ESI class and molecules with a high *m/z* ratio, confirming that ESI allows the detection of metabolites with higher molecular weights.[2] ESI has been favored because metabolites are easier to identify[2,38] and the method also covers a greater metabolic range, e.g. as shown during investigations of the antioxidant capacity or phenolic composition of grape berries, juices and wines.[39–42] However, moderately polar metabolites and even more for strongly polar metabolites are subject to extreme matrix effects when an ESI source is used.[12]

The chromatography conditions we applied were based around the typical C18 stationary phase and a gradient of water and methanol, resulting in the elution of strongly polar molecules such as sucrose and tartaric acid at the beginning of the run. These molecules were therefore ideal to compare the performance of APCI and ESI in terms of ionizing polar metabolites. ESI showed a greater sensitivity for these two standard compounds, with LODs and LOQs lower than the APCI process, suggesting that ESI would be better for the detection of sucrose and tartaric acid traces in plant matrices. However, APCI achieved a broader linear range for both sucrose and tartaric acid, suggesting this method may be better to assess the levels of these two metabolites in different matrices, thus allowing a comparison among samples. Similar considerations might be referred for the investigated moderately polar metabolites, such as caftaric acid, epicatechin and quercetin 3-*O*-glucoside. A matrix effect, higher with ESI, was measured for both ion sources for the quantitative analysis of sucrose, tartaric acid, caftaric acid, epicatechin and quercetin 3-*O*-glucoside, in agreement with previous reports showing that sugars and other molecules with a low retention time (<3 min) are significantly underreported when ESI is used as the ion source, probably also reflecting the large number of molecules eluting in that zone.[12] The matrix effect can either enhance or suppress ion peaks due to the presence of molecules that facilitate or inhibit ionization. These include salts, acids or bases at high concentrations,[43–45] as well as carbohydrates, lipids, amines and peptides, which may interfere with droplet size reduction during ionization.[46,47] However, our results indicated that APCI is less susceptible to matrix effects than ESI, as previously reported for the quantification of sugars in human serum.[32,33] The greater linear range and more limited matrix effects associated with APCI makes this an attractive method for the simultaneous analysis of strongly and moderately polar metabolites in fruit extracts, which are characterized by a high content of sugars and strongly polar aliphatic organic acids, but a lower content of moderately polar metabolites. The more limited matrix effects associated with APCI made it possible to observe a trend in sugar accumulation and tartaric acid depletion in the berries during ripening, as well as vintage-specific effects, which were difficult or impossible to discern using the ESI method.

The tight correlation between ESI and moderately and weakly polar molecules included a correlation with adducts. It is not yet clear how adducts form during ESI, and the process may take place in the liquid droplets or, less likely, in the vapor state.[48] An intriguing hypothesis is that adducts form immediately before the droplets are disrupted, because the rapidly declining droplet volume facilitates interactions between solute molecules. In our case, chloride and formic acid molecules in the chromatography solvents could interact with berry metabolites during droplet evaporation, leading to the formation of adducts during negative ionization. Unfortunately, there is no validated approach to predict the amount of a specific adduct that will be formed, either based on the molecular structure of the analyte or the ionization conditions.[49] Moreover, the abundance of adducts varies substantially among experimental replicates so the resulting data are unreliable.[50]

APCI produced fewer adducts than ESI in our experiments, a phenomenon that has been reported previously with APCI in positive ion mode.[48] Two hypotheses have been proposed to explain this phenomenon, based on the observation that protonation (and probably also deprotonation) occurs in the vapor phase during APCI. The first hypothesis is that sodium ions form inefficiently in the vapor state because sodium is difficult to vaporize, and the second hypothesis is that sodium adducts are formed by weak interactions that are destroyed by the heated APCI interface.[48] We can neither confirm nor exclude either of these hypotheses because the positive and negative modes of APCI involve different processes.[51] However, several studies have shown that APCI also produces adducts in negative ion mode,[34,51–53] so further investigations are required to determine the mechanism of adduction. However, we observed a reproducibility of adduct formation among the different samples and even between the two technical replicates of a single sample.

The employment of a standard RP C18 column leads to poor retention and separation of highly polar compounds. In the last past decade, different strategies have been applied to overcome this issue and the addition of polar groups, including amides or carbamates, to the classical RP columns allowed a better separations of polar compounds.[54] For examples, polar embedded phase or polar endcapped phase

columns have recently been exploited to study water-soluble metabolites of bacterial extracts,[55] intracellular metabolites involved in central carbon metabolism in *Escherichia coli*,[56] and amino acids, organic acids, sugars and sugar alcohols in plants.[57] Therefore, as future perspectives, a comparison between APCI and ESI on polar metabolites separated with more appropriate chromatographic columns should be performed. Finally, the commercial availability and the raising of new published research exploiting hydrophilic interaction liquid chromatography (HILIC) columns might make the use of APCI a new attractive way for the analysis of polar metabolites.

In conclusion, untargeted metabolomics using both LC/APCI-MS and LC/ESI-MS can overcome the disadvantages that affect each of the two ion sources when applied individually, and also offers a successfully strategy to monitor specific metabolites. We found that APCI provided insight into the metabolic composition of berries during ripening because it was more efficient for the ionization of sugars and tartaric acid, allowing their dynamic profiles to be monitored over time. The profiles of these molecules could not be visualized clearly when combining the same chromatography method with an ESI source.

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