NMR spectroscopy and chemometrics-based analysis of grapevine

Ali, K.

CHAPTER 5

Monitoring biochemical changes during grape berry development in Portuguese cultivars by NMR spectroscopy

Kashif Ali[a], Federica Maltese[a], Ana Margarida Fortes[b], Maria Salomé Pais[b], Young Hae Choi[a], and Robert Verpoorte[a]

[a] Natural Products Laboratory, Institute of Biology, Leiden University, The Netherlands
[b] Plant Systems Biology Lab, ICAT, Center for Biodiversity, Functional and Integrative Genomics, FCUL, 1749-016 Lisboa, Portugal

Abstract

^{1}H nuclear magnetic resonance (NMR) was applied for the metabolic profiling of grapes from three Portuguese cultivars including 'Trincadeira', 'Aragonês', and 'Touriga Nacional', at four developmental stages. Two kinds of extraction methods including deuterated NMR solvent extraction and solid phase extraction (SPE) were used for the metabolomic analysis and all the metabolites detected in ^{1}H NMR were elucidated by two-dimensional NMR techniques as well as the in-house NMR chemical shift database. Multivariate data analyses were also performed to identify overall metabolic differences. Trincadeira was found different from the other two cultivars, having relatively low phenolic contents. The initial stages showed comparatively high phenolics and organic acid contents like caftaric and malic acid while the later stages showed higher glucose and fructose levels. Veraison was found to be a metabolically critical stage of berry development. On the basis of these findings distribution of metabolites among different cultivars at different developmental stages is discussed.

Introduction

Grapevine (*Vitis spp*) is one of the most economically important and widely cultivated fruit crops across the world. In addition to their economic importance, an increasing number of health benefits have been attributed to grapes and wine. For instance, grapes are known to have antioxidant, cardioprotective, anti-inflammatory and anti-cancer activities (Ali et al. 2010). Due to this knowledge regarding the development and maturation of grape berries is of great economical interest. Climacteric fruit such as tomatoes and apples have been well studied but comparatively less is known about the development and ripening of non-climacteric fruits e.g. grapes and strawberry (Giovannoni 2004; Given et al. 1988). Considerable scientific efforts have been made to understand the complex series of physical and biochemical changes of grape berries during their development cycle (Coombe 1992). Today, the major concerns of the viticulturists are size, coloration, control of ripening, acidity, and volatile and non-volatile contents of the grape berry.

Chemical characterization of the phenotype of an organism has become the focal point of many researchers in recent years. The analysis of these low molecular weight

compounds seems to reflect the physiological activities of an organism or tissue under different conditions. The observable chemical profile or fingerprint shown by the plant tissue, 'metabolome', is highly complex and consists of a vast variety of chemicals which differ in their structure and function. Due to this, it is unlikely that a single analytical method could provide information about all the metabolites, considering their chemical diversity, and at the same time be unbiased, rapid, reproducible, and stable over time, while requiring only simple sample preparation. Many platforms are being used for the high throughput analysis of plant metabolites, but vary according to their sensitivity (Kopka et al. 2004).

Apart from its routine use in the identification, characterization, and structure elucidation of molecules, nuclear magnetic resonance (NMR) spectroscopy is now increasingly popular in the area of metabolome analysis (Son et al. 2009a). In combination with different multivariate data analyses tools, such as principal components analysis (PCA), NMR has been used for the fingerprinting or metabolic profiling of various sample types (Brescia et al. 2002; Charlton et al. 2002). This combination has been very useful for the characterization of different plant species (Kim et al. 2005), and cultivars as well (Ali et al. 2009). In the study of grapevine, recently, the coupling of metabolic analysis with transcriptional analysis has also been applied to the profiling of two grape cultivars with different resistance capabilities to pathogenic fungi (Figueiredo et al. 2008). In the case of berries, NMR coupled to multivariate analyses has been used to study the effects of growing areas, vintage, and soil (Pereira et al. 2005), and the influence of microclimate on metabolic profiles of grape berries (Pereira et al. 2006a). Many of grape berry metabolic profiling studies have focused mainly on the amino acid or sugar contents while ignoring the phenolic composition of the sample. This may be due to the low signal quality or signal overlapping of phenolics in the NMR spectra.

In this study we sought to obtain a metabolic profile of the different developmental stages of grape berries from three different Portuguese cultivars. To highlight these differences, one- and two-dimensional ^{1}H NMR techniques, coupled with principal component analysis (PCA), projections to latent structures-discriminant analysis (PLS-DA), and orthogonal projections to latent structures (OPLS) analysis were applied.

Materials and methods

Grape cultivars and sampling

Three elite Portuguese cultivars i.e. Trincadeira, Touriga Nacional, and Aragonês, were used in this study. Five biological replicates of each cultivar of 80-100 berries from 8-10 plants were collected in 2008 corresponding to the developmental stages of EL 32 (green), 35 (veraison), 36 (ripe), 38 (harvest) (EL refers to the modified Eichhorn and Lorenz developmental scale as described by Coombe 1995). Each biological replicate contained berries from a single row of plants. Four rows distant 3 to 10 m from each other were used for each variety. Plants from the three varieties were growing in the vineyard 15 to 30 m apart.

Extraction and NMR spectroscopy

A sample of 100 mg of lyophilized grape berries was extracted according to Kim et al. (2010a). Solid phase extraction and NMR spectroscopy parameters were used as explained in Chapter 4.

Data analysis and statistics

The ^{1}H NMR spectra (from both SPE and direct extraction) were automatically reduced to ASCII files. Spectral intensities were scaled to internal standard and reduced to integrated regions of equal width (0.04) corresponding to the region of δ 0.0–10.0. The regions of δ 4.85–4.95 and δ 3.2–3.4 were excluded from the analysis because of the residual signal of D_2O and CD_3OD, respectively. Bucketing was performed by AMIX software (Bruker) with scaling on internal standard. Principal component analysis (PCA) with scaling based on Pareto while projections to latent structures-discriminant analysis and orthogonal projections to latent structures analysis with scaling based on Unit Variance were performed with the SIMCA-P+ software (v. 12.0, Umetrics, Umeå, Sweden). The ANOVA for the ^{1}H-NMR signals was performed by MultiExperiment Viewer (v. 4.0) (Saeed et al. 2003).

Results

Visual analysis of ^{1}H NMR spectra

The corresponding berry weight for each stage at the time of sample collection is shown in Figure 1A. In this study four different developmental stages (EL 32, 35, 36, 38) of the grape berries from three different Portuguese cultivars were analyzed. For metabolic profiling of the berries two kinds of extraction methods were used including extraction directly with deuterated NMR solvents and solid phase extraction (SPE) with C18 resins. In the ^{1}H NMR spectrum, the area between δ 0.8-4.0 corresponds to aliphatic compounds and amino acids with some resonance for the organic acids. The region of δ 4.0-5.5 is considered to be the carbohydrate region and the remaining part, i.e. δ 5.5-8.5, is known as the phenolic region. ^{1}H NMR spectra resulted from both extraction method were analyzed and compared. Figure 2 shows a comparison among the ^{1}H NMR spectra of direct extraction and SPE. The ^{1}H NMR spectra from deuterated extraction method were found dominated by the signals of sugars, organic and amino acids, with very low intensity of the phenolic compounds. In an attempt to amplify the resonances related to phenolics SPE was used and, there was improved signal intensity in the phenolic region of the spectra.

Different developmental stages of Trincadeira were analyzed and compared. It indicates, deuterated extraction showed a pattern in the appearance of both primary and secondary metabolites. A gradual decline in the phenolics amount can be easily observed with the berry growth, along with some organic acids, especially tartarate and malate. On the other hand amino acids and sugars are more accumulated in grape berries at the later stages of their development. Similar developmental stages of all varieties were also compared. The comparison clearly suggested that these cultivars are different in their metabolic profile. Trincadeira can be characterized by low phenolics but higher sugars and organic acids while Touriga Nacional and Aragonês were found with higher phenolics contents with some amino acids. The distribution of these metabolites among the cultivars and their developmental stages will discuss later in details.

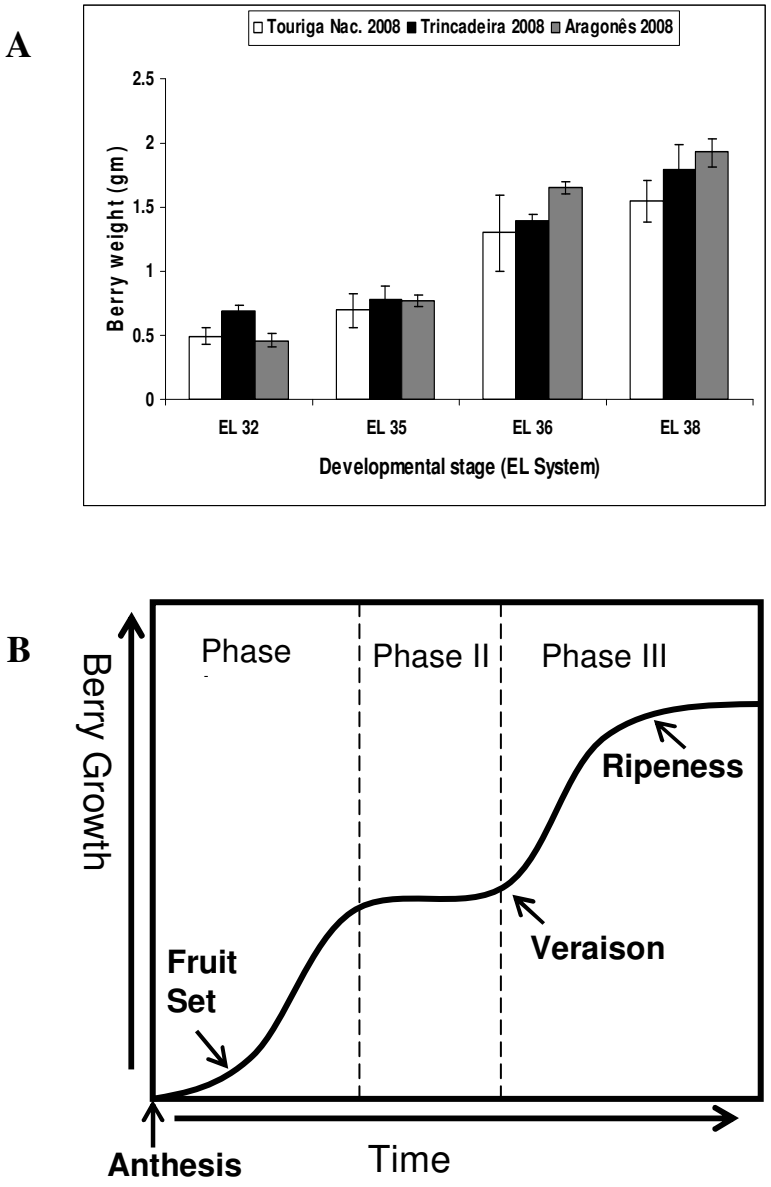

Figure 1. Average berry weight (A) at the time of sampling. Double sigmoid growth curve (B) of berry development showing different phases of growth (Redrawn and modified from Coombe 1992).

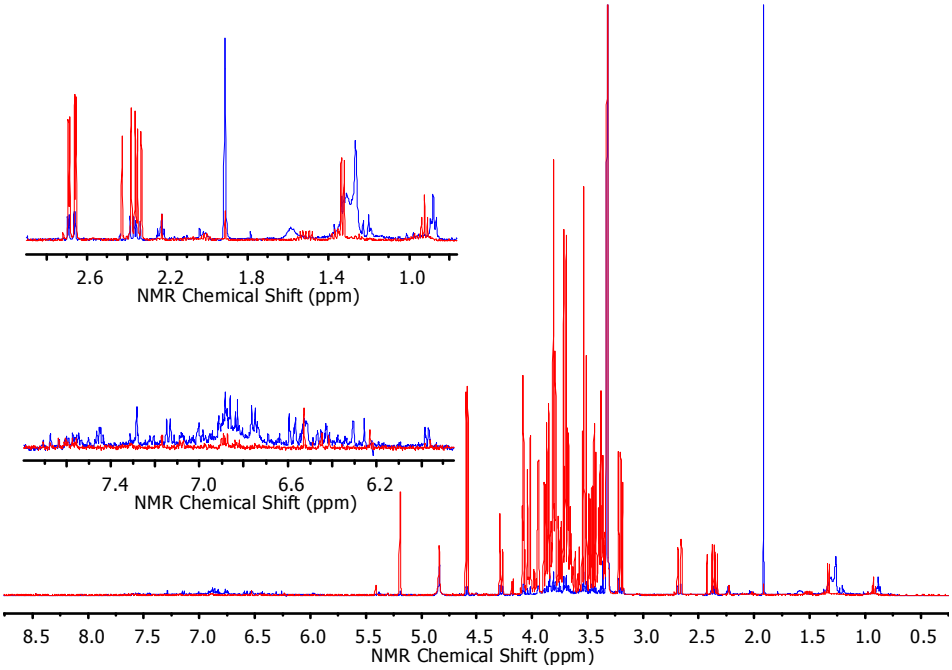

Figure 2. Comparison of ^{1}H NMR spectra of Direct Extraction (red) and Solid Phase Extraction (blue). The amino acid (δ 0.5- δ 3.0) and phenolic (δ 5.8- δ 7.8) regions are shown with signal amplification. It can be easily observed that direct extraction resulted in low phenolics but higher sugars, amino and organic acid signals while SPE resulted with more signals intensities for phenolics.

Figure 3A shows a comparison between ^{1}H NMR spectra (from SPE) of different developmental stages of Trincadeira grapes. It can be observed that in all its developmental stages the appearance of metabolites followed a pattern. The green stage (EL 32) can be identified by the highest intensity of phenolics which gradually decrease along the ripening process of the berries, while there is little difference in the amino acid region in all stages of development. The remaining two cultivars also showed the same pattern. Figure 3B shows a comparison of the phenolic contents of all three cultivars at green stage. Among the cultivars, Touriga Nacional was characterized by the maximum intensity of signals as compared to the other two cultivars while Trincadeira was found to have the lowest signal intensity in the phenolic region of the ^{1}H NMR spectra. Visual analysis the ^{1}H NMR spectra showed that all the three cultivars differed in their metabolic contents for the same developmental stage.

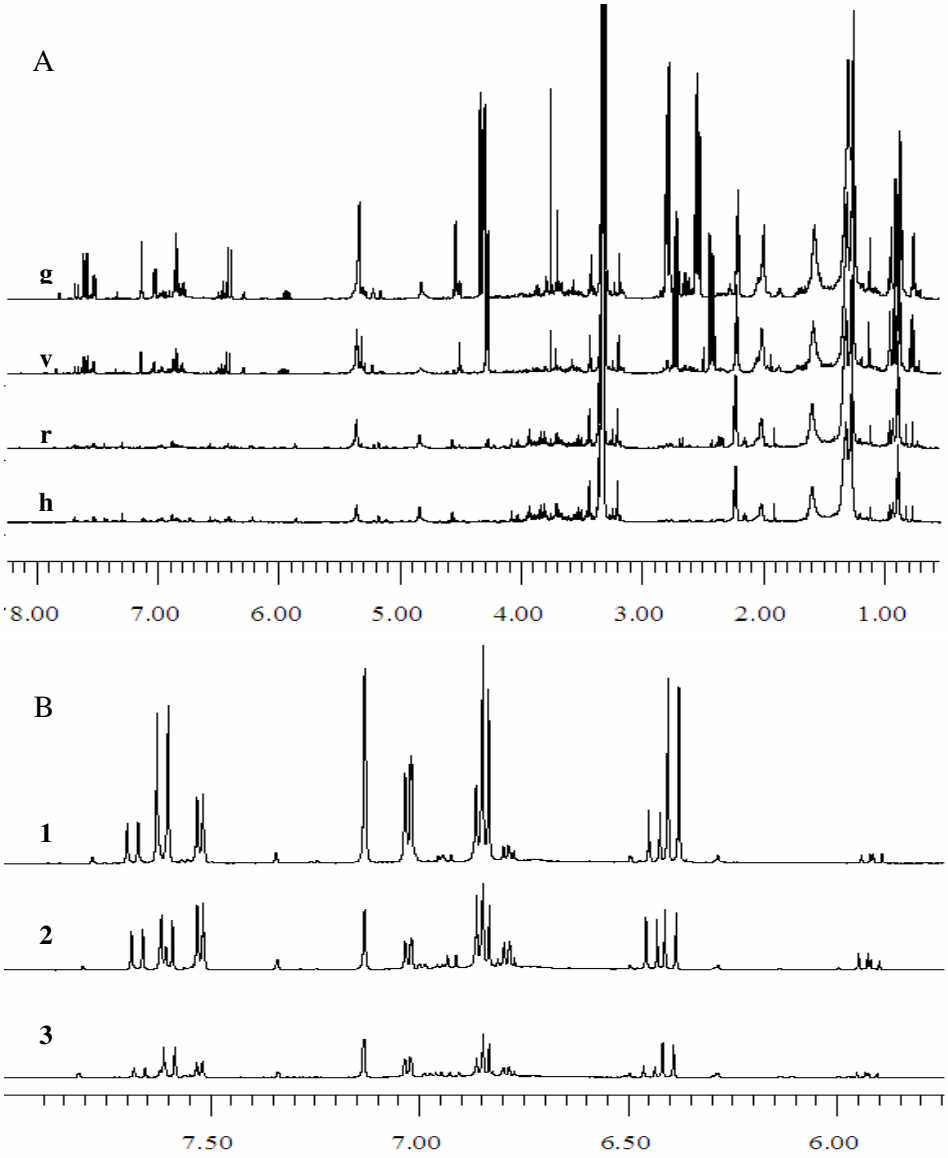

Figure 3. ^{1}H NMR spectra (A) of green (g), veraison (v), ripe (r), and harvest (h) stages of Trincadeira. Phenolic contents (B) in Touriga Nacional (1), Aragonês (2), and Trincadeira (3) cultivars at green stage.

Table 1. ^{1}H NMR Chemical Shifts (δ) and Coupling Constants (Hz) of Grape Metabolites Identified by References and Using 1D and 2D NMR Spectra (CD_3OD-KH_2PO_4 in D_2O, pH 6.0).

Compounds	**Chemical Shifts (δ)**
Valine	1.01 (d, *J*=7.0), 1.06 (d, *J*=7.0), 2.28 (m)
Leucine	0.96 (d, *J*=7.5), 0.98 (d, *J*=7.5)
Alanine	1.48 (d, *J*=7.4), 3.73 (q, *J*=7.4)
GABA	1.90 (m), 2.31(t, *J*=7.5), 3.01 (t, *J*=7.5)
Proline	2.35 (m), 3.37 (m)
Methionine	2.15 (m), 2.65 (t, *J*=8.0)
Threonine	1.32 (d, *J*=6.5), 3.51 (d, *J*=5.0), 4.27 (m)
Glutamic acid	2.13 (m), 2.42 (m), 3.71 (dd, *J*=7.0, 1.9)
α-Glucose	5.17 (d, *J*=3.78)
β-Glucose	4.58 (d, *J*=7.89)
Fructose	4.08 (d, *J*=7.80)
Sucrose	5.39 (d, *J*=3.94)
2,3-butanediol	1.14 (d, *J*=6.47)
Acetic acid	1.94 (s)
Choline	3.20 (s)
Succinic acid	2.53 (s)
Citric acid	2.56 (d, *J*=17.6), 2.74 (d, *J*=17.6)
Tartaric acid (free)	4.30 (s)
α-Linolenic acid	0.95 (t, *J*=7.5)
Ascorbic acid	4.52 (d, *J*=2.0)
Malic acid	2.68 (dd, *J*= 16.6, 6.6), 2.78 (dd, *J*=16.6, 4.7), 4.34 (dd, *J*=6.6, 4.7)
Formic acid	8.45 (s)
Fumaric acid	6.52(s)
Gallic acid	7.03 (s)
Syringic acid	3.89(s), 7.31(s)
Vanillic acid	3.90 (s), 6.77 (d, *J*=8.2), 7.22 (m)
(+) - Catechin	2.49 (dd, *J*=16.1, 8.2), 2.83 (dd, *J*=16.0, 5.4), 4.04 (m), 4.55 (d, *J*=7.5), 5.91 (d, *J*=2.2), 6.75 (d, *J*=8.0)
(-) - Epicatechin	2.72 (dd, *J*=16.8, 2.8), 2.85 (dd, *J*=16.7, 4.6), 5.91 (dd, *J*=10.0, 2.3), 6.96 (d, *J*=2.2)
Quercetin-3-*O*-glucoside	5.30 (d, *J*=7.6), 6.27 (d, *J*= 2.0), 6.49 (d, *J*=2.0), 6.95 (d, *J*=8.6), 7.56 (dd, *J*=8.5, 2.0), 7.81 (d, *J*=2.0)
Myricetin	6.28 (d, *J*= 2.0), 6.51 (d, *J*=2.0), 7.30 (s)
trans-Caftaric acid	5.34 (s), 6.41 (d, *J*=16.0), 6.88 (d, *J*=8.4), 7.02 (dd, *J*=8.4, 2.0), 7.12 (d, *J*=2.0), 7.62 (d, *J*=16.0)
trans-p-Coutaric acid	5.42 (s), 6.45 (d, *J*=16.0), 6.87 (d, *J*=8.8), 7.51 (d, *J*= 8.8), 7.65 (d, *J*=16.0)

trans-Fertaric acid	3.89 (s), 5.38 (s), 6.32 (d, *J*=16.0), 6.89 (d, *J*=8.4), 7.01 (dd, *J*=8.4, 2.0), 7.19 (d, *J*=2.0), 7.56 (d, *J*=16.0)
cis-Caftaric acid	5.34 (s), 5.92 (d, *J*=13.0), 6.71 (d, *J*=8.4), 6.81 (d, *J*=13.0), 7.03 (dd, *J*=8.4, 2.0), 7.44 (d, *J*=2.0)
cis-p-Coutaric acid	5.41(s), 5.94 (d, *J*=13.0), 6.73 (d, *J*=9.2), 6.86 (d, *J*=13.0), 6.93 (d, *J*=9.2), 7.61 (d, *J*=9.2),

Identification of metabolites

The metabolites identified cover a wide range of diversity and include amino acids, organic acids, carbohydrates, hydroxycinnamates, hydroxybenzoates, and flavonoids. The high signal intensities in the amino acid region were helpful to elucidate a number of amino and organic acid signals. The amino acids alanine, threonine, valine, proline, methionine, leucine, and γ-amino butyric acid (GABA) were identified in berries by comparison with the reference spectra of these compounds. The signals in the carbohydrate regions were highly clustered and overlapped. This region showed the signals of the anomeric protons of β-glucose, α-glucose, fructose, and sucrose. Although the direct extraction showed these sugars as major compounds, they were still quite visible as SPE extraction. Other compounds, including choline, 2,3-butanediol, and acetic acid, were also identified in this region. A number of signals were assigned to the organic acids like succinic acid, fumaric acid, formic acid, citric acid, malic acid, and tartaric acid. The tannins (+) – catechin and (-) – epicatechin were also identified (Table 1).

The flavonoids quercetin and myricetin were also identified in the aromatic region (Figure 4A). The quercetin signal at δ 6.49 of H-8 was correlated in the ^{1}H-^{1}H COSY spectrum with the signal at δ 6.27 of H-6 and a signal at δ 6.95 of H-5′ with one at δ 7.56 of H-6′. Similar correlations for the signals of myricetin at δ 6.51 of H-8 with δ 6.28 of H-6 also showed ^{1}H-^{1}H COSY correlations (Figure 4B). The upfield shift of C3 signal (around δ 134.0) in HMBC spectra for quercetin showed the binding of a glucose molecule to quercetin resulting in the identification of quercetin-3-*O*-glucoside.

The aromatic part of the ^{1}H NMR spectra also showed some signals of hydroxybenzoates such as gallic acid, syringic acid, and vanillic acid. The presence of characteristic doublets of 16.0 Hz in the range of δ 6.39-6.50 and δ 7.59-7.70 represent the H-8′ and H-7′ (olefinic protons) of *trans*-hydroxycinnamic acids, respectively

(Figure 4A). The ^{1}H-^{1}H COSY spectra also confirmed the correlation between H-8′ and H-7′ of these compounds, with the coupling with carbonyl carbon at δ 168.3 in the HMBC spectra. In the ^{1}H NMR spectra of grape berry samples, these resonances were assigned to three different hydroxycinnamic acids moieties which include *trans*-caffeoyl, *trans*-coumaroyl, and *trans*-feruloyl derivatives. The ^{1}H-^{1}H COSY spectra (Figure 4B) showed correlations among signals like δ 6.41 with δ 7.62; and δ 7.02 with δ 6.88 of caffeoyl; δ 7.51 with δ 6.87; and δ 6.45 with δ 7.65 of coumaroyl; δ 6.46 with δ 7.56 of feruloyl derivative. These derivatives were found to be conjugated with tartaric acid via an ester linkage. The three singlets for tartaric acid were observed in the region of δ 5.32-5.44 in ^{1}H NMR spectrum, being shifted downfield from the typical tartaric acid signal at δ 4.30 due to their bonding to the carboxylic function of cinnamic acids which was confirmed by their correlation with the signal at the region of δ 167.5-168.5 in the HMBC spectra. Based on these assignments, these compounds were identified as *trans*-caftaric acid (caffeic acid conjugated with tartaric acid), *trans*-fertaric acid (ferulic acid conjugated with tartaric acid), and *trans*-coutaric acid (coumaric acid conjugated with tartaric acid).

Along with the *trans*- forms, the *cis*- forms of these conjugated cinnamic acids, i.e. *cis*-caftaric acid and *cis*-coutaric acid, were also detected. The *cis*- forms showed an upfield shift of the signals for H-8′ and H-7′ along with the reduction in the coupling constant from 16.0 Hz to 13.0 Hz. Two clear doublets of 13.0 Hz at δ 5.92 and δ 5.94 were detected for the H-8′ in the *cis*-configuration (Figure 4A). The ^{1}H-^{1}H COSY spectra also confirmed this by showing the correlation of these signals with the respective H-7′ protons at δ 6.81 and δ 6.86 (Figure 4B). It was also confirmed by the correlation of this signal with the carbonyl resonance at δ 167.2 in the HMBC spectra. All of these assignments (Table 1) were done by comparing the spectra of more than 500 common metabolites in our in-house library.

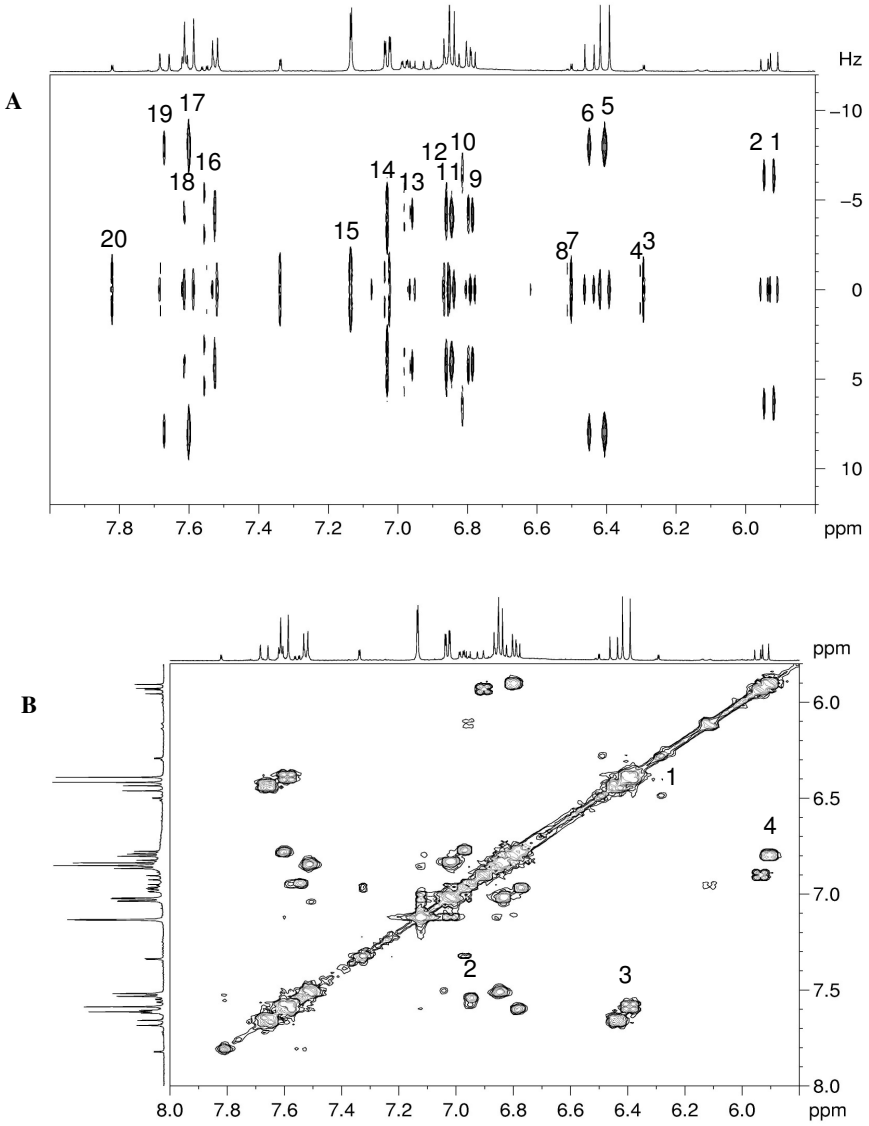

Figure 4. *J*-resolved spectra (A) and ^{1}H-^{1}H COSY spectra (B) of Trincadeira green stage (δ 5.8-8.0). *J*-resolved spectra (A) shows the signals labeled as 3, 7, 13, 16, 20, correspond to H-6, H-8, H-5', H-6', H-2', respectively, of quercetin-3-*O*-glucoside; 4 and 8 to H-6 and H-8 of myricetin; 5, 12, 14, 15, 17 correspond to H-8', H-5, H-6, H-2, H-7', respectively, of *t*-caftaric acid; 6, 11, 19 correspond to H-8', H-3 and H-5, H-7', respectively, of *t*-coutaric acid; 1, 10 correspond to H-8', H-7' of *c*-caftaric acid; 2, 18 correspond to H-8', H-2 and H-6 of *c*-coutaric acid; 9 corresponds to H-5 of vanillic acid. 1H-1H COSY spectra (B) shows correlations among the signals of H-6 with H-8 (1) and H-5' with H-6' (2) of quercetin-3-*O*-glucoside; H-8' and H-7' (3, 4) of *trans*- and *cis*-caftaric and coutaric acid, respectively.

Multivariate data analyses (MvDA)

Principal component analysis (PCA) is one of the most common MvDA methods and is used to reduce the dimensionality of a multivariate dataset. The application of supervised analyses like projections to latent structures-discriminant analysis (PLS-DA) and orthogonal projections to latent structures (OPLS) are considered to be the next step in MvDA. These analyses, unlike the unbiased system used for PCA, are performed with pre-input information regarding the data. The most important information obtained from these analyses is the correlation between data sets which correspond, in this study, to different developmental stages and cultivar types. Like PCA, the differences or similarity among the samples can be detected by using the score plot while the signals responsible for those differences or similarities can be identified by the loadings. The ^{1}H NMR data (bucket table), from both direct extraction and SPE, were used as variables for all the multivariate analyses applied in this study.

Direct extraction and MvDA

The ^{1}H NMR data from direct extraction were first subjected to different multivariate data analyses methods in order to get precise knowledge about the primary metabolites during grape development. The PCA score plot (Figure 4A) showed very tight clustering among the replicates. All the developmental stages of three cultivars were completely separated. Samples from the green stage are clustered on the positive side of PC1 and PC2 while the veraison stage samples are grouped on the negative side of PC2 but having the positive PC1 values. Both ripe and harvest are on the negative side of PC1 but having negative and positive PC2 scores, respectively. By examining the score plot, it is evident that all the developmental stages of grape berry are different in their metabolic profile. It was also interesting to note that green from Trincadeira and veraison from Aragonês were a bit separated from the green and veraison stages of other cultivars. By examining the loading plot, it was evident that the green and veraison stages were characterized by higher levels of phenolics and organic acids whereas the ripe and harvest stages were found with more amounts of amino acids and sugars. The green and veraison stages were concentrated by phenolics along with some amino acids like alanine, proline, GABA, with organic acids like malate, tartarate, and fumarate. The

ripe and harvest stages showed elevated amounts for the compounds like leucine, valine, acetate, and succinate, with sugars like glucose and fructose.

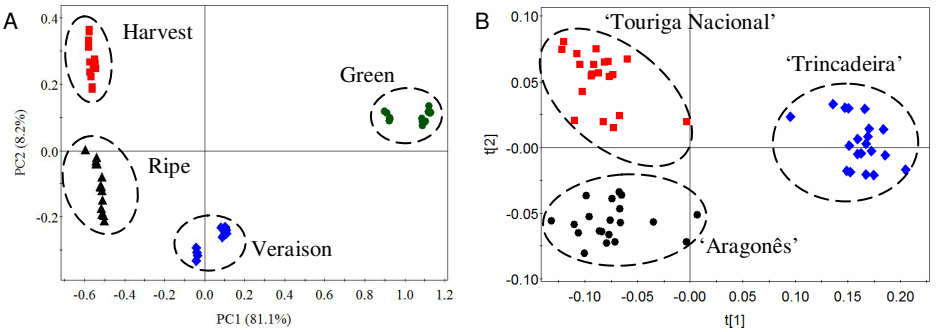

Figure 4. Multivariate data analyses based on the ^{1}H NMR spectra from the direct extraction. Score plot (A) of PCA, Score plot (B) of OPLS with classification based on cultivars. (A) shows general differentiation of the developmental stages of grapes representing Green, Veraison, Ripe, and Harvest stage of grape ripening. (B) shows the separation based on the type of cultivar.

The MvDA based on ^{1}H NMR data of direct extraction was found effective in highlighting the differences among the three cultivars, mainly by primary metabolites. The OPLS score plot (Figure 4B) based on ^{1}H NMR data from direct extraction shows complete separation among the samples from the three cultivars. Samples from Trincadeira are clustered together on the positive side of component 1, while Touriga Nacional and Aragonês are on the negative side of the same component having positive and negative component 2 scores, respectively. Unlike PLS-DA, the score plot of OPLS didn't show any separation among the samples based on the developmental stages. Loading plot revealed that Trincadeira has high signal intensities for malate, ascorbate, glucose, and fructose. Aragonês showed elevated concentration of succinate, tartarate, and fumarate, while Touriga Nacional was characterized by higher resonances for compounds like citrate, gallate, alanine, valine, GABA, and glutamate.

Solid phase extraction and MvDA

The ^{1}H NMR data (from SPE) were subjected to PCA to highlight the differences or similarities among the samples. The PCA score plot showed some good clustering among the replicates with the exception of one outlier of sample AV (Aragonês veraison) possibly due to artifacts produced during the extraction procedure (Figure 5A).

Samples were grouped according to the grape cultivar and also on the basis of developmental stages. As shown in the figure, the green stages for all three varieties are well separated from each other and have negative PC1 scores. This showed that all the three cultivars are quite different in their green stage. The veraison stages for two varieties, i.e. Aragonês and Touriga Nacional, are grouped together (on the negative side of PC1) while the veraison for Trincadeira was very well separated from the other two (on the positive side of PC1). This reveals that the veraison stage of Trincadeira is metabolically quite different from the other two cultivars. Samples from the ripe and harvest stages of all three cultivars were grouped close to each other having positive PC1 scores. This suggests that the level of metabolites in these two stages does not differ significantly.

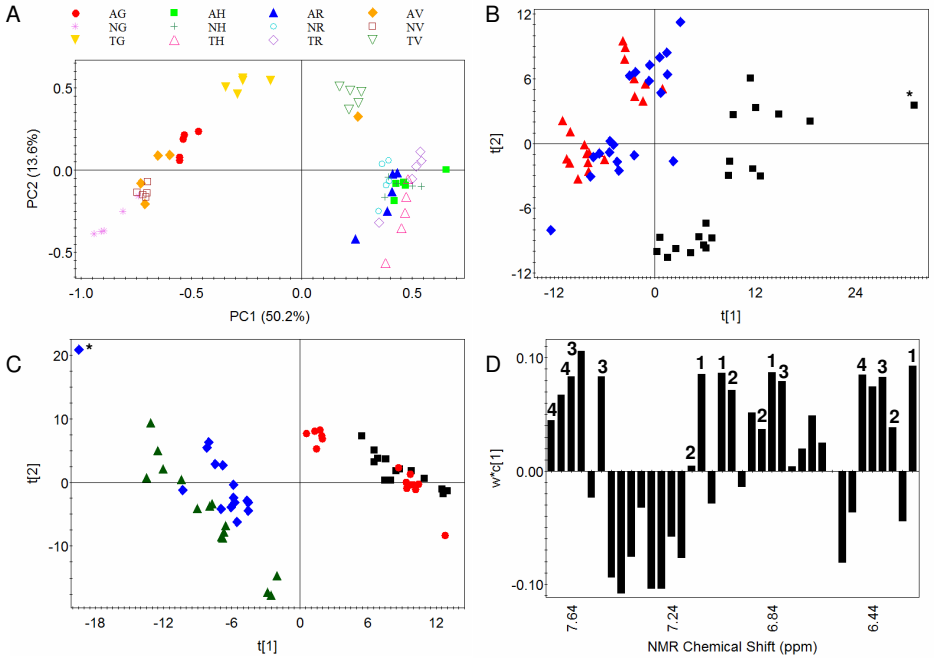

Figure 5. Multivariate data analyses based on Methanol fraction of SPE. Score plot (A) of PCA, Score plot (B) of PLS-DA with classification based on cultivars and black, blue, and red color present 'Trincaderia', 'Aragones', and 'Touriga Nacional', respectively. Score plot (C) of PLS-DA with classification based on development stages and black, red, blue, and green presents Green, Veraison, Ripe, and Harvest stages. Loading column plot (D) shows signals of compounds found higher in the green and veraison stages, 1: Caftaric acid, 2: Fertaric acid, 3: *p*-Coutaric acid, 4: Quercetin-3-*O*-glucoside. Samples with '*' are the outliers.

The green and veraison stages of Trincadeira were characterized by high levels of malic acid, choline, and succinic acid. While the green and veraison stage of Aragonês and Touriga Nacional showed higher levels of citric acid and quercetin glucoside along with elevated signals of hydroxycinnamic acid derivatives such as caftaric acid and coutaric acid, and sucrose. As the score plot clustered the ripe and harvest stages, the signals for their separation were identified as acetic acid, GABA, glucose, and the amino acids like valine and proline.

Another objective of this analysis was to distinguish all developmental stages of all the cultivars. To highlight the differences based on cultivar type, the samples were classified into three classes (Trincadeira, Aragonês, and Touriga Nacional) and PLS-DA was applied. The PLS-DA score plot showed quite interesting results (Figure 5B). Harvest and ripe Trincadeira grapes were separated by component 2 while green and veraison stages of Trincadeira showed different component 2 scores than ripe and harvest. All four stages of Trincadeira were also distinguished from the stages of the other two cultivars (by component 1). The ripe and harvest stages of Trincadeira were clearly separated from the other two cultivars. In the case of Aragonês and Touriga Nacional, the green and veraison stages were clearly separated from the ripe and harvest of the same cultivars by component 2, having negative and positive component 2 scores, respectively.

The loading plot showed that while the harvest stage of Trincadeira has high levels of valine, methionine, glucose, and acetic acid the ripe stage shows more amino acids such as threonine and proline. Succinic acid, malic acid, and choline were found to be higher in the green and veraison stages of Trincadeira. The ripe and harvest of Aragonês and Touriga Nacional had similar metabolic profiles exhibiting higher amounts of the amino acids leucine, alanine, and GABA. The green and veraison stages of these cultivars were also similar showing relatively higher levels of phenolics, similar to what was shown by PCA.

According to the ^{1}H NMR data from SPE, the Aragonês and Touriga Nacional cultivars were found to have a very similar metabolic profile. In an attempt to find some differences between them, we used PLS-DA but omitting Trincadeira data this time. By examining the score and loading plots (not shown in figures), it was easily observed that again the green and veraison stages of both the cultivars were separated from their

respective ripe and harvest stages but it was difficult to discriminate between green and veraison stages of the same cultivar. Higher resonances for proline, choline, and fertaric acid were observed in Aragonês, whereas the levels of caftaric acid and quercetin glucoside, for example, were higher in the green and veraison stage of Touriga Nacional. The ripe and harvest stages for both the cultivars were grouped close to each other and characterized by high levels of glucose, leucine, alanine, and valine. Touriga Nacional was found to have a higher total content of anthocyanins than Aragonês and Trincadeira. These measurements were done for several clones of the three varieties grown in different regions of Portugal. The same results were obtained for the grapes from the 3 varieties analyzed in this study and grown in the same vineyard (Ana M. Fortes, unpublished).

In order to gain more insight into the metabolic differences between the different stages of grape berry development, data from the SPE and direct extraction were again subjected to PLS-DA. This time, the samples were classified into four classes i.e. green, veraison, ripe, and harvest. The score plot for PLS-DA (SPE extraction) showed clear separation of green and veraison stages from the ripe and harvest stages of all three cultivars by component 1 (Figure 5C). Here again the veraison and green stages of Trincadeira were barely separated from the other two but in general these stages were clustered on the positive side of the component 1. This showed that these two stages shared their metabolic contents with some differences. For the ripe and harvest stages, no tight clustering was observed and these stages were grouped on the negative side of the component 1.

The ripe and harvest stages were characterized by high signals for the compounds valine, leucine, catechin, and glucose. The green and veraison stages of all the three cultivars showed higher phenolic contents including compounds such as caftaric and coutaric acid or the flavanoid quercetin glucoside. Some organic acids which include malic acid, tartaric acid, and citric acid, were also found in higher levels as well as sucrose. Figure 5D shows the loading column plot for the component 1. The signals of hydroxycinnamic acids and quercetin glucoside were clearly identified and found to be higher in the green and veraison stages.

Relative quantification and distribution of metabolites

The ^{1}H NMR data set (bucket table) from both SPE and direct extraction was subjected to ANOVA in order to confirm the results obtained from MvDA. The compounds were also relatively quantified in each stage of the grape berry development of all the three cultivars. The ANOVA confirmed the participation of different metabolites in the discrimination of different stages with high statistical significance (p<0.01). Figure 6 shows the graphs representing the compounds with their relative quantities at different developmental stages. These quantities were measured on the basis of the mean peak areas of the characteristic signals of these compounds. The phenolics like catechin, quercetin, caftaric and coutaric acid were quantified using NMR data from SPE while the other compounds like amino acids, organic acids, and sugars have been quantified using the NMR data from direct extraction method.

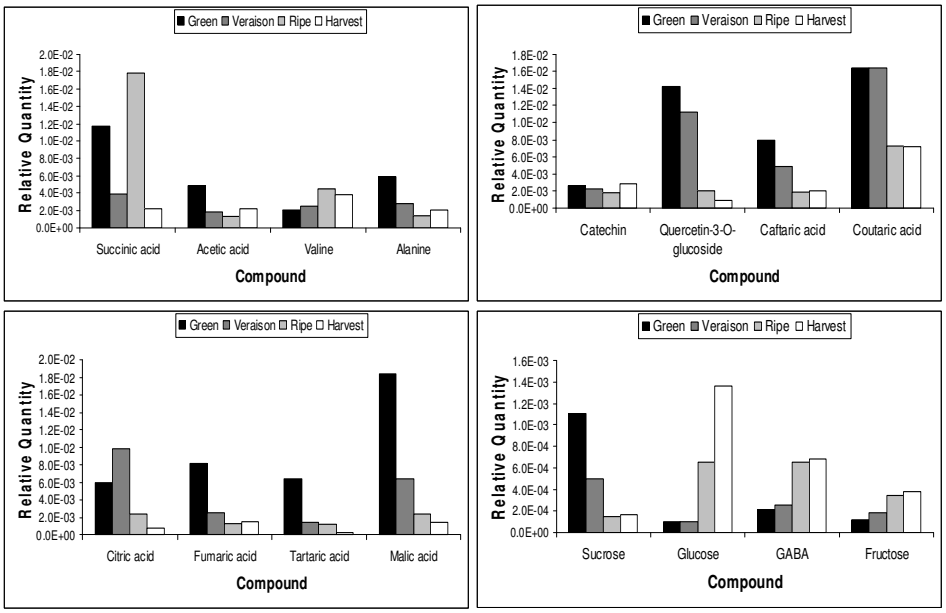

Figure 6. Relative quantification of compounds based on the mean peak area of the associated signals. The bars related to the same metabolites are siginificantly different (p<0.01) after ANOVA between green, veraison, ripe, and harvest groups.

It is obvious from Figure 6 that every stage is dominated by a series of specific metabolites. The first two stages in the grape berry development cycle i.e. green and veraison have significantly higher levels of different phenolics and organic acids

followed by a decline in their concentrations. This pattern of variation of phenolics and organic acids could be due to their catabolism, their participation in the synthesis of new metabolites, or to berry enlargement. In the green stage malic acid, tartaric acid, fumaric acid, quercetin glucoside, and caftaric acid, and in the veraison stage citric acid was found in relatively high concentration. Coutaric acid was found nearly in equal amounts in both stages. The ripe and harvest stages were found to contain considerable quantities of valine, catechin, GABA, glucose, and fructose. Valine was present in high levels in the ripe stage while glucose, fructose, GABA, and catechin were increased in the harvest stage of berry development.

Discussion

Biochemistry of grape berry development

The time required for the growth of berries in *Vitis vinifera* has been defined differently by different authors. As a grape berry grows, it goes through a complex series of physical and biochemical changes including modifications in size, composition, color, texture, flavor, and pathogen resistance. The growth pattern of a developing grape berry exhibits a double sigmoid growth pattern (Figure 1B) which can be divided into three phases (Coombe 1992). At the beginning (phase I), the berry growth is rapid, mainly because of cell division followed by cell expansion. The biosynthesis of compounds such as malate, tartarate, tannins, and hydroxycinnamates, occur mostly during this phase and reach maximum concentrations around 60 days after flowering (Possner and Kliewer 1985). Phase II which comes 7-10 weeks after flowering is considered as the lag phase of the growth. The length of this phase is specific for each cultivar type. Whilst there is no increase in berry size during this phase, it has been postulated that sugar accumulation starts at this time (Coombe 1992). Phase II is followed by another sigmoid curve which starts with the onset of ripening (veraison). In the third phase, the berries undergo most dramatic changes in composition and morphology. The berry size is doubled, with initiation of color development (anthocyanin accumulation in red grapes), along with increase in sweetness (mainly glucose and fructose) and decline in acidity. A large number of aroma and flavor compounds have already been synthesized by the end of this phase. At the time of harvest, the acid:sugar ratio along with the phenolics content of the berry is of utmost importance because of the role of these

compounds in berry taste and ultimately quality of wine. In the following sections, the metabolic fate of these compounds during berry development will be discussed, supported by the findings of the present study.

Sugars in grape berry

As mentioned above, sugar concentration is often used as an indicator for the assessment of ripeness and also to mark the harvesting time. In grapevines, carbohydrates (mainly sucrose) are produced by photosynthesis in leaves and then transported to the berries via phloem (Swanson and Elshishiny 1958). After this transport, any change in sugar concentration is mainly due to water loss. Apart from being a carbon and energy source, altered sugar concentration is important as a regulatory signal for gene expression (Conde et al. 2006). The existence of a positive correlation between sugar and pathogen related proteins accumulation during berry development is a clear indication of the participation of sugars in pathogen resistance mechanisms (Salzman et al. 1998).

In contrast to organic acids, the accumulation of hexose sugars (glucose and fructose) begins during phase II and continues from then on. This accumulation mainly occurs in the vacuole in the form of fructose and glucose after enzymatic breakdown of the sucrose. These sugars are then transferred to different cell organelles by monosaccharides transporters (Conde et al. 2006). Our data confirms the previous findings as shown in Fig. 5. Sucrose was the major sugar in the green stage while glucose and fructose seems to be accumulated in the later ripe and harvest stages.

Organic acids in grape berry

As mentioned earlier, the ratio of sugar:acid is of key importance in determining grape quality and plays an important role in stability of wine. The two main organic acids in grapes are tartaric acid and malic acid which together account for 69 to 92% of all organic acids present in grapevine leaves and berries (Kliewer 1966). These acids are produced from different biosynthetic pathways. Whilst tartaric acid is synthesized from ascorbic acid (Loewus 1999), and in malic acid synthesis, β-carboxylation of phosphoenolpyruvate (PEP) is the critical pathway. It has been suggested that malic acid may either be transformed into glucose and fructose or used as a carbon and energy

source. In grape berry, both the tartaric and malic acid syntheses have been reported to occur until veraison declining in the later stages (de Bolt et al. 2006). Our results are in accordance with these findings, since as can be observed in Figure 6 the highest levels of different organic acids including citric, fumaric, tartaric acid, and malic acid were detected in the green and veraison stages and decreased in ripe and harvest stages. It is interesting to note that malate is higher in Trincadeira whereas citriate is increased in the other two varieties. Together with a different content in sugars, these results highlight the different primary metabolism of Trincadeira.

Phenolics in grape berry

Phenolics are secondary metabolites of high importance. These compounds are known to play key roles in determining the quality traits of grapes and wine as they contribute to the wine color, flavor, astringency, and bitterness (Chamkha et al. 2003). Many of them are also involved in plant protection and are known to have anti-growth activities against pathogens. As most of them also have strong antioxidant activity, they are also important for human health. The phenolics that have been reported in grape berries are tannins, flavan-3-ols, anthocyanins, hydroxycinnamates, and flavonols (Ali et al. 2010). Accumulation of anthocyanins starts at veraison and is one of the main signs of berry ripening along with sugar level increment. Previous studies demonstrated that the condensed tannins are synthesized very early in berry development and very little change in tannin level has been observed from veraison to harvest (Harbertson et al. 2002). Although the total hydroxycinnamates showed different concentration levels in different *Vitis vinifera* cultivars, ranging from 16-430 mg/L (Singleton et al. 1986), they follow the same pattern consisting in maximum levels prior to veraison and then a decline in the later stages. This decline may reflect the catabolism of these phenolics, their utilization in the synthesis of other compounds (anthocyanins and/or flavonoids) or dilution due to berry enlargement. Our study on the berries showed similar findings. The hydroxycinnamates, caftaric and coutaric acid were found to accumulate in the early stages of development followed by a sharp decline after veraison. The levels of quercetin glucoside also followed the same pattern possibly due to the utilization of its precursors (dihydrokaempferol and/or dihydroquercetin) in the production of anthocyanins.

Conclusions

Grape berries are undoubtedly among the most important fruit species because of their use in wine making. Grape biochemistry shows a great diversity in terms of structure and function ranging from simple amino acids and sugars to highly complex polymers of condensed tannins. The understanding of grape berry development and the metabolic fate of different classes of compounds is imperative in order to control and improve different quality traits of grapes and ultimately wine flavor. Since grape berries mimic a complicated chemical factory, the analytical tools used to understand this complex fruit require some special characteristics. In the work presented here, ^{1}H NMR has proved to be a very effective tool to fully reveal the metabolic composition of complex tissues. With the use of different multivariate data analyses, such as principal component analysis (PCA) and projections to latent structures-discriminant analysis (PLS-DA), associated to ^{1}H NMR some genuine differences among the different development stages and among the cultivars used in this study have been detected. The different stages of grape development mainly differ in their phenolic profile along with significant fluctuations in organic acid and sugar contents. In the light of the results obtained from this research it can be concluded that the initial stages, green and veraison, are metabolically very different from ripe and harvest. Organic acids such as malic acid together with the phenolics like caftaric acid and quercetin glucoside were highly accumulated in berries during early stages followed by an increase in glucose, fructose, and catechin during the later stages. Veraison was proved to be the key stage since during this stage the grape berries undergo some dramatic metabolic changes. All of these findings were found to be in accordance with previous reports. The technique applied here is highly reproducible and effective in analyzing a wide range of compounds of the grape metabolome. With the emergence of new analytical tools, with more sensitivity and precision, our understanding regarding the physiology of grape development will certainly increase as there is still a lot to be understood at the level of genomics, proteomics, and especially metabolomics.