NMR spectroscopy and chemometrics-based analysis of grapevine

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CHAPTER 4

Pre-analytical method for NMR-based grapes metabolic fingerprinting and chemometrics

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Abstract

Different pre-analytical methods were compared and used for the metabolic profiling of five grape cultivars from Portugal in combination with multivariate data analyses. The extraction used with deuterated water and methanol was found effective in the case of amino acids, organic acids, and sugars. For secondary metabolites like phenolics, solid phase extraction with C-18 cartridges showed good results. Principal component analysis, in combination with NMR spectroscopy, was applied and showed clear distinction among the cultivars. Primary metabolites like choline, sucrose, and leucine were found discriminating for 'Alvarinho', while elevated levels of alanine, valine, and acetate were found in 'Arinto' (white varieties). Among the red cultivars, higher signals for citrate and γ-amino butyric acid (GABA) in 'Touriga Nacional', succinate and fumarate in 'Aragonês', and malate, ascorbate, fructose and glucose in 'Trincadeira', were observed. Based on the phenolic profile, 'Arinto' was found with higher levels of phenolics as compared to 'Alvarinho'. 'Trincadeira' showed lowest phenolics content while higher levels of flavonoids and phenylpropanoids were found in 'Aragonês' and 'Touriga Nacional', respectively. It is shown that the metabolite composition of the extract is highly affected by the extraction procedure and this consideration has to be taken in account for metabolomics studies.

Introduction

The metabolome of a plant tissue is highly complex and consists of a variety of different chemicals with different functions in the plant's physiology. This great chemical diversity makes any metabolome analysis unlikely to generate information regarding all the metabolites. For metabolome analysis, it is generally preferred that the method should be unbiased, rapid, reproducible, and stable over time with easy sample preparation. Many platforms are available for the high throughput analysis of plant metabolites, varying in their selectivity and sensitivity. In recent years chemical characterization of an organism's phenotype has become the center of interest for many researchers. Low molecular weight compounds efficiently depict the tissue's or organisms physiological activities, e.g. under certain stress conditions (Kopka et al. 2004).

For metabolomics, an accurate snap shot of the metabolome requires the reliable extraction of metabolites from the biological matrices (Beckonert et al. 2007). A single, high throughput extraction step is often complicated by chemical and physical properties and the diverse range of concentrations at which metabolites exist (Colquhoun 2007). Based on sample chemistry and aim of the research, many extraction protocols for metabolomics studies have been published (Lisec et al. 2006; De Vos et al. 2007; Kruger et al. 2008; Kim et al. 2010a). For samples with special characteristics like high sugar content (grape berries, for instance), the analytical method needs to be combined with a more specific sample preparation in order to detect minor compounds. Solid Phase Extraction (SPE) has been successfully used in many studies (Fraccaroli et al. 2008; Zou et al. 2007; Cai et al. 2003) and is an effective sample handling technique with advantages like high recovery, high pre-concentration factors, low organic solvent consumption, simplicity, and easy operation (Zhao et al. 2007).

Nuclear Magnetic Resonance (NMR) spectroscopy is traditionally considered as a prime tool in identification, characterization, and structure elucidation of molecules. In the metabolomics studies, NMR is now getting very popular (Son et al. 2009b; Zulak et al. 2008). Apart from its low sensitivity, the use of NMR to study the metabolome offers great advantages such as being non-destructive, simple sample preparation, shorter analysis time, and ease of quantitation. Its non-selective nature and no need for any sample pretreatment are the two striking benefits that make NMR an ideal choice for broad range metabolite profiling (Dixon et al. 2006). NMR has been used in metabolic profiling of various types of samples in combination with different multivariate data analyses tools (Belton et al 1998; Brescia et al. 2002; Charlton et al. 2002; Duarte et al. 2002). Most studies on grape berries were focused on the amino acids and sugar contents of grapes as the complexity and low resolution NMR spectra of grape phenolics along with unavailability of reference NMR data hampered the analysis of grape phenolics.

In this study, five different grape cultivars from Portugal were used which include three red cultivars, i.e. ‘Trincadeira’, ‘Touriga Nacional’, and ‘Aragonês’, and two white cultivars, i.e. ‘Alvarinho’ and ‘Arinto’. These varieties are known to be used in the production of traditional Portuguese wines. All the cultivars are from the vintage of 2007. Traditionally Portuguese wines are a blend of three to four different wines but the incomparable characteristics of the studied grape varieties, monovarietal wines are also produced with them. Among the red varieties, ‘Trincadeira’ produces elegant balanced wines with excellent acidity, soft tannins, with intense aromas of plums and blackberries. ‘Touriga Nacional’, one of the most important native varieties in Portugal, known to produce dark and full-bodied wines having similar floral notes as purple violet blossoms. ‘Aragonês’ has fine and delicate aromas of pepper and berries and produces full-bodied, inky, and highly aromatic wines. Among the white varieties, ‘Alvarinho’ is considered one of the noblest white varieties in Portugal; for this reason was chosen for producing the first Portuguese monovarietal wines and is one of the most used varieties for Vinho Verde production. It is cultivated mostly in northwest of Portugal and produces small and little compacted berry clusters. The wines present intense citrus and tropical flavors with fresh acidity. ‘Arinto’, also known as Pedernão, is a late ripening variety that produces small berries giving rise to pale wines, aromatic with green tonality and excellent acidity. This grape variety can be used to produce monovarietal citrus colored wines, intensely floral and fruity when young, with high acidity. This variety is highly productive, assuring a perfect balance between acidity, structure, freshness and minerality. Other than for monovarietal wine production, ‘Arinto’ grapes are also used for blended wines production.

This study is a comparison of already published extraction protocols by our group and a new method based on SPE, for metabolomics. These methods were compared using NMR spectroscopy and multivariate data analyses with the aim to optimize the pre-analytical method for the grape metabolomics. The metabolic characterization of Portuguese grape cultivars based on different extraction methods is also targeted.

Material and Methods

Sampling and extraction

Five elite Portuguese cultivars i.e. three red varieties ‘Trincadeira’, ‘Touriga Nacional’, and ‘Aragonês’, and two white varieties ‘Arinto’ and ‘Alvarinho’ growing in Plansel´vineyards (Montemor-o-Novo, Southern Portugal) were used in this study. Five biological replicates of each cultivar of 80–100 berries from 8 to 10 plants were collected in the 2007 season corresponding to the developmental stage of EL38 (harvest) (EL refers to the modified Eichhorn and Lorenz developmental scale as described by Coombe et al. 1992. Each biological replicate contained berries from a single row of plants. Four rows distant 3–10 m from each other were used for each variety. Plants from the five varieties were growing in the vineyard 15–40 m apart. Berries were frozen immediately in liquid nitrogen and transported to the lab in dry ice. Then seeds were removed and pulp and skin grinded together in liquid nitrogen followed by lypholization for 4 days at -40 ºC.

Four different extraction procedures were compared. In the first procedure (M1), a sample of 100 mg of lyophilized grape berries was mixed with 2 ml of extraction solvent composed of water and methanol in the ratio of 2:8, respectively. At room temperature, the suspension was ultrasonicated for 20 minutes and centrifuged at 3500 rpm at 25°C for 20 minutes. The supernatant was collected in a round bottom flask. The same procedure was repeated two more times and the supernatants were pooled together and evaporated by a rotary evaporator. The dried extract was redissolved in 5 ml of deionized water and then partitioned by adding a similar volume of ethyl acetate. The ethyl acetate extraction was repeated two more times and the extracts were pooled together. The residual water was removed by treating the extract with Na_2SO_4 and then the extract was evaporated using a rotary evaporator and subjected to solid phase extraction.

In the second procedure (M2), 100 mg of dried grape berries was extracted according to Kim et al. (2010a). Briefly, each sample was extracted with 1.5 ml of a mixture of KH_2PO_4 buffer of (pH 6.0) in D_2O containing 0.005% of trimethyl silyl propionic acid sodium salt (w/w) (TMSP) and methanol-d_4 (1:1). The third extraction (M3) was carried out by simply removing D_2O from the second procedure and 100 mg of sample was extracted by only 1.5 ml of deuterated methanol. In the fourth extraction (M4), 100 mg of the dried berries were extracted according to Choi et al. (2005). Briefly, 100 mg of ground material was placed in a centrifuge tube. Then 5mL of a 50% water/methanol mixture and 5 mL of chloroform were added to the tube followed by vortexing for 30 sec and sonication for 1 min. The tube was then centrifuged at 3000 rpm for 20 min. This procedure was repeated twice. Both fractions were dried with a rotary vacuum evaporator. Only the water/methanol fraction was redissolved in 1.5 ml mixture of KH_2PO_4 buffer of (pH 6.0) in D_2O containing 0.005% of trimethyl silyl propionic acid sodium salt (w/w) (TMSP) and methanol-d_4 (1:1) and subjected to NMR spectrscopy.

Solid phase extraction (SPE)

The ethyl acetate extract (from M1) of grape berries was subjected to solid phase extraction (SPE) by using SPE C-18 cartridges (Waters, Milford, MA, USA). The ethyl acetate extract was redissolved in 1 ml of deionized water. The SPE was done by first activating the cartridge with the elution of 10 ml of methanol followed by 10 ml of water. Then the redissolved (not completely) grape berries extract (1 ml of deionized water) was applied to the cartridge and eluted successively with 5 ml of water, and 5 ml of methanol containing 0.01% (v/v) acetic acid. Both elutes were collected in round bottom flasks and evaporated under vacuum. Both methanol (M1m) and water (M1w) fractions were used for further NMR analysis.

^{1}H NMR spectroscopy

The methanol fraction eluted from SPE was redissolved in 1 ml of deuterated solvent containing 1:1 ratio of methanol-d_4 and D_2O (KH_2PO_4 buffer, pH 6.0) with 0.005% TMSP (w/v). An aliquot of 800 µl of sample was transferred to the 5 mm NMR tube. ^{1}H NMR spectra were recorded at 25 °C on a 500 MHz Bruker DMX-500 spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 500.13 MHz. MeOH-d_4 was used as the internal lock. Each ^{1}H NMR spectrum consisted of 128 scans requiring 10 min and 26 s acquisition time with the following parameters: 0.16 Hz/point, pulse width (PW) = 30° (11.3 µs), and relaxation delay (RD) = 1.5 s. A pre-saturation sequence was used to suppress the residual H_2O signal with low power selective irradiation at the H_2O frequency during the recycle delay. FIDs were Fourier transformed with LB = 0.3 Hz. The resulting spectra were manually phased and baseline corrected, and calibrated to TMSP at 0.0 ppm, using XWIN NMR (version 3.5, Bruker). 2D *J*-resolved NMR spectra were acquired using 8 scans per 128 increments for F1 and 8 k for F2 using spectral widths of 5000 Hz in F2 (chemical shift axis) and 66 Hz in F1 (spin–spin coupling constant axis). A 1.5 s relaxation delay was employed, giving a total acquisition time of 56 min. Datasets were zero-filled to 512 points in F1 and both dimensions were multiplied by sine-bell functions (SSB = 0) prior to double complex FT. *J*-resolved spectra tilted by 45°, were symmetrized about F1, and then calibrated, using XWIN NMR (version 3.5, Bruker). ^{1}H–^{1}H correlated spectroscopy (COSY) and heteronuclear multiple bonds coherence (HMBC) spectra were recorded on a 600 MHz Bruker DMX-600 spectrometer (Bruker). The COSY spectra were acquired with 1.0 s relaxation delay, 6361 Hz spectral width in both dimensions. Window function for COSY spectra was sine-bell (SSB = 0). The HSQC spectra were obtained with 1.0 s relaxation delay, 6361 Hz spectral width in F2 and 27,164 Hz in F1. Qsine (SSB = 2.0) was used for the window function of the HSQC. The HMBC spectra were recorded with the same parameters as the HSQC spectra except for 30,183 Hz of spectral width in F2. The optimized coupling constants for HSQC and HMBC were 145 Hz and 8 Hz, respectively.

Data analysis and statistics

The ^{1}H NMR spectra 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 by AMIX software (Bruker). The regions of δ 4.85–4.95 and δ 3.25–3.35 were excluded from the analysis because of the residual signal of D_2O and CD_3OD, respectively. Principal component analysis (PCA) with scaling based on Pareto was performed with the SIMCA-P+ software (v. 12.0, Umetrics, Umea, Sweden).

Results

Visual analysis of ^{1}H NMR spectra

A representative ^{1}H NMR spectrum can be divided into three distinct regions. Figure 1 shows these three regions of the NMR spectra of the grape berries, extracted according to the different procedures as previously explained. It can be easily observed that M2, M3, and M4 had resulted in more or less similar ^{1}H NMR spectra. The results showed that these extracts were dominated by high concentrations of primary metabolites like amino acids, sugars, and organic acids. It is also obvious from the figure that, apart from the methanol fraction of SPE (M1m), all the other extractions methods for grape berries showed low signal intensity in the phenolic region. The methanol fraction of SPE was also found with good signal intensity for amino and organic acids. Since the low intensity of phenolics signals hampered in their identification, SPE proved to be a suitable alternative with single step separation of sugars from the crude extract of berries. It was also observed, in ^{1}H NMR spectra (not shown), that water elution effectively washes out most of the sugars while the methanol fraction was rich in phenolics showing high signal intensity and enabling relatively easy identification. All the extracts from different extraction methods were used for 2D NMR analyses and metabolite identification.

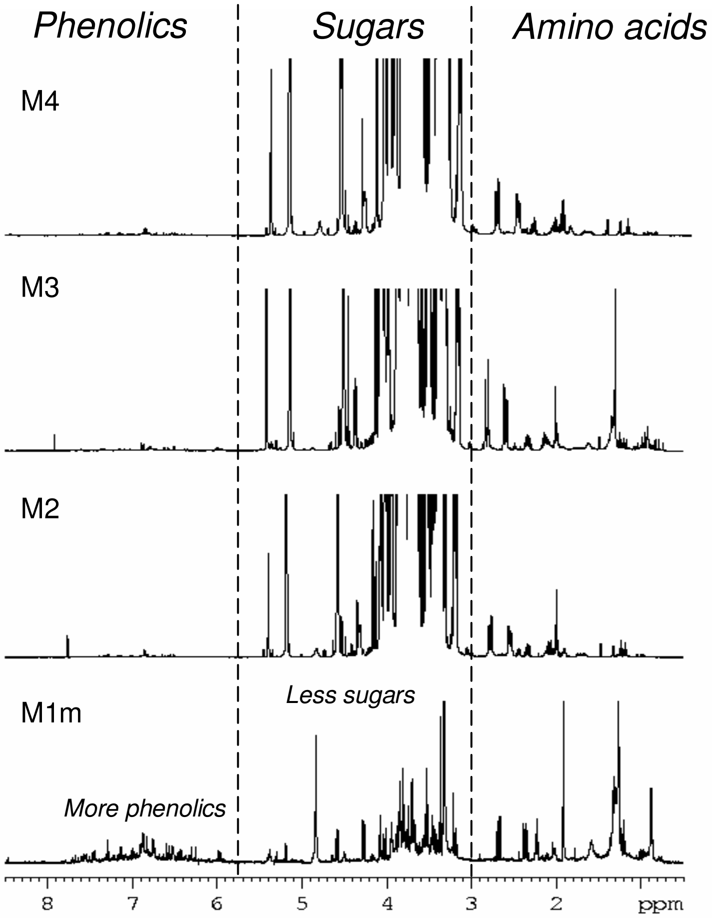

Figure 1. Comparison of ^{1}H NMR spectra resulting from different extraction protocols.

Metabolite identification

Apart from low sensitivity, signal overlapping is another major problem in metabolite identification in NMR based metabolomics. Particularly, with the samples like berries which are characterized by a very high sugar content. This problem is usually overcome by the use of different 2D techniques like *J*-resolved, ^{1}H-^{1}H COSY, HMBC, and HSQC, which provides extra information regarding compound structure. 1D ^{1}H NMR spectroscopy in combination with different 2D techniques of grape berries resulted in the identification of more than twenty five metabolites which covers a wide range of structural and functional diversity. The identified compounds belong to different classes like amino acids, organic acids, carbohydrates, hydroxycinnamates, hydroxybenzoates, and flavonoids. All of the NMR signal assignments discussed in detail in Chapter 5 (next chapter) were done by comparing the 1D and 2D NMR spectra of common metabolites in our in-house library.

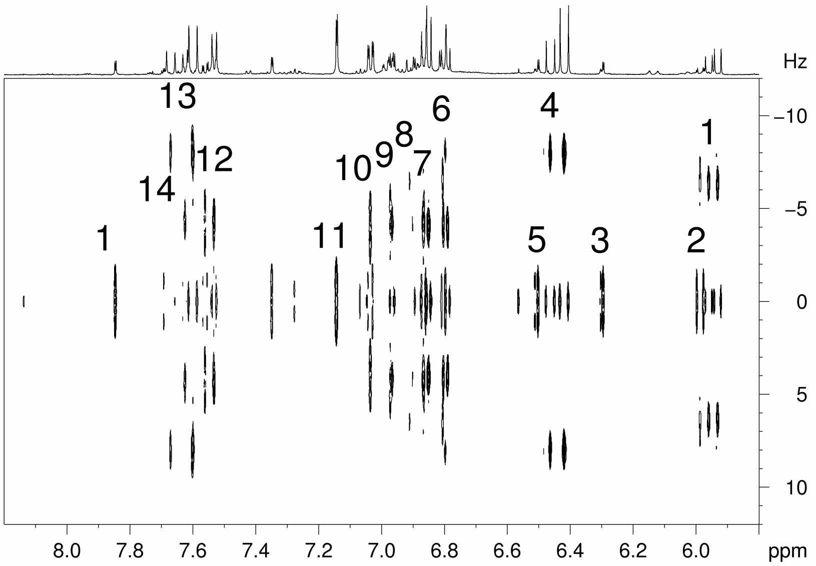

Figure 2. *J*-resolved spectra of 'Trincadeira' variety from δ 5.8-8.2. Figure shows signals like 1, H-8' of *c*-caftaric and coutaric acid; 2, H-6 of catechin and epicatechin; 3, H-6 of quercetin and myricetin; 4, H-8' of *t*-caftaric and coutaric acid; 5, H-8 of quercetin and myricetin; 6, H-7' of *c*-caftaric acid; 7, H-3 of *t*-coutaric acid; 8, H-5 of *t*-caftaric acid; 9, H-5' of quercetin; 10, H-6 of *t*-caftaric acid; 11, H-2 of *t*-caftaric acid; 12, H-6' of quercetin; 13, H-7' of *t*-caftaric and coutaric acid; 14, H-2 and H-6 of *c*-coutaric acid; and 15, H-6' of quercetin.

Amino acids, sugars, and organic acids

A number of amino acids have been identified due to high signal intensity in that region. Resonances for alanine, valine, leucine, proline, and methionine, have been observed and assigned. Although the region in ^{1}H NMR spectra corresponding to carbohydrates is highly clustered and overlapped, the signals for the anomeric protons of β-glucose at δ 4.58, α-glucose at δ 5.17, and sucrose at δ 5.39 can be observed. Many organic acids like succinic acid at δ 2.53, fumaric acid at δ 6.52, formic acid at δ 8.45, citric acid at δ 2.56, and δ 2.74, malic acid at δ 2.68, δ 2.78, and δ 4.34, and tartaric acid at δ 4.35, have also been identified in the grape berries.

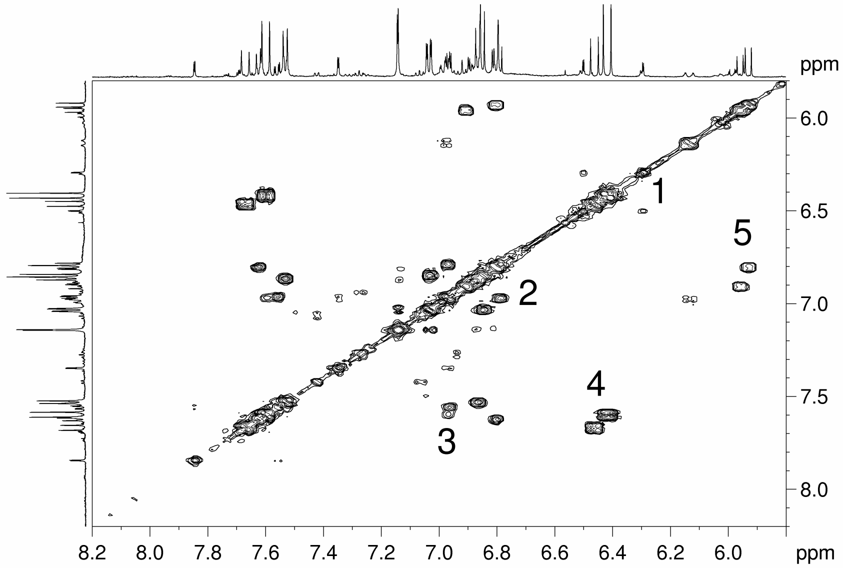

Figure 3. ^{1}H-^{1}H COSY spectra of 'Trincadeira' variety from δ 5.8-8.2. Figure shows correlations among the signals of H-6 with H-8 (1) and H-5' with H-6' (3) of quercetin-3-*O*-glucoside; H-5 and H-6 of *t*- and *c*-caftaric and coutaric acid; H-8' and H-7' (4, 5) of *t*- and *c*-caftaric and coutaric acid, respectively.

Flavonols and flavan-3-ols

Signals for flavonols like quercetin and myricetin were identified in the phenolic region of the ^{1}H NMR spectra. The ^{1}H-^{1}H COSY spectrum showed correlations among the quercetin signals (Figure 3). The resonance at *δ* 6.49 of H-8 was correlated with the signal at *δ* 6.27 of H-6, and a signal at *δ* 6.95 of H-5′ with *δ* 7.56 of H-6′. Similar correlations were also observed for the signals of myricetin at δ 6.51 of H-8 with δ 6.28 of H-6 in the ^{1}H-^{1}H COSY spectrum. The upfield shift of the C-3 signal (around δ 134.0) in the HMBC spectrum of quercetin illustrates the binding of a glucose molecule to quercetin resulting in the identification of quercetin-3-*O*-glucoside. Compounds like (+)- catechin and (-)- epicatechin were also identified in the phenolic region.

Phenylpropanoids

The *J*-resolved spectrum of the aromatic part shows the presence of characteristic doublets with a coupling constant of 16.0 Hz, which represent the H-8′ and H-7′ (olefinic protons) of *trans*-hydroxycinnamic acids (HCA), in the range of δ 6.39-6.50 and δ 7.59-7.70, respectively (Figure 2). These assignments were confirmed by the ^{1}H-^{1}H COSY spectrum (Figure 3) which showed correlations between these two protons. The HMBC spectrum also provides evidence by showing the coupling of H-8′ with the carbonyl carbon at δ 168.3 of the ^{13}C spectrum. The HCAs moieties identified in grape berries are *trans*-caffeoyl, and *trans*-coumaroyl derivatives. These derivatives were found to be conjugated with tartaric acid via an ester linkage. The singlets of tartaric acid are downfield shifted to δ 5.32-5.44 from the typical tartaric acid signal shift at δ 4.80 due to their bonding to the carboxylic function of cinnamic acids. This conjugation was also confirmed by their correlation with the signal at δ 167.5-168.5 in the HMBC spectra. Based on these assignments, these compounds were identified as *trans*-caftaric acid (caffeic acid conjugated tartaric acid), and *trans*-coutaric acid (coumaric acid conjugated tartaric acid).

The *cis*- forms of these conjugated cinnamic acids, i.e. *cis*-caftaric acid and *cis*-coutaric acid, were also detected along with their *trans*- forms. The H-8′ and H-7′ in the *cis*-configuration showed signals shifted upfield to around δ 5.90 and δ 6.85, respectively, along with the reduction in coupling constants to 13.0 Hz (Figure 2). The coupling of H-8′ and H-7′ in the ^{1}H-^{1}H COSY spectra (Figure 3) along with the correlation with the carbonyl carbon resonance at δ 167.2 in the HMBC spectra confirmed this identification.

Apart from HCAs, different hydroxybenzoic acids (HBA) were also identified in grape berries. The aromatic part of the ^{1}H NMR spectra also showed some signals of HBA like gallic acid and syringic acid.

Multivariate data analysis

Principal component analysis (PCA) is a clustering method used to reduce the dimensionality of a multivariate dataset. It is an unbiased and unsupervised method, in which the principal components can be presented in a graphical form and any grouping or outliers among the samples can be easily observed in a score plot of PCA. The corresponding loadings plot actually shows the variables (NMR chemical shifts) responsible for separation or grouping on the score plot and by identifying these variables with the help of 1D and 2D NMR, the identification of the responsible metabolites can be made. To highlight the metabolic differences based on grape cultivars and extraction methods, the bucket data were differently arranged. In the first arrangement, all the cultivars are compared based on each extraction method (Figure 4) while in the second arrangement, all the extraction methods are compared based on each cultivar (Figure 5). The ^{1}H NMR data were further subjected to principal component analysis using SIMCA-P+ (ver. 12.0) software but before that data were mean centered and then scaled using Pareto scaling in which the square root of the standard deviation was used as a scaling factor.

Cultivar differentiation and MvDA

In order to underline the metabolic characteristics of each grape cultivar, PCA is applied. The score plots for PCA based on different extraction procedures are shown in Figure 4. A clear distinction among the grape cultivars with tight clustering of the replicates can be easily observed suggesting metabolic differences among grape varieties and robustness of the extraction procedures, respectively. All the plots showed clear separation of samples from red (‘Trincadeira’, ‘Aragonês’, ‘Touriga Nacional’) and white (‘Alvarinho’, ‘Arinto’) grapes. The PCA score plot of samples extracted with method M3 is shown in Figure 4D. PC1 was found responsible for the separation of red and white grapes. As indicated, samples from ‘Trincadeira’ and ‘Touriga Nacional’ are separated by PC2, having positive and negative PC2 scores, respectively. The ‘Aragonês’ is clustered nearly in between these two cultivars with negative PC2 scores while their PC1 scores are nearly zero. The samples from ‘Alvarinho’ and ‘Arinto’ are grouped on the positive side of PC1 with a slight overlapping.

Samples extracted with method M2 and M4 are projected on PCA score plot in Figure 4C and E. It is evident from the score plots that the samples from all the cultivars followed nearly a similar pattern of clustering. Among the red varieties, ‘Trincadeira’ and ‘Touriga Nacional’ are grouped close to each other (on positive side of PC1) while the remaining ‘Aragonês’ is on the negative side of PC1. The white cultivars (‘Alvarinho’ and ‘Arinto’) are clustered close to each other, on the positive side of PC2.

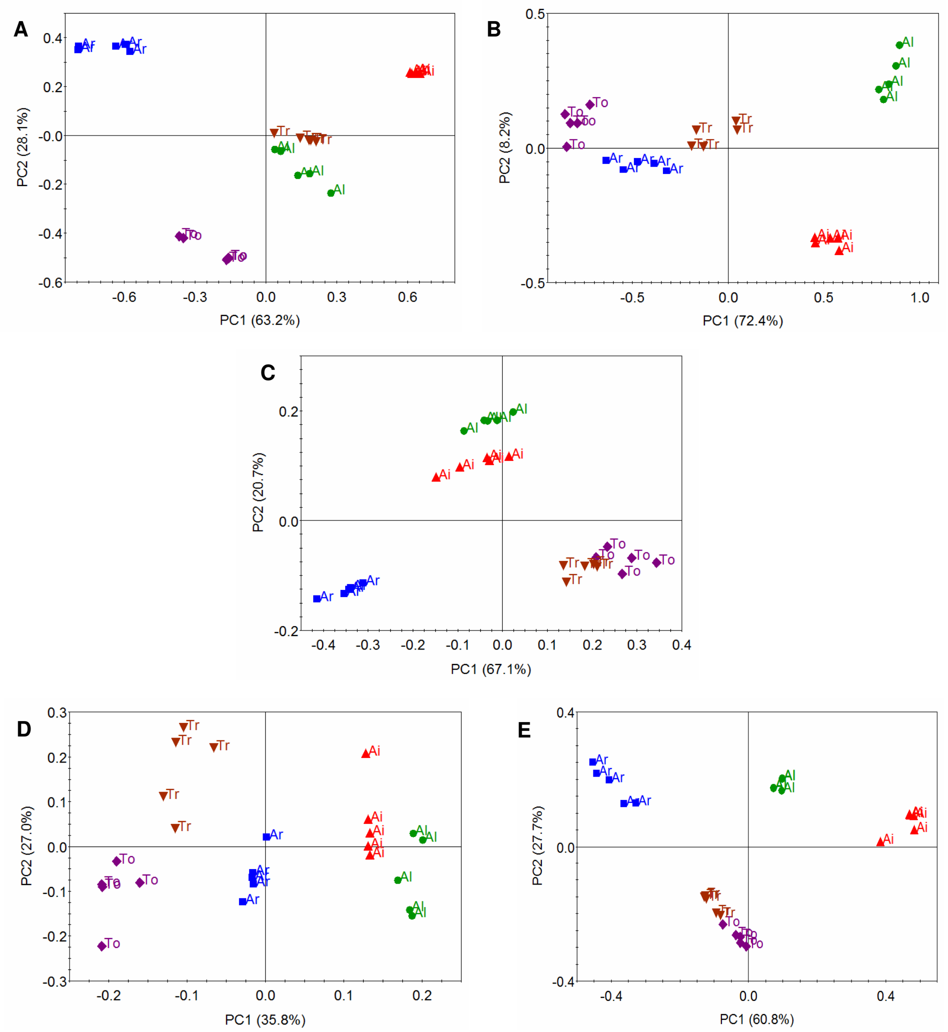

Figure 4. PCA score plots based on ^{1}H NMR data to compare five grape cultivars. Score plot A, B, C, D, and E presents data for extraction method M1w, M1m, M2, M3m and M4, respectively. Codes assigned to each cultivar are ‘Trincadeira’ (Tr), ‘Aragonês’ (Ar), ‘Touriga Nacional’ (To), ‘Alvarinho’ (Al) and ‘Arinto’ (Ai).

The SPE (M1) fractions of the grape samples are projected on PCA score plots (Figure 4A and B). The score plot for the methanol fraction showed separation of red grapes from white grapes by PC1. The white varieties, ‘Alvarinho’ and ‘Arinto’, are separated by PC2 having positive and negative PC2 scores, respectively. The red cultivars are clustered close to each other on the negative side of PC1. The score plot for the water fraction of SPE is the only projection which showed the overlapping of red and white grape samples i.e. 'Trincadeira' and 'Alvarinho'. The other red varieties, 'Aragonês' and 'Touriga Nacional', are on the negative side of PC1, having positive and negative PC2 scores, respectively. Samples from 'Arinto' are on the positive side of PC1 and 2.

The application of different extraction methods enables us to highlight the metabolic differences existing among the different grape cultivars. The ^{1}H NMR spectra revealed that the extracts resulting from M2, M3, and M4, were dominated by primary metabolites (amino acids, sugars, organic acids) which are involved in separation or grouping on the PCA score plots. By examining the loadings plots it was observed that 'Trincadeira' has high signal intensities for malate, ascorbate, glucose, and fructose. 'Aragonês' showed elevated concentrations of succinate, tartarate, and fumarate, while 'Touriga Nacional' was characterized by higher resonances for compounds like citrate, valine, and GABA. Different metabolites were found to be higher in white cultivars, e.g. alanine and acetate for 'Arinto' while elevated resonances for sucrose, glutamate, choline, and leucine were found in 'Alvarinho'. It should be noted that many volatile compounds biosynthetically derive from amino acids (Hernandez-Orte et al. 2006).

As shown by ^{1}H NMR spectra, the extracts from method M1 (which involved SPE) showed more phenolics than any other method used. Hence the PCA based on M1m showed clustering of cultivars mainly based on their phenolic profiles. The major difference among the red and white cultivars is mainly due to the higher phenolics content in red grapes. Among the white cultivars, 'Arinto' was characterized by higher concentration of hydroxybenzoic acid and vanillic acid as compared to 'Alvarinho'. Benzoate is a precursor of important benzenoid compounds such as many volatiles found in aroma of flowers and fruits. Content in benzenoids is high in 'Arinto' together with terpenes (Cabrita et al. 2006), which is, in fact, a variety known to provide both citrus and tropical aromas and flavors. 'Trincadeira' was found with the lowest phenolics concentration among the red cultivars which is in agreement with the results of Cabrita et al. (2006). The difference between 'Aragonês' and 'Touriga Nacional' is mainly based on the elevated signals of flavonoids and phenylpropanoids, respectively.

Both ‘Aragonês’ and ‘Touriga Nacional’ are known to give highly aromatic wines though the later one provides a more intense fruity aroma.

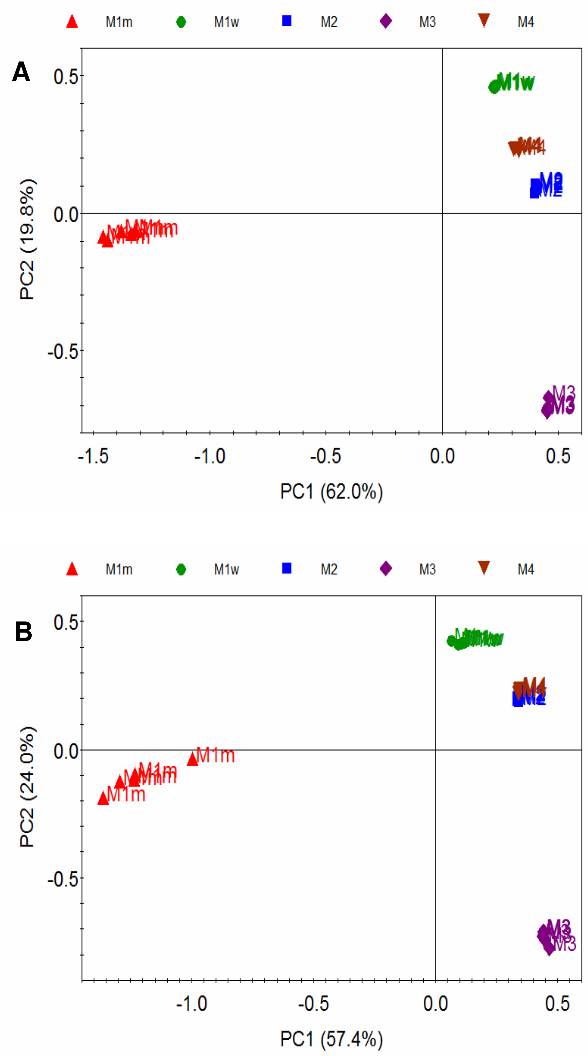

Figure 5. PCA score plots based on 1H NMR data to compare different extraction methods. Score plot (A) presents only one red (‘Trincadeira’) while score plot (B) presents one white (‘Arinto’) cultivar.

Extraction methods and MvDA

With the aim to emphasize the metabolic differences based on extraction methods for each cultivar, PCA is employed and the resulting score plots are shown in Figure 5. As the figure reveals, the extracts and clustered according to the types and proportion of solvents involved in the extraction methods. The replicates show tight clustering indicating the robustness of the methods used. As it can be easily observed, different extracts from the different red and white grape cultivars behaves nearly similar and resulted in a almost analogous PCA projections. The 'Trincadeira' (red) and 'Arinto' (white) grape variety show clustering among the extracts of M2, M4, and M1w. Also in the case of above mentioned cultivars, the extracts from M1m and M3 are not only grouped separately from the others but also from each other (Figure 5). The same is true for the other red and white varieties indicating that regardless the varietal metabolic difference, the extraction methods have resulted in the same clustering on the score plots (not shown in Figure 5).

By examining the corresponding loading plots and ^{1}H NMR spectra from the different extraction methods, differences existed among the metabolite composition of the extracts have been underline. It is evident that for all the grape cultivars, methods M2, M4, and M1w had resulted in similar metabolic contents as the extracts grouped closer to each other on the score plots. Metabolites like glucose, fructose, malate, succinate, choline, glutamate, methionine, proline, alanine, and GABA, are found to be more concentrated in the M2 and M4 extracts, which however contained small amounts of phenolics. The M3 extract is characterized by elevated levels of fatty acids, leucine, valine, threonine, and ascorbate. Phenolics like caftaric acid, coutaric acid, and quercetin-3-*O*-glucoside, are the metabolites found at high level in the methanol fraction of SPE (M1m) whereas the water fraction showed signals of sugars with some amino acids like alanine, methionine, and proline.

Discussion

NMR spectroscopy offers a means of non invasive structural analysis of metabolites in the sample. In the present study, using ^{1}H NMR in combination with several 2D NMR techniques, several key metabolites of the grape metabolome have been identified. The metabolites identified cover a vast structural diversity and include amino acids, organic acids, sugars, flavonoids, hydroxycinnamates, and hydroxybenzoates. This clearly suggests the immense analytical capacity of NMR spectroscopy to analyze diverse metabolites as compared to other platforms for such type of metabolomics studies (Verpoorte et al. 2008).

NMR in combination with multivariate data analyses has been widely used as a fingerprinting tool for plants. The system has proved very effective for the metabolic characterization of not only cultivars but species as well (Ali et al. 2009). Regarding grape berries, many reports have been published related to effects of geography (Son et al. 2009a; Pereira et al. 2005), microclimate influence (Pereira et al. 2006a), and vintage and soil effect on grape metabolome (Pereira et al. 2006b). These reports clearly suggest the enormous potential of this approach in metabolic characterization of plants which in turn can be very useful in explaining different physiological behavior and distinctive characteristics of plants species. In the current study, metabolic characterization of five grape cultivars has been performed which not only resulted in the discrimination of red grapes from white grapes but also underlined the differences among the red and white grape cultivars. The combination of NMR spectroscopy with the multivariate data analysis reported here is found effective and can be useful in the studies involved in metabolic phenotyping.

The methods used and compared in this study are not really suitable for the analysis of non-polar metabolites. Also NMR spectroscopy is not an adequate method for the analysis of volatiles or lipids (non-polar) metabolites (Kim et al. 2010a). In the current study, different extraction methods were also compared and some interesting observations were made. Among the extraction methods, SPE was found very effective in extracting characteristic grape phenolics but less effective in the case of other metabolites. This indicates that SPE could be a better option for the studies targeted towards phenolics as many of the previous reports (Son et al. 2009b; Pereira et al. 2005; 2006a; 2006b) were not as successful in identifying characteristic grape phenolics. This procedure is simple and easy but can be criticized because of longer time required as multiple steps are involved. The method involving deuterated water and methanol (M2) was found effective in the case of other metabolites like organic acids, amino acids, and sugars. This method offers great advantages as it is fast, easy, and able to extract a vast array of metabolites. The drawback of this method is the high signal intensity of sugars that may hamper visualization of less concentrated secondary metabolites. The results explained above indicate the importance of the extraction procedure in metabolomics studies.

Conclusions

Metabolic profiling of five Portuguese cultivars has been performed by using NMR spectroscopy in combination with multivariate data analysis. This approach enable us to highlight the genuine differences among the cultivars as several diverse classes of metabolites were identified by comparing the spectra of analyzed samples to a library of NMR spectra of standards run under identical conditions. While comparing different extraction procedures for grape metabolomics, two potential candidates emerged i.e. SPE and direct extraction using deutrated solvents. SPE was found most effective in the case of less concentrated phenolics while the direct extraction give good results in the case of metabolites like organic acids, amino acids, and sugars. It was shown that distribution of metabolites in the extract was highly influenced by the solvent(s) used for extraction or the solubility or polarity of the metabolites. This information clearly shows the importance of such a comparative analysis before metabolomics study. In drawing conclusions of a single extraction solvent metabolomics analysis, one should always be careful. Differences can be observed for certain compounds, but that are not necessarily the only compound that differ among the samples. For comprehensive profile of differences in levels of metabolites, more than one extraction method should be used. At the same time, the present study shows that it is possible to distinguish grape varieties with a single solvent extraction method.