

¹H NMR-Based Metabonomics for the Classification of Greek Wines According to Variety, Region, and Vintage. Comparison with HPLC Data

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A sensitive and simple method was developed for the classification of wines according to variety, geographical origin, and vintage using NMR-based metabonomics. Polyphenol-rich extracts were prepared from 67 varietal wines from the principal wine-producing regions of Greece, using adsorption resin XAD-4. 1D ¹H NMR spectra obtained from the corresponding extracts were segmented, integrated, and normalized, and the data were subjected to principal component analysis. The chemometric classification of wines according to their phenolic profile allows discrimination between wines from different wineries of the same wine-producing zone and between different vintages for wines of the same variety.

KEYWORDS: Wine classification; NMR; principal component analysis (PCA); HPLC; polyphenols

INTRODUCTION

Wine is an important agricultural product closely related to human history and civilization. Moreover, it is a product of significant commercial value; thus, it has been a target of economic fraud. Adulteration may be practiced by grape juice fortification with sugars and/or colorants to increase alcohol content and color intensity or by mixing high-quality wine produced in restricted areas (denomination of controlled origin, DOC) with wine of reduced quality, often originating from other geographical regions or countries. Therefore, there is significant interest in developing accurate methods for wine characterization that could be used to prevent adulteration and to classify wine from different geographical origins or countries (1, 2). At present, wine analysis is based on a variety of analytical techniques with various degrees of sensitivity and specificity such as high performance liquid chromatography (HPLC), gas chromatography (GC), mass spectrometry (MS), near-infrared spectroscopy (NIR), and nuclear magnetic resonance spectroscopy (NMR) (3, 4). These techniques have often been used in combination in order to obtain maximum information (5, 6). ¹H NMR spectroscopy is a method of choice for assessing complex mixtures because it allows the simultaneous monitoring of a variety of compounds. In combination with multivariate statistical analysis techniques it has been extensively used in metabonomic studies of biofluids and tissues (7), as well as in food classification and origin determination (8–12). Pattern recognition and related multivariate statistical approaches such as the unsupervised principal component analysis (PCA) and the supervised partial least-squares discriminate analysis (PLS-DA) can be used to discern significant patterns in complex data sets and aim at classifying objects by identifying inherent patterns in a set

of indirect measurements (13). In particular, NMR spectroscopy has been applied for grape and wine analysis and classification according to geographical origin, variety, and vintage (6, 9, 14) as well as metabolite evolutions during alcoholic fermentation (15–17). Recently an NMR–PCA method has been reported for the classification of wines based on their primary constituents profile (amino acid and sugar etc.) without any or with minimum sample preparation (10, 18–20). Direct wine analysis often results to chemical shift variations of various metabolite signals due to differences in pH values necessitating peak alignment procedures to be employed (21). On the other hand, there is strong evidence that the phenolic composition of wines can be used as a metabolic fingerprint for the classification of wines according to variety, vintage, and soil (22, 23). The phenolic compounds of wines are not only responsible for some very important organoleptic characteristic of the wine, such as color, astringency, and bitterness, but also possess significant biological properties such as anticarcinogenic, antiviral, and cardioprotective activities (24–26).

The presence of phenolic secondary metabolites in grapes and consequently in wines is strongly affected by a number of factors, such as grape variety, soil, climate, agricultural practices, UV irradiation, weather conditions, infections, and maturation stage (27, 28). Furthermore, wine phenolic composition is influenced by vinification techniques and wine aging. Thus, it is of great interest to investigate whether the phenolic profile of a wine could be used as an index for the classification of wines of a certain variety, geographical region, and vintage.

A facile and efficient extraction of the phenolic compounds of wine is crucial for method development and can be performed by adsorption–desorption processes using highly efficient sorbents such as XAD type resins (29, 30). The chemical structure of the resin material favors adsorption by weak interactions of molecules with moieties of high electron density, such as aromatic

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rings. In contrast, sugars or polar lipids cannot establish this kind of interaction and are eluted with the water flow during the rinsing phase. The adsorbed phenolic compounds can then be recovered by elution with EtOH, giving an enriched extract.

Using of the above procedure we introduce herein a simple and convenient method for the classification of the principal red and white Greek wines, according to variety, region, and vintage, on the basis of their phenolic profile. The method relies on the application of PCA and PLS-DA techniques on the data acquired from the ^1H NMR spectra of the enriched phenolic extracts of wines prepared using adsorption resin XAD-4.

MATERIALS AND METHODS

Reagents. MeOH used for the extraction of polyphenols was purchased from J. T. Baker and it was of HPLC grade. MeOD 99.9% was purchased from Sigma-Aldrich, and distilled water was prepared from a distillation apparatus. Resins XAD-4, XAD-7HP, and XAD-16 were purchased from Rohm and Haas. Reference compounds gallic acid, (+)-catechin, (–)-epicatechin, *p*-coumaric acid, ferulic acid, 4-hydroxybenzoic acid, chlorogenic acid, tryptophol, *trans*-caffeic acid, syringic acid, *trans*-cinamic acid, kaempferol, quercetin, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, and *trans*-resveratrol were purchased from Sigma-Aldrich. *trans*-Caftaric acid was isolated with preparative HPLC and quantified using the *trans*-caffeic acid curve.

Sample Collection. Wine samples were collected from principal Greek grape varieties, red (Agiorgitiko and Mandilaria) and white (Moschofilero and Asyrtiko), cultivated in Nemea region in Peloponnesus and the island of Santorini for two successive vintages, 2005 and 2006. Agiorgitiko variety is grown mainly in the appellation d'origine contrôlée (AOC) region Nemea, producing high-quality red wines. Moschofilero is a distinct aromatic grape variety with pink skin cultivated mainly in the Peloponnesus, producing white wines. Asyrtiko is considered to be the most important Greek white grape variety. It was first cultivated on Santorini, a volcanic island in the southern Aegean Sea with extreme weather conditions and limited rainfall, where it has developed a unique character producing excellent AOC wines. Asyrtiko is also the main constituent of Vinsanto, a naturally sweet wine that has been produced on Santorini since medieval times. Mandilaria is a red grape variety characteristic of the Aegean Sea islands and Crete. It is usually blended with other red grape varieties to produce AOC wines.

Among the varieties studied, Agiorgitiko is of special interest because it is the principal Greek variety producing wines with appellation of origin name, and thus it may be a target of economic fraud. Furthermore, the Nemea region shows a wide geological diversity with vineyard altitudes varying between 200 and 850 m. For this reason an attempt was made to cover the whole area of Nemea to construct the metabolic fingerprint for this type of wine. The variety and origin of the 67 analyzed wines are summarized as follows (see also Table S1 of the Supporting Information): ARN1, Agiorgitiko red wine from Cooperative of Nemea Winery, 2006 vintage ($n = 20$); ARN2, Agiorgitiko red wine from Lafazanis Winery, Nemea, 2005 ($n = 9$); ARN3, Agiorgitiko red wine from Lafazanis Winery, Nemea, 2006 ($n = 4$); ARN4, Agiorgitiko rosé wine from Cooperative of Nemea Winery, 2006 vintage ($n = 2$); MWN1, Moschofilero white wine from Lafazanis Winery, Nemea, 2006 ($n = 3$); MRS1, Mandilaria red wine from Cooperative Santorini, 2005 ($n = 3$); MRS2, Mandilaria red wine from Cooperative Santorini, 2006 ($n = 3$); AWS1, Asyrtiko white wine from Cooperative Santorini, 2005 ($n = 10$); AWS2, Asyrtiko white wine from Cooperative Santorini, 2006 ($n = 13$).

Each sample was collected from a different vinification tank in each winery 5 months after the beginning of the vinification procedure (February 2006 and 2007) before any mixing for commercial use, to ensure representative statistical variance of the variety and geographical origin as well as differences in the vinification procedures. All samples were stored at 2 °C prior to analysis.

Sample Preparation. One hundred and fifty milliliters of each wine sample was diluted with the appropriate volume of distilled water so that the final alcoholic grade was about 6.0% v/v. The corresponding solution was loaded on a glass column, filled with 15 g of XAD resin, previously prepared with sequential passing of 30 mL of EtOH and 30 mL of distilled

water. The flow rate was set at 1.5 mL/min. The column was then washed with water to remove sugars and dried with air. The phenolic fraction was then collected with elution of the column with 60 mL of EtOH at a flow rate of 2.0 mL/min, and the solvent was evaporated under vacuum at 40 °C until dryness. Finally, each extract was dissolved in 700 μL of MeOD for NMR analysis.

^1H NMR Spectroscopy. Spectra were acquired on a Bruker DRX-400 Avance spectrometer using a single 90° pulse experiment with water suppression. Typically, 64 scans were collected into 64K data points over a spectral width of 12000 Hz with a relaxation delay of 5 s and an acquisition time of 2.7 s. Prior to Fourier transformation (FT), the FIDs were zero-filled to 128K and an exponential weighing factor corresponding to a line broadening of 1 Hz was applied. The spectra were phase corrected interactively using XWINNMR. A baseline correction factor was applied to each spectrum using a simple polynomial curve fitting of the mathematical equation $A + Bx + Cx^2 + Dx^3 + Ex^4$. Baseline correction was carried out manually using each time the appropriate factors (31).

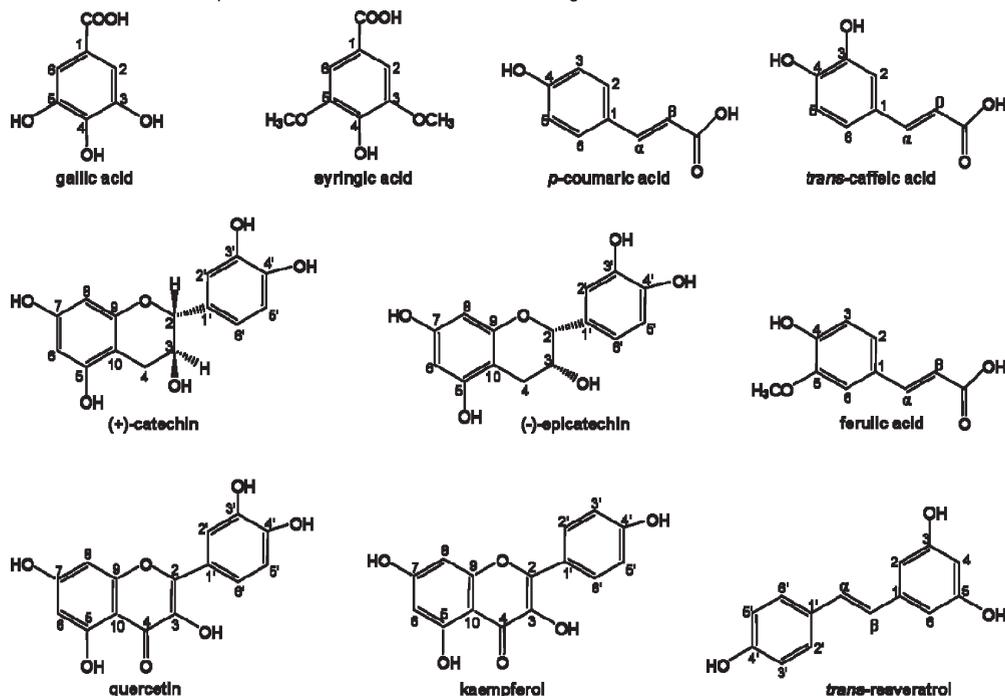
Sample Fortification. Fortification was performed by successive addition of 1.0 mg of 10 pure phenolic compounds shown in **Scheme 1**. ^1H NMR spectra of the corresponding solutions were acquired to assign the corresponding signals in the wine-derived complex phenolic mixture.

Data Reduction of NMR Spectra and Multivariate Analysis. The aromatic region (δ 5.40–10.48) of the wine sample spectra was segmented into 126 chemical shift regions of 0.04 ppm width using the AMIX (Analysis of Mixtures) software package version 2.7 (Bruker Analytische Messtechnik, Karlsruhe, Germany) Each region was integrated and normalized using the total intensity of the aromatic region (δ 5.40–10.48) (31).

Data were further subjected to PCA and PLS-DA (13), using the SIMCA-P 10.5 software package (Umetrics, Umeå, Sweden). Prior to PCA, data were mean-centered and then scaled using both Pareto and unit variance (UV) scaling. Mean-centering implies that the variables are centered, but not scaled. In UV scaling, variables are centered and scaled to unit variance, which means that “long” variables are shrunk and “short” variables are stretched, so that all variables will rest on equal footing using as scaling factor the standard deviation. Pareto scaling is between no scaling and UV scaling using the square root of the standard deviation as the scaling factor.

PCA is a multivariate projection method useful in classifying samples according to their common spectral characteristics. A plot of the first two principal components (scores plot) provides a two-dimensional representation of the information contained in the data set. In addition, a corresponding “loadings plot or coefficient histograms” provides information on the variables that influence any observed clustering of samples. PLS-DA is a supervised method used when clusters are not distinctly separated in the scores plot and groups overlap; PLS attempts to derive latent variables, analogous to PCs, which maximize the covariation between the measured data (X) and the response variable (Y) regressed against. PLS-DA was applied to the groups of samples using NMR spectral data as X matrix and group membership as the response matrix Y . Two different PLS-DA models were applied for further classification of wine samples from each region studied, one for Nemea wines, including all red wines from Agiorgitiko cultivar (ARN1 = class 1; ARN2 = class 2; ARN3 = class 3) (training set I), and the second for Santorini wines, including all white wines from Asyrtiko cultivar for 2005 and 2006 vintages (training set II). The confidence level for membership probability was considered to be 95%; observations at < 5% are considered to be outliers. The overall predictive ability of the model is assessed by cumulative Q^2 representing the fraction of the variation of Y that can be predicted by the model, which was extracted according to the internal cross-validation default method of SIMCA-P software.

HPLC Analysis. HPLC analysis of wines was carried out with the method already described in a previous paper (32). Briefly, wine samples were directly injected into a Thermo Finnigan 3000 chromatographic system equipped with a quaternary pump, an autosampler, a degasser, and a diode array detector (DAD). Polyphenols were separated on a Lichrosphere C18 column (250 mm \times 4.1 mm, particle size = 5 μm) and a C18 guard column of the same type. The mobile phase consisted of solvent A (2 mM sodium acetate aqueous solution with 3% v/v acetic acid) and solvent B (ACN). Run time was 70 min, and the flow was 1.0 mL/min. The injection volume was 20 μL , and polyphenols were eluted using a gradient

Scheme 1. Structures of the Phenolic Compounds Identified in Wine Extracts through the Fortification Process**Table 1.** Properties of the Tested Adsorption Resins

resin	XAD-4	XAD-7HP	XAD-16
copolymerisate	styrene– divinylbenzene	acrylate– divinylbenzene	styrene– divinylbenzene
specific surface ($\text{m}^2 \text{g}^{-1}$)	750	450	750
porosity (cm cm^{-3})	0.65–0.70	0.55	0.58–0.63
bulk density (g m^{-3})	0.62–0.63	0.62	0.61
particle size (mm)	0.3–1.2	0.3–1.2	0.3–1.2

system. The analysis was monitored at 280, 320, and 360 nm simultaneously. Peaks were identified by comparing their retention time and UV–vis spectra with those of reference compounds, and data were quantified using the corresponding curves of the reference compounds as standards. All standards were dissolved in synthetic wine matrix consisting of a $\text{H}_2\text{O}/\text{EtOH}$ (85:15) solution with 0.3% w/v tartaric acid. Results were expressed in milligrams per liter of wine.

Identification of Pure Compounds. *trans*-Caftaric acid was unambiguously characterized on the basis of its physical and spectral data, as previously described (32).

Statistical Analysis. Statistical analysis was performed using Statistica 7.0. Differences between HPLC results were located using a *t* test, and significance was determined at $p < 0.05$. HPLC data were reported as the mean \pm SD of the individually analyzed samples.

RESULTS AND DISCUSSION

Extraction Method. Three resins, namely, XAD-4, XAD-7HP, and XAD-16, were examined for their ability to adsorb low molecular mass polyphenols. The optimum conditions for polyphenol extraction were first determined by evaluating the quality of the aromatic part of the corresponding NMR spectrum of the extract. The properties of the adsorbents are presented in Table 1.

The ^1H NMR spectra of the wine extracts prepared from the three different resin types (Figure 1S of the Supporting Information) revealed that the sample obtained from XAD-4 extraction showed an aromatic spectral area with distinctively sharper peaks and better signal-to-noise ratio compared to the spectra from XAD-7HP and XAD-16. The lower signal width observed for the XAD-4 extract resulted in significantly less overlapping and better resolution. Spectra from both XAD-7HP and XAD-16

samples are characterized by broad signals in the baseline due probably to polymeric species. XAD-4 has been successfully used in the past for the recovery and isolation of phenolic compounds from plant materials (29,30). Our results demonstrate that XAD-4 resin is more selective for low molecular weight polyphenols than the other two resins examined, producing enriched phenolic extracts of high purity.

These properties make XAD-4 particularly useful for sample preparation from white wines, which have a significantly lower phenolic concentration than red ones.

At this point it should be noted that the alternative minimum sample preparation with no extraction and straightforward freeze-drying results in spectra dominated by the carbohydrate signals, whereas the aromatic area resonances are very weak and relatively broad (Figure 1S,D of the Supporting Information). Furthermore, as has previously been stated, the conditions of the freeze-drying method are not easily controlled, resulting in low reproducibility (33).

Therefore, XAD-4 resin was selected for the preparation of all wine extracts with the aforementioned procedure.

^1H NMR Spectra and Multivariate Analysis. The aromatic region (δ 5.8–8.10) of the ^1H NMR spectra of wine extracts exhibits characteristic signals arising from the phenolic content of the wines as shown in Figure 1. Due to signal overlapping, assignment of the resonances was possible only for the major phenolic compounds after fortification. Detailed assignment of the various protons of the reference compounds (Scheme 1) in the spectra is provided as Supporting Information and is summarized in Table 2.

The aromatic area of ^1H NMR spectra (δ 5.40–10.48) was segmented, integrated, and normalized, and the data were subjected to PCA. Data were centered and scaled using UV or Pareto scaling. The latter resulted in the most adequate models, and the analyses presented hereafter were all performed after Pareto scaling.

Initially, PCA was performed over all of the wine samples and revealed a quite clear discrimination between samples of different cultivars and geographical origins. The PC1/PC2 scores plot

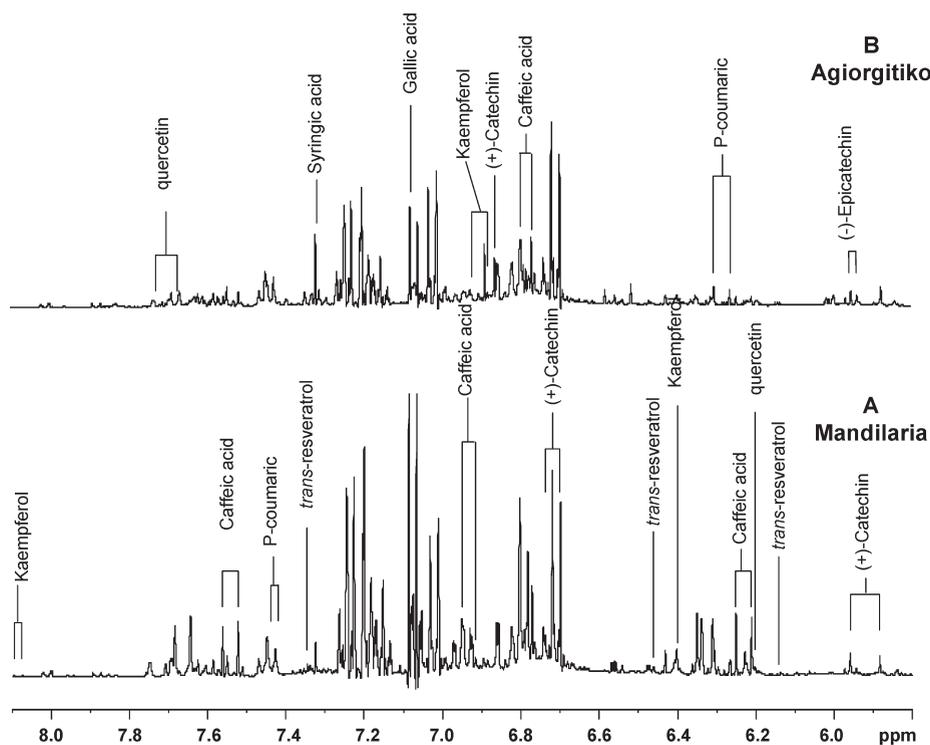


Figure 1. Representative 400 MHz ^1H NMR spectra (δ 5.80–8.10) of (A) Mandilaria and (B) Agiorgitiko wines along with the assignment of resonances resulting following spiking of 10 standard polyphenols. (For resolution reasons the spectra presented were processed using Gaussian multiplication of the FID (LB = -2, GB = 0.1 prior to Fourier transform).)

Table 2. ^1H NMR Chemical Shifts of the Phenolic Components Detected in Wines^a

no.	compound	$\delta^1\text{H}$ (multiplicity, assignment)
1	gallic acid	7.08 (s, H2, H6)
2	(+)-catechin	6.84 (d, H2'), 6.76 (d, H5'), 6.72 (dd, H6'), 5.93 (d, H6), 5.85 (d, H8), 4.57 (d, H2), 3.98 (td, H3), 2.85 (dd, H4ax), 2.50 (dd, H4eq)
3	(-)-epicatechin	6.97 (d, H2'), 6.79 (m, H5'), 6.77 (m, H6'), 5.94 (d, H6), 5.92 (d, H8), 4.81 (s, H2), 4.18 (m, H3), 2.85 (dd, H4ax), 2.74 (dd, H4eq)
4	quercetin	7.74 (d, H2'), 7.64 (dd, H6'), 6.89 (d, H5'), 6.40 (d, H8), 6.19 (d, H6)
5	kaempferol	8.08 (d, H2', H6'), 6.91 (d, H3' H5'), 6.41 (d, H8), 6.18 (d, H6)
6	trans-caffeic acid	7.53 (d, H- α), 6.22 (d, H- β), 7.04 (d, H2), 6.93 (dd, 6), 6.78 (d, H5)
7	p-coumaric acid	7.60 (d, H- α), 6.28 (d, H- β), 7.45 (d, H2, H6), 6.80 (d, H3, H5)
8	trans-resveratrol	7.35 (d, H2', H6'), 6.45 (d, H2, H6), 6.16 (t, H4)
9	syringic acid	7.32 (s, H2, H6), 3.87 (s, OMe3, OMe5)
10	ferulic acid	7.67 (d, H- α), 6.47 (d, H- β), 7.17 (d, H2), 7.38 (dd, H3), 7.53 (d, H6), 3.90 (s, OMe5)

^as, singlet; d, doublet; dd, doublet of doublet; m, multiplet; td, triplet of doublet.

(Figure 2) shows that four major distinct clusters are formed corresponding to the four different varieties studied. Moreover, a clear separation between the two geographical regions of production is observed: on the left side of the plot, wines produced in the appellation of Nemea are positioned, whereas on the right side, wines from Santorini Island are located. On the other hand, discrimination between red and white wines is also possible, with white wines being placed on the upper right part of the scores plot. Moreover, the rosé wines produced in Nemea from the Agiorgitiko cultivar are placed between the white and red wines from the same region. Wines from the Mandilaria cultivar also show a tendency to separate according to vintage, although the number of samples is limited.

To examine the validity of the method, four different wine extracts were prepared separately from the same Mandilaria–Santorini initial wine sample. Their spectroscopic data were analyzed along with all other samples, and their final position on the PCA scores plot was identical, suggesting a satisfying reproducibility of the method (data not shown).

Examination of the loadings plot suggested that the variables referred to the resonances of (+)-catechin, gallic acid, syringic

acid, (-)-epicatechin, quercetin, trans-resveratrol, p-coumaric acid, and trans-caffeic acid contributed to the discrimination of wines (see Supporting Information Figure 2S).

In a second step the possibility to discriminate between wineeries and vintage years was further examined. Figure 3A presents the PCA of the Nemea Agiorgitiko red wines studied. A tendency to separate wines from the two different wineeries is observed. Most of the ARN1 samples are placed on the right side of the vertical line representing PC2, whereas ARN2 and ARN3 wines are placed on the left side. Simple PCA comparing the two vintage years 2005 and 2006 did not result in discrimination between the two groups (data not shown). Further PLS-DA was applied for these data, giving a complete separation between 2005 and 2006 vintages ($Q^2 = 0.55$) (Figure 3B). The PLS-DA model was further validated using a test set consisting of four ARN1 and three ARN2 samples. All test set samples were correctly assigned, validating the discriminant model. The 2005 vintage was characterized by heavy rainfall in the appellation of Nemea during harvest time, resulting in overhydration of grape berries and thus lower concentration of polyphenols, which could be a possible explanation for the separation of the vintages.

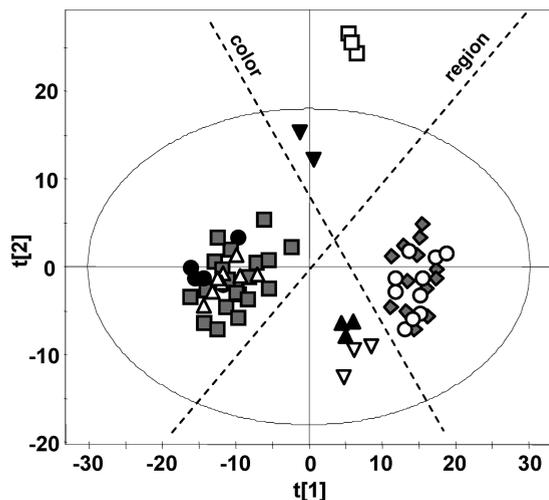


Figure 2. PCA scores plots derived from ^1H NMR spectra of extracts of red and white wines from Santorini Island and Nemea region: (■) ARN1; (Δ) ARN2; (●) ARN3; (∇) ARN4; (□) MWN1; (\blacktriangle) MRS1; (∇) MRS2; (○) AWS1; (\blacklozenge) AWS2.

Similarly, PLS-DA was applied for data obtained from Asyrtiko wines, also resulting in a complete separation between 2005 and 2006 vintages ($Q^2 = 0.49$) (Figure 3C). The PLS-DA model was further validated using a test set of three AWS1 and three AWS2 samples. All test set samples were correctly assigned apart from a sample from 2006 vintage, which was placed close to the 2005 samples.

To clarify the discrimination reasons between the different cultivars, PCA was also applied between Agiorgitiko and Mandilaria (red) wines (data not shown). In the first plot the PC2 axis separates wines according to color and region. Examination of the loadings suggests that the separation of Mandilaria and Agiorgitiko wines is achieved due to spectral domains belonging to the resonances of gallic acid, syringic acid (δ 7.32), ($-$)-epicatechin, *trans*-caffeic acid, and other unidentified phenolic compounds. Further studies for the identification of the type and structure of the polyphenolic compounds present in wine are in progress using LC-NMR-MS analysis.

HPLC Analysis. The average concentration for the phenolic compounds identified in each wine group for the 2006 vintage group using HPLC-DAD is presented in Table 3. Among the major polyphenols detected in wines was the monomeric flavanol (+)-catechin, averaging between 55.39 ± 18.86 and 70.98 ± 4.07 mg/L for red wines and between $4.01 (\pm 0.57)$ and $16.92 (\pm 2.61)$ mg/L for white wines. Its isomeric form, ($-$)-epicatechin, was also detected but in lower concentrations. All red wines (ARN1 and MRS2) were rich in (+)-catechin and ($-$)-epicatechin, with no significant differences in their concentrations ($p > 0.05$). On the contrary, significantly higher concentrations of these flavanols were detected in the white wines from Santorini (AWS2), compared to the white wines from Nemea region (MWN1) ($p < 0.05$), with (+)-catechin levels being almost 2-fold higher than in rosé wines (ARN4) ($p < 0.05$).

Among the hydroxybenzoates, gallic acid was the most abundant phenolic acid in wines and the phenolic compound with the highest concentration in general. In Mandilaria wines, in particular, the average concentration of gallic acid was 102.00 ± 4.72 mg/L. Syringic acid was present in lower concentrations with the higher concentration being observed for ARN1 wines (5.84 ± 1.20 mg/L), whereas no significant differences were found for ARN3, ARN4, and MRS2 wines ($p > 0.05$).

From the hydroxycinnamates, caffeic acid and its ester caffeoyl-tartaric acid (*trans*-caftaric acid) were detected in considerable

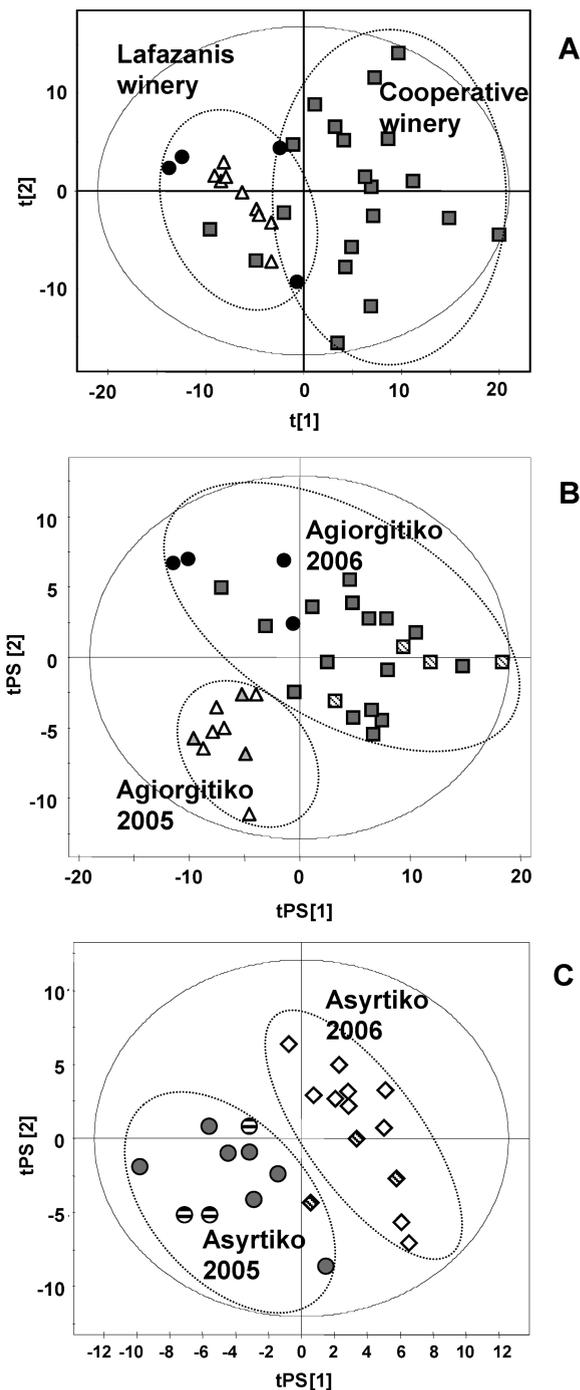


Figure 3. Multivariate analysis plots exhibiting classification of samples according to winery and vintage year: (A) PCA scores plots derived from ^1H NMR spectra of red wine (Agiorgitiko) extracts produced in Nemea region (clustering of samples according to wineries is shown by dashed line ellipses added on the plot for clarification purposes); (B) PLS-DA scores plot for red Agiorgitiko wines from Nemea region exhibiting classification according to vintage year; (C) white Asyrtiko wines from Santorini Island exhibiting classification according to the vinification year. Classification of test set samples was predicted using the model created by the training set using the following key: (A) (■) ARN1; (Δ) ARN2; (●) ARN3; (∇) ARN4; (□) MWN1; (\blacktriangle) MRS1; (∇) MRS2; (○) AWS1; (\blacklozenge) AWS2; (B, C) (■) ARN1 samples of the training set; (Δ) ARN2 samples of the training set; (●) ARN3; (◻) ARN1 samples of the test set; (\blacktriangle) ARN2 samples of the test set; (○) AWS1 samples of the training set; (\blacklozenge) AWS2 samples of the training set; (◻) AWS1 samples of the test set; (\blacklozenge) AWS2 samples of the test set.

Table 3. Average Phenolic Composition of 2006 Vintage Wine Samples (Milligrams per Liter)^a

	gallic acid	(+)-catechin	(-)-epicatechin	trans-resveratrol	syringic acid	trans-caffeic acid	trans-caftaric acid	p-coumaric acid	Q-galactoside	Q-glucoside	quercetin	kaempferol
Nemea												
ARN1 (n = 20)	50.30 ± 16.0.5b	55.39 ± 18.86a	28.45 ± 11.46a	0.82 ± 0.31b	5.84 ± 1.20a	5.36 ± 1.37a	30.65 ± 7.67d	1.72 ± 1.20bc	4.65 ± 2.03c	1.51 ± 0.79c	1.59 ± 1.28b	t
ARN3 (n = 4)	59.59 ± 6.1b	67.15 ± 9.70a	34.07 ± 5.33a	1.03 ± 0.17b	4.52 ± 0.40b	4.89 ± 0.67b	48.73 ± 2.31b	0.95 ± 0.24bc	7.67 ± 1.10b	3.14 ± 0.31b	1.50 ± 1.20b	t
ARN4 (n = 2)	1.11 ± 0.12d	8.95 ± 0.87c	4.04 ± 0.08b	0.40 ± 0.06c	3.36 ± 0.77b	2.63 ± 0.11c	16.35 ± 0.62e	0.89 ± 0.18c	1.91 ± 0.18d	1.33 ± 0.16c	1.25 ± 0.39bc	t
MWN1 (n = 3)	1.20 ± 0.26d	4.01 ± 0.57d	1.84 ± 0.71c	0.18 ± 0.10c	0.20 ± 0.09c	1.71 ± 0.22d	6.96 ± 1.32f	0.45 ± 0.11d	1.35 ± 0.12d	0.92 ± 0.67c	0.58 ± 0.50c	t
Santorini												
MSR2 (n = 3)	102.00 ± 4.72a	70.98 ± 4.07a	39.99 ± 3.33a	1.55 ± 0.16a	4.37 ± 0.74b	6.69 ± 0.56a	63.32 ± 2.88a	2.91 ± 0.24a	13.74 ± 1.13a	7.52 ± 1.79a	4.35 ± 0.61a	0.48 ± 0.12a
AWS2 (n = 13)	3.27 ± 0.43c	16.92 ± 2.61b	3.69 ± 1.43bc	0.30 ± 0.17c	0.22 ± 0.10c	4.21 ± 0.95b	96.19 ± 6.43c	1.88 ± 0.54b	4.58 ± 0.87c	2.89 ± 1.30b	1.60 ± 0.76b	0.37 ± 0.06a

^a Entries with no letters in common are significantly different ($p < 0.05$) in the same column. Q = quercetin; t = traces.

amounts in both red and white wines. *trans*-Caftaric acid was the most abundant, with MSR2 wines having the highest concentration among red wines, averaging 63.32 ± 2.88 mg/L, and AWS2 among white wines with an average concentration of 36.19 ± 6.43 mg/L. *p*-Coumaric acid was also present in lower concentrations, whereas ferulic acid was detected in amounts lower than the quantification limit.

From the flavonol group the glycosides quercetin-3-*O*-galactoside and quercetin-3-*O*-glucoside were detected in considerable amounts, especially in Santorini wines, with the first being the most abundant. MSR2 wines contained significantly higher amounts of Q-3-*O*-galactoside and Q-3-*O*-glucoside in comparison to ARN1, ARN3, and ARN4 wines ($p < 0.05$), with concentrations averaging 13.74 ± 1.13 and 7.52 ± 1.79 mg/L, respectively. Similarly, AWS2 wines were significantly richer in quercetin glycosides than MWN1 wines ($p < 0.05$).

The aglycon quercetin was present in considerably lower amounts than its glycosylated forms, with average concentrations between 1.50 ± 1.20 and 4.35 ± 0.61 mg/L for red wines and between 0.58 ± 0.50 and 1.60 ± 0.76 mg/L for white wines. Kaempferol was detected in very low amounts in Santorini wines, whereas in Nemea wines it was found in traces and quantification was not possible. The relatively high flavonol content of Santorini wines could be an indication of stress factors affecting the plants, such as UV irradiation.

Finally, the stilbene *trans*-resveratrol was detected in relatively low amounts in both Nemea and Santorini wines. Mandilaria wines exhibited the highest *trans*-resveratrol content, with an average concentration of $1.55 (\pm 0.16)$ mg/L ($p < 0.05$).

These results are in a general agreement with previous studies concerning the phenolic profile of Greek wines (34–37).

HPLC results indicate that wines from the Mandilaria variety exhibit a higher phenolic content among red wines and Asyrtiko among white, which is attributed to a combination of plant genome and region. As already mentioned, Santorini is a volcanic island with extreme weather conditions, and vines grow under water stress, resulting in the accumulation of polyphenols in the plant tissues. Other studies on the phenolic composition of varietal Greek wines have also revealed the exceptional phenolic potential of Asyrtiko cultivar, a variety cultivated mainly on Santorini Island, which produces wines distinctive for their rich phenolic content among white wines (36). Also, Mandilaria wines showed an elevated phenolic profile compared to Agiorgitiko wines (37).

Some interesting comparisons can also be made between Agiorgitiko wines from different wineries. Agiorgitiko wines from both wineries show a similar pattern in their phenolic composition; ARN3 wines, though, appear to have an elevated phenolic content compared to ARN1 wines. In particular, statistically important differences ($p < 0.05$) were found in the content of quercetin glycosides and the phenolic acids: syringic, *trans*-caffeic, and *trans*-caftaric acid, revealing that factors such as soil composition, infections, and agricultural and vinification practices result in the production of wines with unique phenolic composition, even within a restricted geographical area. This observation is also supported by the large deviation observed for ARN1 wines produced by the cooperative of Nemea, which covers a large number of vineyards in the Nemea region.

PCA was conducted on HPLC data, and the most adequate models were obtained after Pareto scaling (Figure 4). PCA revealed a good separation between wines of different varieties. Red wines are mainly placed on the positive side of PC1 axis; Mandilaria wines are placed in the upper right corner of the ellipse, whereas for Agiorgitiko wines some spreading has been observed on both sides of the PC2 axis. There is also a good discrimination

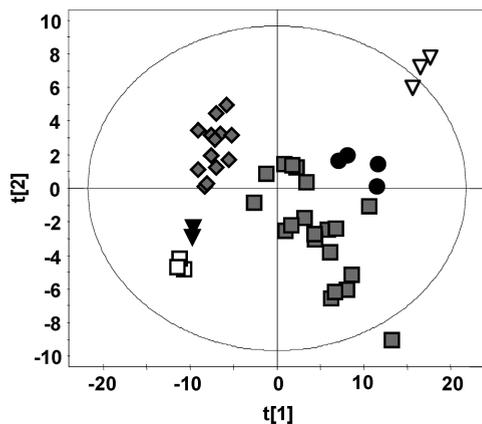


Figure 4. Scores plots concerning PCA of HPLC-derived concentrations of 12 polyphenols in red and white wine samples from Santorini Island and Nemea region for 2006 vintage: (■) ARN1; (●) ARN3; (▼) ARN4; (□) MWN1; (▽) MRS2; (◆) AWS2.

between Agiorgitiko produced in the different wineries. White and rosé wines are all located on the negative side of the PC1 and PC2 axis. Rosé wines from Agiorgitiko are placed between the white and red wines from the same region, in accordance with the results produced by ^1H NMR data. PCA of the HPLC data failed to separate wines according to origin, as was accomplished with ^1H NMR data, and separation was mainly achieved by color. The loadings plot (data not shown) suggests that gallic acid along with (+)-catechin and (−)-epicatechin dominate the first component, exhibiting the higher distance from the plot origin. The concentration of these polyphenols in red wines exhibits higher differences compared to white wines (Table 3) and characterizes the different locations of red and white wines along PC1. *trans*-Cafaric acid dominates the second component correlated positively with quercetin-3-*O*-galactoside. The location of Mandilaria red wines from Santorini correlates with the increased concentration of *trans*-caftaric and gallic acid and to a lesser extent to quercetin-3-*O*-galactoside. Compared to the ^1H NMR data (Figure 2S of the Supporting Information), gallic acid and also quercetin dominate the separation between the two different regions' red wines, whereas differences in syringic acid characterize the position of white wines in the PCA scores plot (Figure 2).

In general, discrimination of wines was satisfactory with both ^1H NMR and HPLC data used, and there was a good correlation between the two techniques applied. Wine metabolomics based on their phenolic profile is usually performed with HPLC data, the most popular method of analysis for phenolic compounds (35, 38). It could be argued that the first is advantageous over the second in both accuracy and simplicity, because ^1H NMR spectra contain all of the information of the aromatic compounds present in a wine extract and there is no need for standards to identify and quantify certain peaks in the chromatogram. Moreover, NMR spectra can be obtained with higher reproducibility than chromatograms, which need careful choice of analytical conditions (column, solvents, etc.). Furthermore, PCA using the chromatographic data is more time-consuming because the total run time for each chromatogram is quite long and the data need to be further processed to complete the quantification of the phenolic compounds identified.

Overall, wines can be classified according to variety, region, and year of production on the basis of their phenolic extract obtained by XAD resin and monitored by NMR in combination with multivariate analysis chemometric methods.

Our results show that XAD resin can be successfully applied for the isolation of the phenolic compounds from wines and the

production of enriched phenolic extracts, even for white wines, which have a very low phenolic concentration compared to red wines.

Polyphenols constitute a metabolic fingerprint of grapes and, consequently, wines and can be used for their classification. The phenolic composition of a wine appears to be characteristic of the variety and the year of production, as well as the vinification technique, allowing discrimination between wines of different vintages and wineries.

XAD polyphenol extraction combined with NMR spectroscopy and multivariate analysis is a rapid method capable of detecting differences between similar mixtures of organic molecules derived from diverse plants or fruits. The method can highlight differences in simple PCA scores plots, reducing the necessity for more laborious measurements of mixture constituents, and it could be applied in various problems of food product classification and authentication.

ACKNOWLEDGMENT

We are grateful to S. Lafazanis (Lafazanis Winery), I. P. Malisioris (Nemea Cooperative Winery), and P. Kanelopoulos (Santorini Cooperative Winery) for invaluable help during the sample collection.

Supporting Information Available: Table S1 and Figures 1S and 2S. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Received for review June 22, 2009. Revised manuscript received October 12, 2009. Accepted October 12, 2009. The present study was supported by DSBEPRO 05, GGET (2006–2007).