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RESEARCH ARTICLE

Classification and Chemometric Study of Southern Italy Monovarietal Wines Based on NMR and HPLC-DAD-MS

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Abstract There is increasing interest in development of methods for wine characterization to guarantee traceability and product safety. Metabolomics represents a valuable tool to trace the metabolic fingerprint of complex mixtures. Wines from 22 pure varieties (autochthonous from Southern Italy and grown in the same area of Apulia) were genetically identified and metabolically characterized using both NMR and HPLC-MS. NMR associated with chemometrics was used to identify amino acid (proline, alanine, and tyrosine), organic acid (malic, lactic, acetic, and succinic acids), alcohol (2-phenylethanol), sugar (sucrose), and polyphenol (gallic acid) contents. Minor components of wines were determined using HPLC-MS analyses. Wines showed potential health-promoting effects with good amounts of phenolic compounds. In particular, consistent levels of *trans*-resveratrol and flavonoids were found in Negro Amaro, Uva di Troia, Ancellotta, and Montepulciano wines.

Keywords: NMR, HPLC-DAD-MS, monovarietal wine, metabolome, polyphenol

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Introduction

Although wine is a product of important commercial value, one of the problems still facing the wine producing sector is proper characterization and identification of wines, especially wines derived from autochthonous grape varieties. At present, certificates of origin merely contemplate a series of specifications and regulations that are more concerned with growing conditions (type of soil, yield per hectare, and pruning) rather than characteristics of the final wine product.

There is increasing interest in development of accurate methods of wine characterization to prevent product adulteration and to guarantee traceability and safety of products (1,2). Classification and characterization of wine is feasible through qualitative and quantitative constituent information for alcohols, sugars, amino acids, volatile compounds, polyphenols, and trace substances. Metabolic examination of wine is based on diverse analytical techniques with different degrees of sensitivity and specificity, such as HPLC, MS, near-infrared spectroscopy (NIR), and NMR (3,4). These techniques have been extensively used in metabolomic studies together with multivariate statistical methods in order to obtain maximum information (5-7).

NMR has an exceptional place in chemical analyses of food products. Apart from routine use for identification, characterization, and structural elucidation of molecules, NMR is now increasingly popular in the area of metabolome analysis (8). The non-selective nature of NMR makes it an ideal tool for simultaneous profiling of a broad range metabolites. The most promising features of NMR are a non-destructive nature and simple and rapid sample preparation. A major advantage of this technique is that samples can be analyzed as such and quantification is only dependent on mole concentrations of compounds. However,

identification of individual metabolites is challenging in complex mixtures, such as wine, due to signal overlap and shifting phenomena, and only medium to highly abundant metabolites can be detected (9,10). MS offers quantitative analyses with great selectivity and sensitivity, and with the potential to identify metabolites. Furthermore, MS combined with a time-dimension separation technique (HPLC or GC) can reduce the complexity of mass spectra, provide isobar separation, and deliver supplementary details about physical and chemical properties of compounds (11).

In this study, wines made from autochthonous pure grape varieties of Southern Italy and grown in a small hilly area of Apulia, Rutigliano, Bari, Italy and vinified following identical winemaking techniques were genetically and metabolically characterized. For preparation of a comprehensive description, the 2 complementary approaches of metabolic fingerprinting (indirect or untargeted approach) and metabolic profiling (direct or targeted approach) were used. Metabolic fingerprinting using 1D NMR was aimed at recognition of a metabolite fingerprint associated with terroir effects (6) on a specific grape variety studied. Metabolic profiling focused on quantitative analysis of selected metabolites and was achieved using HPLC-UV-MS/MS for the main nutraceutic polyphenols and NMR for the most abundant compounds (12). In addition, the chemometric methods of unsupervised principal components analysis (PCA) and supervised partial least-squares discriminant analysis (PLS-DA) were applied to discern patterns in complex datasets obtained, and to identify similarities and/or differences among samples. The final goal of this work was application of a metabolomic approach to obtain a comprehensive description of a wine product from the region with a high wine vocation.

Materials and Methods

Samples Analysis was conducted using 2011 vintage wines obtained from 10 white grape and 12 red grape plant varieties (Table 1) collected from 5 regions of Southern Italy (Campania, Puglia, Basilicata, Calabria, and Sicilia) prior to 2001. Plants were moved to a trial site on a hilly area in Rutigliano, Puglia at long. 17º00'25"E, at lat. 40º57'29"N at an elevation of approximately 185 m and grown in a sandy-clay soil composed of 50% sand, 12% slit, and 38% clay, with a root zone depth of 1 m. All vines were planted on 1103 Paulsen (*Vitis berlandieri-Vitis rupestris*) rootstock and trained to a tendone trellis system, with a planting density of 4,000 vines/ha with a vine spacing of 2.5 m between rows and 1 m within a row.

Wines were produced in 3 replicates and under the same conditions. Briefly, grapes were harvested manually, destemmed, gently pressed and maintained in 100 L capacity stainless steel vats with a commercial sulfiting agent (20 g/100 kg of must, corresponding to 10 mg/L of free SO_2) ($K_2S_2O_5$-Nº CE 240-795-3, EVER s.r.l.; Pramaggiore, Venice, Italy). The maceration period was 10 days (red varieties) with 2 daily manual reconstitutions at 20ºC. Must was decanted from solids and transferred to 80 L capacity stainless steel vats. White grape must was clarified using bentonite (0.5 g/L), gelatine (0.1 g/L), and commercial enzymes with a pectinolytic activity (15 mg/L).

To initiate alcohol fermentation, the active yeast *Saccharomyces cerevisiae* 206 at a dosage of 0.25 g/L was added to must. The fermentation temperature was approximately 17ºC for white wines, and 20ºC for red wines. At the end of alcohol fermentation, wines were chilled to 15-20ºC for 20 days during which a spontaneous malo-lactic fermentation of red wines occurred. Finally, $K_2S_2O_5$ (corresponding to 35 mg/L of free SO_2 on average) was added to all wines prior to bottling. All wines were 12 months old at the time of analysis.

Chemicals Formic acid and HPLC grade water were purchased from J.T. Baker (Deventer, Holland). LC-MS grade solvent acetonitrile was purchased from Riedel-de Haën (Steinheim, Germany). HPLC reference standards were purchased from Extrasynthese (Genay, France). For NMR analyses, commercial reagent grade chemicals and solvents were purchased from Sigma-Aldrich (Milan, Italy).

Microsatellite analysis Variety true-to-type assessment was performed based on DNA fingerprinting as previously described (13). Briefly, genomic DNA was extracted using a Qiagen DNeasy Plant Mini kit (Qiagen, Valencia, CA, USA) and PCR was performed for amplification of the 13 microsatellites ISV2, ISV3, ISV4, VVS2, VVMD5, VVMD7, VVMD25, VVMD27, VVMD28, VVMD32, VrZAG62, VrZAG79, and VMCNG4b9 (Vitis Microsatellite Consortium). Amplicons were analyzed using a CEQ™ 8000 Series Genetic Analysis System (Beckman Coulter S.p.A., Milan, Italy) and detected sizes were scored following the codex of the International Organization of Vine and Wine (OIV) (Table 1). Resulting DNA fingerprints were compared with values included in the CRA-UTV database and with values registered in the European Vitis Database (available at: http://www.eu-vitis.de/index.php).

^{1}H-NMR spectroscopic analysis For NMR sample preparation, 100 μL of D_2O containing 3-trimethylsilyl-propionic acid sodium salt (TSP) (10 mM) as internal standard was added to 900 μL of a wine sample. For stability of chemical shifts, the pH was adjusted to 3.2 using 1 N HCl and 1 N NaOH solutions. From this prepared mixture, 600 μL was placed into a 5 mm Wilmad

NMR tube (Wilmad Labglass Inc., Vineland, NJ, USA). NMR was performed using a Bruker Avance 400 Ultrashield spectrometer (Bruker Biospin, Rheinstetten, Germany) equipped with a 5-mm inverse triple resonance probe with Z-gradient coils and a Bruker Automatic Sample Changer (B-ACS 120; Bruker Biospin). All spectra were acquired at 298.0 K. Manual tuning, matching, locking, and shimming were performed for each sample analysis. The 90° hard pulse P (90°) was set at 11.5 μs (pl1= –0.8 dB), and the 25 Hz pre-saturation pulse was adjusted to 57.99 dB. Two ^{1}H NMR analyses were performed for each sample.

***Experiment 1 (water pre-saturation pulse program-ZGPR)*:** A standard single-pulse experiment was carried out using continuous wave irradiation during the relaxation delay (RD) for pre-saturation of the water resonance (RD -P90°- acquisition of the free induction decay, FID). A 25 Hz RF field was used for pre-saturation. The RD and acquisition time (AQ) were set to 4 s and approximately 3.28 s, respectively, resulting in a total recycle time of approximately 7.28 s. After application of DS=4 dummy scans, NS=4 free induction decays (FIDs) were collected into a time domain (TD) of 65536 (65K) complex data points using a spectral width (SW)=19.9947 ppm and a receiver gain (RG)=1 (unit-less number). FID values were multiplied by an exponential function corresponding to LB =2 Hz prior to Fourier transformation.

***Experiment 2 (water suppression pulse program–NOESYGPPS)*:** One-dimensional ^{1}H NMR pulse sequence FID acquisition with suppression of the water and ethanol signals at RD t_{G1}-P (90°) 4 μs-P (90°)-tm t_{G2}-P (90°) was recorded. Settings for the parameters RD, P (90°), AQ, and TD were similar as for experiment 1 at DS=4 dummy scans and NS=32 scans with the mixing time (tm) set to 10 ms. A shaped pulse was applied during RD with a frequency spectrum of 8 highly selective bands to achieve highly selective suppression of the water signal and the 7 individual lines of the ethanol triplet and quartet, leaving the rest of the spectrum undistorted. Therefore, the receiver gain could be increased to RG=16, resulting in an increased signal-to-noise ratio, compared to the ZGPR analysis. The additional defocusing gradients G1 and G2 were applied at t_{G1} and t_{G2}=1 ms to improve the signal suppression quality. FID values were multiplied by an exponential function corresponding to LB=0.3 Hz prior to Fourier transformation.

***Metabolite identification*:** Identification of wine metabolites was performed on the basis of 2D NMR analyses, as previously reported (9,10).

***Metabolite quantification*:** The sensitivity of the NMR technique under experimental conditions based on recording of ^{1}H-NMR spectra of standard compounds (sucrose, malic acid, and succinic acid) generally present in wine and prepared at concentrations of 100, 75, 50, 25, 17.5, and 10 mg/L was determined. Concentrations were calculated based on integrals of suitable peaks as:

$$(C)_X=(C)_{TSP}\ N_{TSP}\cdot I_X/N_X\cdot I_{TSP}$$

where $(C)_X$ is the concentration of metabolite X, I_X and I_{TSP} are NMR signal intensities of X and TSP, respectively, N_X is the number of protons per molecule giving rise to the integrated signal, and N_{TSP}=9 (14). Compounds at concentrations ≥10 mg/L were detected with good correlation between measured and expected values (data not shown).

Chemometrics-Multivariate statistical data analysis AMIX 3.9 software (Bruker BioSpin, Italy) was used for statistical analysis of NMR spectral data. The 2 multivariate analyses of PCA and PLS were performed using input variables obtained via bucketing of NOESYGPPS spectra. Bucketing was done within 0.5-10 ppm, dividing the region into 238 sequential segments (bins) of 0.04 ppm. An integral value was obtained for each segment. Regions of residual water and ethanol were excluded.

HPLC-DAD-MS/MS analysis Separation and identification of phenolic compounds were carried out using an HPLC 100 (Agilent Technologies, Palo Alto, CA, USA) interfaced with a diode array detector (model G1315B DAD system; Agilent) and an XCT-trap Plus mass detector (model G2447A; Agilent). After filtration through 0.45 mm pore size regenerated cellulose filters (VWR International, Milano, Italy) wine samples were directly injected into a Luna C18 reversed stationary phase column (150×2 mm i.d., particle size 3-μm; Phenomenex, Torrance, CA, USA). A gradient system was used of water/formic acid (99:1, v/v) (solvent A) and acetonitrile (solvent B) of 0 min at 2% B, 10 min at 13% B, 25 min at 15% B, 30 min at 22% B, 50 min at 22% B, followed by washing and re-equilibration of the column during which the column temperature was not controlled, the flow was maintained at 0.2 mL/min, and the sample injection was 3 μL. The flow rate and the elution program were controlled using LC ChemStation 3D software (Hewlett-Packard, Palo Alto, CA, USA). UV-Vis detection wavelengths were set at 520, 360, 320, and 280 nm and spectrophotometric spectra were registered from 250 to 650 nm.

Both positive and negative electrospray modes (ESI) was used for ionization of molecules with a capillary voltage at 4,000 V and a skimmer voltage at 40 V. The nebulizer pressure was 30 psi and the nitrogen flow rate was 8 L/min. The temperature of drying gas was 350°C. In full scan mode, the monitored mass range was from *m/z*= 100 to 1,200 at a scan speed of 13,000 Da/s. MS/MS was performed using helium as a collision gas at a pressure of 4.6×10^{-6} mbar. Fragmentation spectra were obtained with an isolation width of 4.0=*m/z* for precursor ions and a fragmentation amplitude of 0.8 V. Compound identification

was achieved based on combination of UV absorption maxima (λ_{max}), the elution order, and mass spectral information, which were compared with pure standards, when available, and interpreted with the help of structural models (15,16). Finally, quantification of selected polyphenols was carried out using the external standard method. Calibration curves were constructed using 5 concentration levels and were fitted to a linear equation. Each point of the calibration curve was the mean value of 3 peak-area measurements. The detection limit (LOD) and quantification limit (LOQ) were calculated on the basis of chromatograms and defined as signal-to-noise (six times SD of baseline) ratio of 3 and 10, respectively.

Statistical analysis AMIX 3.9 software (Bruker BioSpin, Italy) was used for multivariate analysis (PCA and PLS) of NMR spectral data while HPLC data were analyzed statistically by the STATISTICA 6.0 software package (StatSoft Inc., Tulxa, OK, USA), using one-way analysis of variance (ANOVA). Significance was determined at $p<0.05$.

Results and Discussion

NMR wine analysis based on a combination of untargeted and targeted approaches Wines from 22 different grape varieties, the identities of which were confirmed based on DNA microsatellite fingerprinting, were analyzed based on 13 simple sequence repeats (SSRs) for exclusion of varietal misclassification, which is often an issue in studies of *Vitis vinifera* cultivars; sizes are expressed as a relative base pair distance to the shortest allele recorded within gene 081 and arbitrarily chosen as a reference, as recommended in the OIV descriptor list for grape varieties and *Vitis* species (13) (Table 1). In order to obtain metabolic profiles of autochthonous varieties grown in a specific terroir and to point out descriptive information, several steps of multivariate statistical analysis (PCA and PLS-DA) were used.

PCA analysis using the whole spectrum of 22 wines, discarding water and ethanol regions, produced 2 well-separated clusters (Fig. 1). Red wines with positive values were clearly separated from white wines characterized by negative values on PC1 (50.43%). However, both wine types were greatly scattered along PC2 (29.61%). Examination of loadings contributing to PC1 indicated that red wines were generally characterized by higher amounts of lactic acid (chemical shift, $\delta_{b(bucket\_ppm)}$=1.42, δ_b=1.38), succinic acid (δ_b=2.66), acetic acid (δ_b=2.06), medium-chain alcohols (δ_b=0.90), and lower amounts of malic and citric acids (δ_b =2.86; δ_b=2.82; δ_b=2.78; δ_b=2.74; δ_b=2.94; δ_b=2.90) than for white wines. On the other hand, the major contributors to the distribution of wines along PC2 (loading>|0.25|) were proline (δ_b=2.38; δ_b=2.34; δ_b=2.30; δ_b=2.10; δ_b=2.02; δ_b=1.98) and lactic acid. In order to better identify intra-cluster differences, a PCA analysis was performed on red

Table 1. Amplified genomic microsatellites used to establish varietal identification of the wine grape cultivars (*n*=22) analyzed

		MD28		ZAG79		ZAG62		MD7		VVS2		MD27		MD5		MD25		MD32	
B1	NV-ANTINELLO B.	12	20	14	22	20	22	8	16	10	10	4	14	4	24	4	6	37	37
B2	032-BOMBINO BIANCO B.	28	32	14	22	16	26	18	22	22	28	6	20	6	10	6	28	23	37
B3	065-CODA DI VOLPE BIANCA B.	32	42	14	22	20	30	18	18	10	22	6	19	4	14	6	6	23	37
B4	079-FALANGHINA B.	18	42	22	22	14	20	16	18	12	32	6	6	4	6	14	20	17	35
B5	081-FIANO B.	12	28	8	14	14	20	8	8	32	32	8	14	4	6	14	20	23	27
B6	092-GARGANEGA B.	20	32	14	14	26	26	18	22	10	20	4	19	4	10	6	20	15	23
B7	097-GRECO B.	12	20	6	14	28	30	16	18	10	32	10	14	6	10	4	6	17	17
B8	132-MALVASIA BIANCA LUNGA B.	32	39	6	14	22	26	8	22	22	22	4	4	4	18	4	6	17	21
B9	153-MOSCATO BIANCO B.	30	52	14	18	12	22	2	18	10	10	4	20	6	14	6	14	29	37
B10	252-VERDECA B. (179 PAMPANUTO B.)	32	42	6	14	14	22	8	8	10	22	4	14	10	18	4	4	15	17
R1	002-AGLIANICO N.	12	42	8	10	14	14	8	8	28	32	8	14	10	24	14	28	15	21
R2	062-CILIEGIOLO N.	18	30	8	22	20	30	16	32	10	10	4	8	4	14	6	6	17	17
R3	012-ANCELLOTTA N.	18	28	8	10	20	20	8	32	10	32	10	14	10	10	6	18	5	37
R4	150-MONTEPULCIANO N.	18	26	14	14	16	26	18	18	10	22	14	19	4	6	4	6	23	37
R5	163-NEGRO AMARO N.	18	20	22	22	16	28	18	18	22	28	4	6	4	14	6	28	17	37
R6	165-NERELLO MASCALESE N.	12	28	14	22	22	28	8	18	10	20	4	4	4	14	6	6	17	21
R7	046-CALABRESE N.	20	28	14	14	12	28	8	18	20	28	4	6	4	18	6	20	17	17
R8	174-NOTARDOMENICO N.	18	20	2	8	26	28	22	32	10	12	10	10	4	6	6	20	17	37
R9	247-UVA DI TROIA N.	28	32	14	14	14	26	12	22	20	28	14	20	4	10	28	32	15	37
R10	NV-PLAVINA N. (CROATIA)	32	42	0	6	14	26	8	18	10	20	4	14	10	14	4	4	17	29
R11	NV-PRIMUS NERO N..	20	30	2	18	12	20	8	18	12	28	4	10	6	14	14	20	29	37
R12	229-SUSUMANIELLO N.	18	20	14	22	22	26	8	18	10	20	4	14	4	10	6	20	21	27

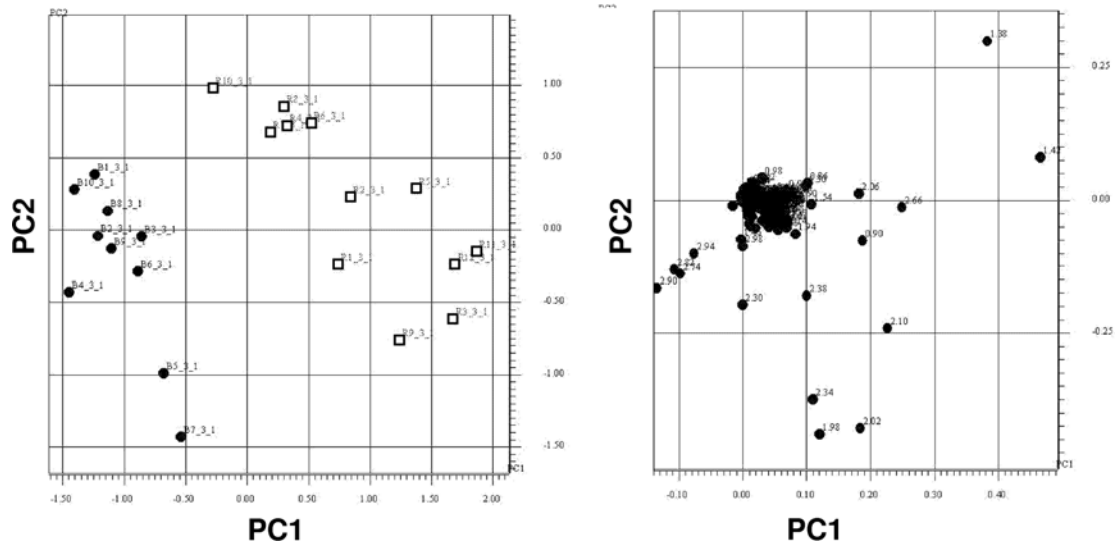

Fig. 1. PCA analysis of 22 red (square) and white (circle) wines (whole spectrum) with exclusion of water and ethanol regions. Score (left) and loading (right) plots of PC1 vs. PC2

and white wine attributes separately (Fig. 2). Due to different proline contents, some clustering along PC1 was observed for red wines with 3 groups centered near −1, 0, and +1, respectively. The spread within each subgroup was caused by different levels of acetic and lactic acids. For white wines, a single group without sub-clustering was observed with 2 outliers, the positions of which were correlated with higher proline resonance values in Greco (B7) wine and acetic and succinic acid values in Coda di Volpe Bianca (B3).

Organic acid compositions of wines are of importance for organoleptic properties, microbiological growth, and wine stabilization since organic acids influence pH. Organic acids originate directly from grapes (tartaric, malic, and citric acids) or derive from alcoholic fermentation, oxidation of ethanol, and malo-lactic fermentation (lactic, succinic, and oxalic acids) (17). Increased levels of malic or lactic acid have been associated with increased perceptions of sourness and astringency, but also with a milkier wine taste, and have been reported as quality features in white and red wines (8,9). In this study, higher concentrations of malic acid were found in white wines, particularly in Greco (B7) and Falanghina (B4) (2,885 and 3,055 mg/L, respectively). Lactic acid was significantly (p<0.05) more abundant in red wines, such as Negro Amaro (R5) (2,714 mg/L) and Susumaniello (R12) (3,120 mg/L) than in white wines. Furtheremore, the level of acetic acid in wines was generally insufficient (<900 mg/L) to negatively affect the wine fragrance (18).

There are different origins for amino acids in wine. Some amino acids are indigenous to grapes and can be partially or totally metabolized by live yeasts at the end of fermentation, or released from dead yeasts. Others are produced by enzymatic degradation of grape proteins (18). However, the amino acid composition is of great importance for wine because amino acids contribute to wine taste and appearance (such as proline, which gives wine an apparent mouthfeel or body), and also have an influence on aromas during aging (9,19). Proline was the most prominent amino acid found in wines with values ranging from 2,700 mg/L in Greco (B7) and Susumaniello (R12), to 105-378 mg/L in Plavina (R10) and Verdeca (B10). Lower concentrations of alanine (up to 90 mg/L in Coda di Volpe Bianca, B3) and tyrosine (up to 156 mg/L in Ancellotta, R3) were also identified.

Subsequent to elimination of buckets corresponding to previously identified metabolites, partial least-squares discriminant analysis (PLS-DA) as a supervised method was applied to NMR data in order to identify contributions of minor components and to emphasize differences between red and white wines. PLS maximized separation between red and white wines and revealed differences in the sucrose (δ_b=5.30), methionine (δ_b=2.62), arginine (δ_b=1.94; δ_b=1.78; δ_b=1.74), isopentanol (δ_b=1.54), alanine (δ_b=1.46), isoleucine, and leucine (δ_b=0.86, overlapped signals) contents (Fig. 3). PLS-DA analysis was also performed in the range 9.5-6.0 ppm (the region of polyphenols) for determination of differences due to polyphenol compounds, the concentrations of which were too low to contribute to the total variance with consideration of the entire spectrum. However, the sensitivity of the analytical technique allowed only detection of few highly concentrated polyphenols, such as gallic acid. A PLS score plot showed a spatial arrangement of samples according to the tyrosine content (δ_b=6.84; δ_b=7.16) along PLS1, and 2-phenylethanol (δ_b=7.36; δ_b=7.32; δ_b=7.28) and gallic acid (δ_b=7.12) along PLS2

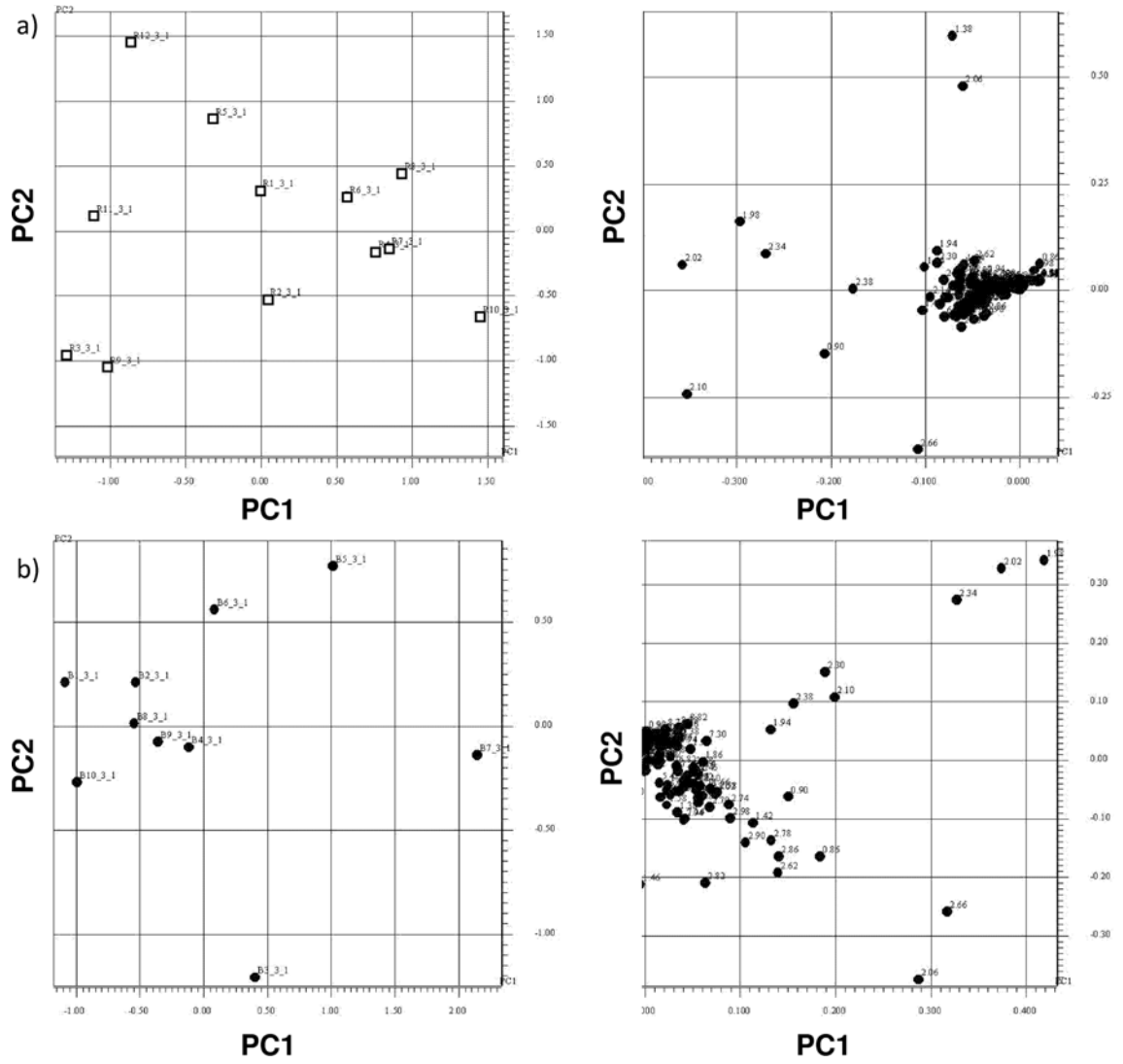

Fig. 2. PCA analysis applied using the whole NMR spectrum (with exclusion of water and ethanol regions) for 12 red (a) and 10 white (b) wines. Score (left) and loading (right) plots of PC1 vs. PC2

(Fig. 4). Mean concentrations of 90 and 42 mg/L for tyrosine in red and white wines, respectively, 65 and 43 mg/L for 2-phenylethanol, and 45 and 20 mg/L for gallic acid were observed. In addition, broad signals in the spectra were responsible for spatial separation of Ciliegiolo (R2), Ancellotta (R3), Uva di Troia (R9), and Primus (R11) wines from all other wines (Fig. 4). These signals were probably due to the presence of precipitated polymeric pigments, a common phenomenon that occurs during red wine-aging (20).

HPLC-DAD-MS/MS identification and quantification of the main nutraceutic polyphenols in wines Over the last 20 years, wine polyphenols have received considerable attention due to biological activities and possible pharmacological applications. Especially, *trans*-resveratrol, quercetins, and catechins together with anthocyanins have appeared to be efficient for prevention or reduction of a wide range of pathologies, such as cancer, cardiovascular disease, and ischemic damage (21,22). An extracted ion chromatogram (EIC) of 9 selected polyphenols in wine identified based on MS and MS/MS spectra is shown in Fig. 5 and Table 2. The most abundant anthocyanins were peonidin-3-*O*-glucoside and malvidin-3-*O*-glucoside, detected as flavilium cations M^+, providing high intensity signals for operation in the positive ion mode (15,23). Conversely, the negative ion mode appeared more selective and more sensitive for ESI-MS analysis of flavonols (quercetin-3-*O*-glucoside and kaempferol-3-*O*-glucoside), flavan-3-ols (catechin, epicatechin, procyanidin B_1, and procyanidin B_2), and *trans*-resveratrol.

Differences in the anthocyanin content were identified among red wines (Table 3). The highest concentrations of malvidin-3-*O*-glucoside (70±10 mg/L) and peonidin-3-*O*-

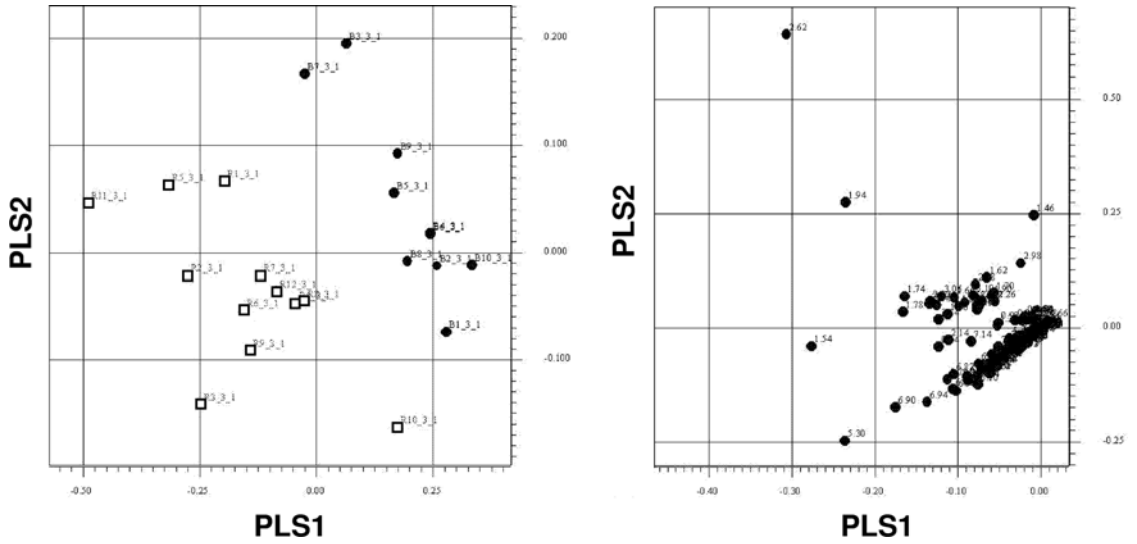

Fig. 3. PLS applied to 88 bucket-reduced NMR spectra (excluding regions of water, ethanol, and other identified metabolites) of 12 red (square) and 10 white (circle) wines. Score (left) and loading (right) plots of PLS1 vs. PLS2

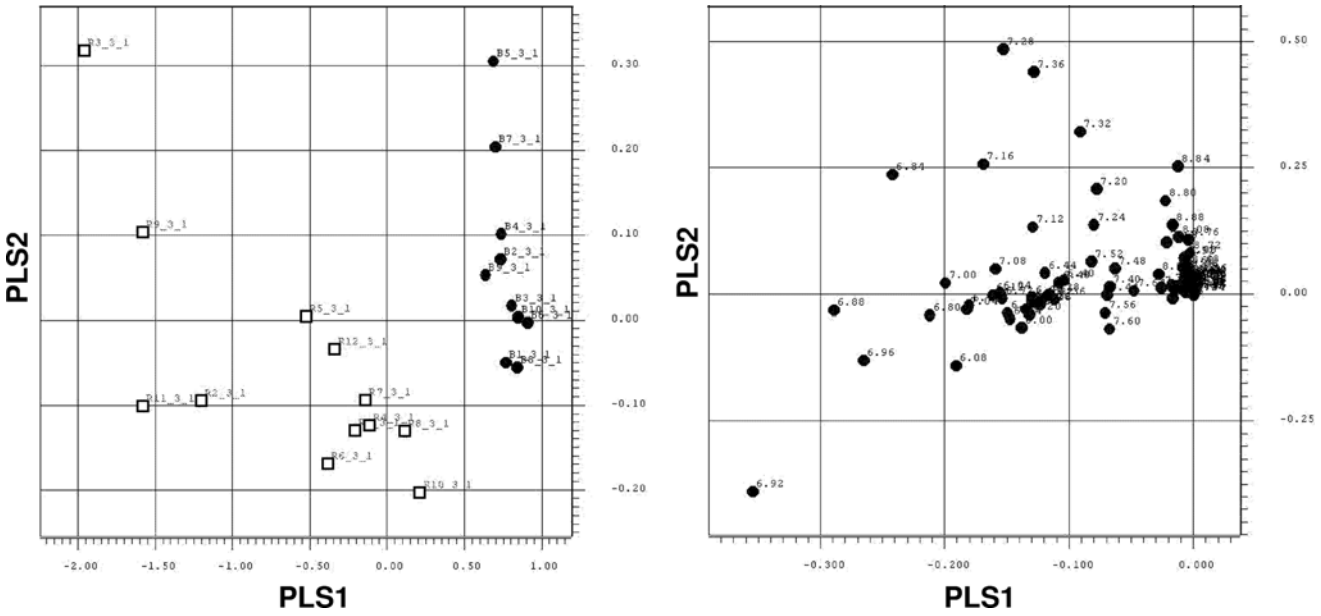

Fig. 4. PLS applied to the 9.5-6.0 ppm NMR region of red (square) and white (circle) wines. Score (left) and loading (right) plots of PLS1 vs. PLS2

glucoside (12.0±1.6 mg/L) were identified in Ancellotta (R3) and Uva di Troia (R9) varieties, respectively. Conversely, Notardomenico (R8), Plavina (R10), Susumaniello (R12), and Nerello Mascalese (R6) wines were characterized by anthocyanin levels up to 10× lower. Concentrations of different anthocyanins can depend on the vintage, together with other factors, such as agronomic and pedoclimatic conditions, with a relatively constant ratio for the same cultivar (18). For Aglianico (R1), Montepulciano (R4), and Calabrese (R7) wines, ratios between malvidin-3-*O*-glucoside and peonidin-3-*O*-glucoside concentrations (15,17, and 32, respectively) were in agreement with previous reports, although absolute concentrations were lower (24-26).

Flavanol and flavonol amounts in white wines were an order of magnitude lower than in red wines, since these compounds are localized in grape skins and seeds. Procyanidin B_1 and B_2 were the dominant compounds among flavanols in red wines with values ranging from 80±13 mg/L in Ancellotta (R3) to 16.0±1.8 mg/L in Montepulciano (R4), and from 6.4±1.0 mg/L in Nerello Mascalese (R6) to 1.1±0.2 mg/L in Susumaniello (R12) wines, in agreement with values reported by Sánchez-Moreno *et al* (26) for selected red wines. Moreover, concentrations of catechin (mean value of 15 mg/L in red wines and 1.4 mg/L in white

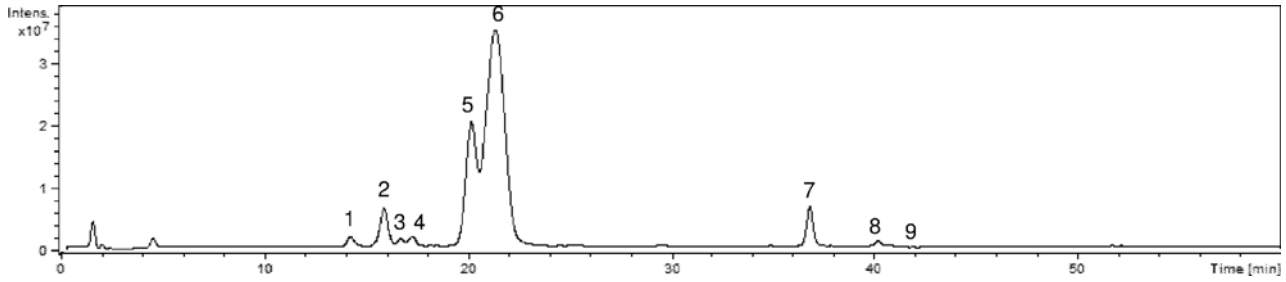

Fig. 5. Extracted ion chromatogram (EIC) of a red wine (Negro Amaro). (1) Procyanidin B1; (2) catechin; (3) procyanidin B2; (4) epicatechin; (5) peonidin-3-*O*-glucoside; (6) peonidin-3-*O*-glucoside; (7) quercetin-3-*O*-glucoside; (8) kaempferol-3-*O*-glucoside; (9) *trans*-resveratrol

Table 2. Characteristics of the main nutraceutic polyphenols found in wines, related to their retention times (RT), spectroscopic absorption ($\lambda_{max.}$), MS and MS/MS patterns

Peak	Compound	RT (min)	λ_{max} (nm)	$M^{+1)}$	$(M-H)^{-2)}$	MS/MS
1	Procyanidin B1	14.3	279		577	289
2	Catechin	15.9	282		289	245
3	Procyanidin B2	16.7	279		577	289
4	Epicatechin	17.3	282		289	245
5	Peonidin-3-*O*-glucoside	20.2	518	463		301
6	Malvidin-3-*O*-glucoside	21.4	527	493		331
7	Quercetin-3-*O*-glucoside	36.8	354		463	$301^{3)}$, $300^{4)}$
8	Kaempferol-3-*O*-glucoside	40.1	282, 330		447	$285^{3)}$, $284^{4)}$
9	*trans*-Resveratrol	42.4	308; 316		227	

[1)] M^+, molecular ion in positive ionization mode
[2)] $(M-H)^-$, deprotonated molecular ion in negative ionization mode
[3)] deprotonated radical aglycone $(Y_0-H)^{-\cdot}$ product ion
[4)] aglycone, Y_0^-, product ion

wines) as well as epicatechin (mean value of 11 mg/L in red wines and 0.8 mg/L in white wines) were generally lower than values in French wines (27). For flavonols, the highest values of quercetin-3-*O*-glucoside and kaempferol-3-*O*-glucoside were identified in Ciliegiolo (R2) (9.0±1.7 mg/L) and Uva di Troia (R9) (8.9±1.4 mg/L), and in Ancellotta (R3) (1.04±0.17 mg/L) and Primus (1.11±0.17 mg/L) wines, respectively. However, the level of quercetin-3-*O*-glucoside and kaempferol-3-*O*-glucoside in tested red and white wines were generally lower than in some Spanish wines (28). Since the extent of maceration with skins and seeds during fermentation is the main factor for efficient extraction of stilbenes from wines (27), the presence of *trans*-resveratrol only in red wines was not surprising in this study. However, analyzed wine samples were especially rich in *trans*-resveratrol, especially Negro Amaro (R5) (2.1±0.4 mg/L), Uva di Troia (R9) (1.51±0.18 mg/L), Ancellotta (R3) (1.4±0.2 mg/L), and Montepulciano (R4) (1.16±0.19 mg/L) wines. The concentrations identified were higher than reported values for Italian red wines (29), perhaps due to the fact that grape variety, climatic conditions (humidity and temperature), vintage, territory of origin, viticulture practice, and soil composition all influence the resveratrol concentration in wine. This statement is confirmed by the behavior of Aglianico (R1) which exhibited in this research lower content of resveratrol and flavonoids respect to other agronomic conditions, in particular trellis system and soil composition (25).

In conclusion, wines made using pure grape varieties all grown in the same area of Apulia, and vinified following the same winemaking technique were both genetically and metabolically characterized. NMR with chemometrics revealed a clear distinction based on organic acid and amino acid contents. Malic acid was more abundant in Greco and Falanghina wines, lactic acid in Negro Amaro, and Susumaniello wines, acetic acid in Coda di Volpe Bianca and Primus wines, and succinic acid in Greco and Ancellotta wines. The highest amount of proline was observed in Greco and Susumaniello wines and the lowest amount in Verdeca and Plavina wines. Primus and Coda di Volpe Bianca wines showed the highest content of alanine, while Ancellotta and Fiano wines were richest in tyrosine. Using HPLC-DAD-MS analysis, consistent levels of potentially health-promoting compounds were found in wines, in particular the Negro Amaro, Uva di Troia, Ancellotta, Ciliegiolo, and Montepulciano varieties, which contained high levels of *trans*-resveratrol, quercetin-3-*O*-glucoside, malvidin-3-*O*-glucoside, and peonidin-3-*O*-glucoside.

Table 3. Content of selected polyphenols in analyzed wines

(unit: mg/L)

Wines	Procyanidin B_1[1)]	Catechin	Procyanidin B_2	Epicatechin[2)]	Peonidin-3-*O*-glucoside	Malvidin-3-*O*-glucoside	Quercetin-3-*O*-glucoside[3)]	Kaempferol-3-*O*-glucoside	*trans*-Resveratrol
R1	46±7[4)]	23±4	2.0±0.3	17±3	1.7±0.3	25±4	1.3±0.3	0.37±0.06	0.29±0.06
R2	61±8	16±2	3.6±0.6	7.9±0.9	4.1±0.7	36±6	9.0±1.7	0.39±0.05	0.36±0.06
R3	80±13	18±3	4.9±0.9	14±2	2.9±0.5	70±10	1.6±0.2	1.04±0.17	1.4±0.2
R4	16.0±1.8	10±2	3.5±0.6	3.8±0.7	2.24±0.15	38±6	2.2±0.5	0.31±0.05	1.16±0.19
R5	17±2	6.5±1.7	3.2±0.4	3.1±0.5	1.4±0.3	43±7	1.8±0.3	0.19±0.04	2.1±0.4
R6	22±4	16±3	6.4±1.0	6.7±0.7	1.0±0.2	6.9±1.1	6.6±1.0	0.21±0.04	0.23±0.04
R7	35±7	15±3	4.1±0.4	4.9±0.9	1.12±0.09	36±5	0.78±0.18	0.32±0.05	0.22±0.06
R8	26±5	8.9±1.5	4.7±0.7	3.9±0.4	0.61±0.15	13±3	1.6±0.3	0.09±0.02	0.76±0.15
R9	67±10	16±3	4.8±0.8	43±6	12.0±1.6	63±10	8.9±1.4	0.47±0.08	1.51±0.18
R10	38±5	22±3	2.1±0.2	18±3	0.12±0.02	14±2	1.3±0.3	0.11±0.02	0.080±0.014
R11	27±5	25±4	3.2±0.5	8.2±1.4	3.0±0.5	53±9	1.4±0.3	1.11±0.17	0.73±0.10
R12	54±9	8.6±1.6	1.1±0.2	4.4±0.6	1.4±0.2	16±2	2.3±0.4	0.047±0.011	0.21±0.03
B1	2.2±0.4	0.78±0.14	0.89±0.12	1.9±0.3	ND[5)]	ND	0.21±0.03	0.049±0.009	ND
B2	3.3±0.6	1.5±0.3	0.81±0.10	1.2±0.2	ND	ND	0.31±0.08	0.061±0.010	ND
B3	5.0±0.6	4.2±0.6	0.65±0.08	2.8±0.4	ND	ND	0.17±0.02	0.035±0.005	ND
B4	3.9±0.6	2.0±0.3	0.72±0.08	0.75±0.16	ND	ND	0.42±0.04	0.092±0.008	ND
B5	4.2±0.8	0.19±0.03	0.70±0.10	0.37±0.04	ND	ND	0.151±0.012	0.050±0.004	ND
B6	3.4±0.6	1.5±0.3	tr.[6)]	1.0±0.2	ND	ND	0.34±0.04	0.087±0.007	ND
B7	4.8±0.7	1.7±0.3	tr.	2.6±0.4	ND	ND	0.38±0.05	tr.	ND
B8	2.1±0.3	0.37±0.06	0.91±0.07	0.88±0.15	ND	ND	0.27±0.03	0.081±0.006	ND
B9	2.3±0.4	1.1±0.2	0.69±0.11	0.52±0.09	ND	ND	0.112±0.014	0.039±0.004	ND
B10	2.4±0.5	0.97±0.19	tr.	1.0±0.2	ND	ND	0.36±0.06	tr.	ND

[1)]quantified as procyanidin B_2 equivalents
[2)]quantified as catechin equivalents
[3)]quantified as kaempferol-3-*O*-glucoside equivalents
[4)]values are expressed as mean±SD (n=3).
[5)]not detected, below the LOD
[6)]trace, below the LOQ

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