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NMR-based metabolomics to identify bioactive compounds in herbs and fruits

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Chapter 7

Application of NMR spectroscopy and chemometrics based approach to examine the effect of red wine polyphenols on TNF-α production

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Abstract

It is very well documented that bioactive compounds from plant derived food may have beneficial effects on human health and can reduce the risk of various chronic inflammatory diseases. In this study, eleven different types of red wine from Portugal were screened for their potential TNF-α inhibition. Nuclear magnetic resonance (NMR) spectroscopy together with multivariate data analysis methods are applied for the metabolite profiling of different red wines. All the fractions from solid phase extraction were subjected to *in-vitro* TNF-α bioassay. Principal component analysis (PCA) was used to see separation among the different SPE fractions. Supervised multivariate data analysis, i.e. Partial least square (PLS), Partial least squares-discriminant analysis (PLS-DA), orthogonal bidirectional OPLS-DA and orthogonal partial least square (OPLS) were used as statistical methods to find correlation between metabolite profile of the extracts and their respective bioactivities. OPLS-DA was found most effective in discriminating the high activity samples from the low and medium activity samples. According to variable importance in the projection (VIP), different phenolic compounds were found to correlate with high activity samples and identified as caftaric acid, quercetin, and (+)-catechin. Among the

different vintages, maximum TNF-α inhibition was found in samples from the 2010 vintage. The results obtained in this study advocate the efficacy of NMR spectroscopy in combination with chemometrics to identify the possible bioactivity in the several crude extracts of red wines.

Keywords: Chemometrics, NMR, red wines, TNF-α,

7.1. Introduction

Inflammation is the response of the body (immune system) against an external stimuli e.g. a pathogenic invasion aiming at the production, activation, and recruitment of immune cells (leucocytes) to sites of infection in order to combat the pathogens. The process of inflammation involves a complex mechanism of cell signaling cascades and is regulated by different pro-inflammatory cytokines and antiinflammatory cytokines. However, besides pathogenic infection, dysregulation of one or more regulatory proteins in the cell signaling pathways can also lead to inflammation and the emergence of wide varieties of chronic inflammatory diseases such as metabolic disorders, diabetes, atherosclerosis and cardiovascular complications, rheumatoid arthritis, Alzheimer's disease and cancer (Neuman, 2007).

Tumor necrosis factor-α is one of the regulatory proteins of the immune system in which dysregulation of this protein can lead to the emergence of the diseases. The proper regulation of TNF-α is necessary to keep the immune system in balance (homeostasis). TNF-α is secreted by immune cells; (mainly) by macrophages and also by T-cells, neutrophils, NK cells, and synovial cells (Vilcek and Lee, 1991). Increased level of TNF-α occurs during the early phase of inflammatory diseases and it contributes significantly to the secretion of other cytokines like IL-1, IL-6, and IL-8 (Cho et al., 2001; Cho et al., 1998). Overproduction of TNF-α can lead to systemic toxicity, thus, suppression of the secretion of TNF-α in this case can be a method for prevention and/or therapy.

The imbalance between prooxidants and antioxidants in an organism leads to a condition known as oxidative stress, which can be a trigger of a chronic inflammatory disease (Rahman et al., 2006). Healthy life style includes a rich antioxidant diet, which moderates the immune system and prevents diseases. Plants are known to be a rich source of antioxidants. Food and beverage from plants contain compounds that may work as TNF-α inhibitor. Wine for example, is a beverage of long tradition and high value and known to contain a complex mixture of compounds at a wide range of concentrations. The compounds in wine cover a diverse range of metabolites including primary (e.g. sugars, organic acids, amino acids) and secondary metabolites (e.g. flavonoids, hydroxycinnamates, hydroxybenzoates, anthocyanins). Wine phenolics have been proved to posses several health promoting activities (Ali et al., 2010a; Halpern, 2008) and nearly all of these beneficial effects associated to wine are due to anti-oxidant and radical scavenging properties of wine phenolics (German and Walzem, 2000). Since grape skin, seeds, and stem are the main source of phenolics in wine, red wines contain much higher concentrations of

these compounds as compared to white wines as skin, seeds, and stem are left in contact with must in red wine making, but is rapidly separated from the must in the case of white wine.

Metabolomics is a powerful tool to be applied on food or beverages such as wines in order to gain more insight in the compounds that are responsible for an interesting pharmacological activity. Metabolomics is an approach aimed for the better understanding of primary and secondary metabolism and can be defined as a metabolic snap shot of a living system (Andrew Clayton et al., 2006). More powerful and sophisticated tools (like mass spectrometry and NMR) for such chemical analyses have been introduced with the advancement in the field of analytical chemistry. Other than the use of NMR spectroscopy in structure elucidation of novel compounds, it is now a popular tool in the area of metabolome analysis (Son et al., 2009). Often criticized for its low sensitivity as compared to other platforms for metabolomics analysis, the most promising features of NMR are its non destructive nature, simple and fast sample preparation, and quantification is easy as for all compounds it is only dependent on the molar concentration. Nuclear magnetic resonance now has an important place in the chemical analyses of food. Some recent publications reported the use of NMR for the studies like quality control, authenticity or geographical characterization of different food products like coffee, olive oil, tomato and orange juices, wine and beer (Charlton et al., 2002; D'Imperio et al., 2007; Le Gall et al., 2001; Nord et al., 2004; Sobolev et al., 2003).

The development of methods and algorithms for the multivariate statistical modeling have contributed much to metabolomics as they opened the way for handling the huge datasets of large-scale metabolic analyses (Crockford et al., 2005). In combination with different multivariate data analyses methods, NMR has been widely used to do metabolic profiling of various samples (Brescia et al., 2002; Charlton et al., 2002). Several other studies have been published using the same combination focusing on the characterization of different plant species (Hye Kyong Kim et al., 2005) and cultivars (Ali et al., 2009), monitoring grape berry growth (Ali et al., 2011), and studying the effects of growing areas, vintage, soil, and microclimate (Pereira et al., 2005; Pereira et al., 2006). NMR based metabolomics was used for the analysis of the extracts of *Hypericum perforatum* (Roos et al., 2004), *Artemisia annua* (Bailey et al., 2004), *Citrus grandis* (Cho et al., 2009), and *Galphimia glauca* (Cardoso-Taketa et al., 2008), to correlate metabolites with the aid of pharmacological activities.

As wine in many countries is considered to be beneficial for health, with antioxidant thought to be responsible for this, we were interested to learn if also other effects could be present. With inflammation being a major disease, the present study was aimed at measuring the effect on an important mediator of inflammation. We therefore measured *in-vitro* TNF-α inhibition of some Portuguese red wines from different vintages. Several wine phenolics and other primary metabolites were identified using 1D and 2D NMR techniques. The correlation of activity data and NMR data using different multivariate data analyses methods in order to identify the active ingredients in red wines is also presented.

7.2. Materials and Methods

7.2.1 Wine samples

Wine samples analyzed in this study were kindly provided by Eng. Inês Aranha and Esporão (http://www.esporao.com).

7.2.2 Solid Phase Extraction (SPE)

A sample of 10 mL of each wine was completely dried under vacuum and redissolved in 1 mL of deionized water before subjected to solid phase extraction (SPE) on SPE-C18 cartridges (Waters, Milford, MA, USA). Prior to its use, the SPE cartridge was preconditioned by elution using 10 mL of methanol followed by 10 mL of water. The sample was subsequently applied to the cartridge and eluted successively with 5 mL of water, 5 mL of methanol:water (1:1) and 5 mL of methanol. 1mL of each fraction was taken into separate 2ml vial, dried under Nitrogen gas and dissolved in DMSO with concentration of 10mg/ml. The remaining fractions were collected, each in a round bottom flask and evaporated using a rotary evaporator except water fraction which was dried using freeze drier. The dried samples were in MeOD for NMR analysis. All the solvents were purchased from Biosolve B.V. (Valkenswaard, the Netherlands).

7.2.3. Growth of cells, Lipopolysaccharides stimulation, and treatments with wine

Human monocyte-like histiocytic lymphoma U937 cells were cultured and treated with extracts as described in chapter 3

7.2.4 ELISA for TNF-α

TNF-α in culture supernatants were determined by quantitative 'sandwich' enzyme-linked immunosorbent assay as described in chapter 3.

7.2.5. Cell viability assay

Cell viability after treatment with different wine samples was determined by using MTT assay (Lee et al., 2007) as described in chapter 3.

7.2.6. ^{1}H NMR Spectroscopy

The three fractions eluted from SPE were redissolved in 1 mL of methanol-d_4. An aliquot of 800 µL of sample was transferred to the 5-mm NMR tube and used for the NMR analysis. The deuterated methanol was purchased from Cambridge Isotope Laboratories, Inc., Andover, MA, USA. ^{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 sec acquisition time with the following parameters: 0.16 Hz/point, pulse width (PW) = 30° (11.3 µsec), and relaxation delay (RD) = 1.5 sec. 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 MeOH- d_4 at 3.3 ppm, using XWIN NMR (version 3.5, Bruker). 2D NMR techniques were performed on a 600 MHz Bruker DMX-600 spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 600.13 MHz. *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 sec 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.

7.2.7 Data analysis

The ^{1}H NMR spectra (from all SPE fractions) were automatically reduced to ASCII files. Spectral intensities were scaled to methanol signal (δ 3.30) and reduced to integrated regions of equal width (0.04) corresponding to the region of δ 0.0–10.0. The regions of δ 4.85–4.95 and δ 3.2–3.4 were excluded from the analysis because of the residual signal of D_2O and CD_3OD, respectively. Bucketing was performed by AMIX software (Bruker) with scaling on total intensity. Principal component analysis (PCA) with scaling is based on Pareto scaling, while projection to latent structures (PLS), PLS-discriminant analysis (PLS-DA), bidirectional orthogonal PLS (O2PLS), and O2PLS-discriminant analysis (O2PLS-DA), is based on Unit Variance scaling, all were performed with the SIMCA-P software (v. 12.0, Umetrics, Umeå, Sweden). The TNF-α level was arbitrarily set as 100 in the positive control and all the other values are normalized to this (% activity) and shown in results. Means and standard deviations were calculated and means comparisons were made with ANOVA at a significance level <0.01.

7.3. Results and Discussion

TNF-α inhibition results from the SPE fractions of the different wine samples are shown in Table 1. Water fraction of Petit Verdot 2008 shows least inhibition while the most active water fraction is of Aragones 2010 wine. The most active methanol fraction of SPE is from the Aragones (2009) while the least active fraction is from Alicante 2008. By comparison among the three SPE fractions maximum TNF-α inhibition was found in the methanol:water fraction. In the fractions, the vintage effect is obvious as Petit Verdot, Touriga Nacional, Aragones, and Alicante from 2010 vintage are significantly more active than the vintages of 2008 (Petit Verdot, Touriga Nacional, and Alicante) and 2007 (Aragones). Among all SPE fractions of different wine types, the Touriga Nacional (2010) showed the maximum inhibition of TNF-α production.

Wine type	Vintage	TNF-α inhibition (%)*		
		Water Fraction	Methanol:Water Fraction (1:1)	Methanol Fraction
Petit Verdot	2008	**7.51±2.72**	56.53±3.14	34.10±6.10
	2010	39.77±0.58	83.82±2.41	37.40±6.96
Touriga Nacional	2008	34.39±2.00	64.84±5.64	28.11±2.04
	2009	20.03±4.16	79.51±4.59	26.39±2.25
	2010	35.93±0.09	**88.81±2.56**	35.23±2.90
Aragones	2007	36.22±2.90	57.86±2.11	34.14±2.28
	2009	**48.93±0.94**	63.71±1,29	**43.08±5.38**
	2010	54.47±3.68	77.83±7.49	24.68±2.65
Alicante	2008	36.77±5.52	**55.14±1.97**	**17.71±2.87**
	2009	25.45±3.76	61.26±3.62	25.93±3.12

2010	38.11±4.92	73.47±1.35	27.69±3.35

* Values are expressed as mean ± standard deviation of *n*=3 experimental replicates. Means in a column differ significantly (*P*<0.01).

NMR spectroscopy of the SPE fractions was used for the metabolic profiling of the different red wines. Figure 1 shows the ^{1}H NMR spectra of water, methanol:water (1:1), and methanol fractions. From the NMR spectra it is clear that the SPE fractions are different from each other in terms of metabolites contained. Sugars and organic acids are mostly found in the water fraction. The methanol fraction shows high signal intensity in amino acids and fatty acids regions with relatively less sugars and no phenolics. The methanol:water fraction shows more signals in the phenolic region (δ 5.50 to

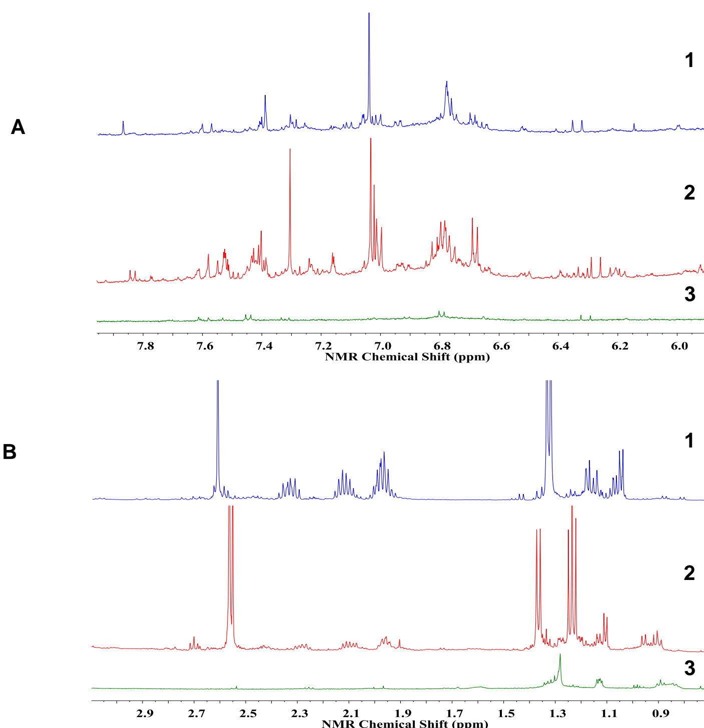

Figure 1: Comparison of 1H NMR spectra of phenolics (A) and amino acids (B) regions of SPE fractions. Both (A) and (B) show water (1), methanol:water (1:1) (2), and methanol (3) fractions from top to bottom.

8.50) with relatively less sugars and amino acids.

1D and 2D NMR techniques like *J*-resolved, ^{1}H-^{1}H COSY, HMBC and HSQC were used to identify the metabolites responsible of activity. Among the flavonoids, quercetin, myricetin, and kaempferol, are identified in the aromatic region. Signal correlation is observed between *δ* 6.49 of H-6 and *δ* 6.27 of H-8, and also between 6.99 of H-5′ and *δ* 7.66 of H-6′ of quercetin in the ^{1}H-^{1}H COSY spectrum. Likewise myricetin signals, δ 6.47 of H-8 with δ 6.25 of H-6, and kaempferol signals at δ 8.04 of H-2′ and H-6′ with δ 6.74 of H-3′ and H-5′, and at δ 6.52 of H-8 with δ 6.28 of H-6 also showed ^{1}H-^{1}H COSY correlations. Compounds like (+)-catechin and (-)-epicatechin were also identified. For both the (+)-catechin and (-)-epicatechin, signals of H-6′ and H-5′ along with signals of H-6 and H-8 showed correlations in ^{1}H-^{1}H COSY spectra. Resonances like δ 6.21 (t, J = 2.1 Hz), δ 6.31 (d, J = 2.1 Hz), δ 6.68 (d, J = 13.3 Hz), δ 6.71 (d, J = 8.5 Hz), δ 6.76 (d, J = 13.3 Hz), and δ 7.18 (d, J = 8.5 Hz) are assigned to resveratrol. This compound is identified as *cis*- isomer of resveratrol as the olefinic protons signals are shifted, i.e. H-8: from δ 6.79 to δ 6.68, and H-7: from δ 6.89 to δ 6.76, with reduced coupling constants (from 16.1 Hz to 13.2 Hz). These olefinic protons are also found to be correlated in the ^{1}H-^{1}H COSY spectrum along with other signal correlations like H-4 (δ 6.21) with H-2 and H-6 (δ 6.30), and H-6′ (δ 7.18) with H-3′ (δ 6.71).

The aromatic part of the ^{1}H NMR spectra also showed some signals of benzoic acid derivatives such as gallic acid, syringic acid, *p*-benzoic acid, and vanillic acid. The presence of characteristic doublets of 16.0 Hz in the range of δ 6.39-6.50 and δ 7.59-7.70 represent the H-8′ and H-7′ (olefinic protons) of *trans*-cinnamic acids, respectively, which are also found correlated in the ^{1}H-^{1}H COSY spectra and also coupled with the carbonyl carbon at δ 168.3 in the HMBC spectra. These metabolites are identified as caffeic acid, and *p*-coumaric acid. These two cinnamic acids derivatives, along with *trans*-feruloyl derivative, were also identified conjugated with tartaric acid through an ester linkage. Based on these assignments, these compounds were identified as *trans*-caftaric acid (caffeic acid conjugated with tartaric acid), *trans*-fertaric acid (ferulic acid conjugated with tartaric acid), and *trans*-coutaric acid (coumaric acid conjugated with tartaric acid). Along with the *trans*- forms, the *cis*- forms of these conjugated cinnamic acids, i.e. *cis*-caftaric acid and *cis*-coutaric acid, were also detected as an upfield shift of the signals for H-8′ and H-7′ along with the reduction in the coupling constant from 16.0 Hz to 13.0 Hz was observed in the J-resolved spectrum. Different amino acids like alanine, threonine, valine, proline, methionine, tyrosine, phenylalanine, glutamic acid, glutamine, arginine, and aspartic acid were identified by comparison with the reference spectra of

these compounds. All the compounds were identified (Table 2) by comparing the spectra with previous reports (Ali et al., 2011; Ali et al., 2009; Choi et al., 2004; Choi et al., 2005; Liang et al., 2006) and 1D and 2D NMR spectra of common plant metabolites in our in-house library.

Table 2: ^{1}H NMR chemical shifts (δ) and coupling constants (Hz) of wine metabolites identified by using 1D and 2D NMR Spectra of the reference compounds (CD3OD-KH2PO4 in D2O, pH 6.0)

Compounds	Chemical Shifts (δ)
Alanine	1.48 (d, *J*=7.4)
Threonine	1.32 (d, *J*=6.5), 3.51 (d, *J*=5.0), 4.27 (m)
Valine	1.01 (d, *J*=7.0), 1.06 (d, *J*=7.0), 2.28 (m)
Proline	2.35 (m), 3.37 (m)
Methionine	2.15 (m), 2.65 (t, *J*=8.0)
Tyrosine	6.85 (d, *J*=8.5), 7.19 (d, *J*=8.5)
Phenylalanine	3.15 (dd, *J*=8.2, 14.5), 3.91 (t, *J*=9.6)
Glutamine	2.14 (m), 2.41 (td, *J*=16.2, 7.5)
Glutamate	2.13 (m), 2.42 (m), 3.71 (dd, *J*=7.0, 1.9)
Arginine	1.75 (m), 3.75 (t, *J*=5.5)
Aspartate	2.80 (m), 3.80 (m)
β-glucose	4.58 (d, *J* = 7.8)
α-glucose	5.17 (d, *J* = 3.7)
Sucrose	5.39 (d, *J* = 3.9)
GABA	1.90 (m), 2.31(t, *J*=7.5), 3.01 (t, *J*=7.5)
Choline	3.20 (s)

Glycerol	3.56 (m), 3.64 (m)
2,3-butanediol	1.14 (d, *J*=6.5)
Acetic acid	1.94 (s)
Succinic acid	2.53 (s)
Fumaric acid	6.52 (s)
Formic acid	8.45 (s)
Citric acid	2.56 (d, *J* = 17.6), 2.74 (d, *J* = 17.6)
Malic acid	2.68 (dd, *J* = 16.6, 6.6), 2.78 (dd, *J* = 16.6, 4.7), 4.34 (dd, *J* = 6.6, 4.7)
Lactic acid	1.40 (d, *J*=7.0)
Tartaric acid	4.35 (s)
cis-Resveratrol	6.21 (t, *J*=2.1), 6.31 (d, *J*=2.1), 6.68 (d, *J*=13.3), 6.71 (d, *J*=8.5), 6.76 (d, *J*=13.3), 7.18 (d, *J*=8.5)
Gallic acid	7.03 (s)
Syringic acid	3.89(s), 7.31(s)
Vanillic acid	3.90 (s), 6.77 (d, *J*=8.2), 7.22 (m)
p-Benzoic acid	6.83 (d, *J*=8.7), 7.94 (d, *J*=8.6)
p-Coumaric acid	6.38 (d, *J*=16.0), 6.84 (d, *J*= 8.8), 7.50 (d, *J*=8.8), 7.59 (d, *J*=16.0)
Caffeic acid	6.24 (d, *J*=16.0), 6.87 (d, *J*=8.4), 7.02 (dd, *J*=8.4, 2.0), 7.12 (d, *J*=2.0), 7.52 (d, *J*=16.0)
(+)- Catechin	2.52 (dd, *J*=16.1, 8.2), 2.83 (dd, *J*=16.0, 5.4), 4.04 (m), 4.55 (d, *J*=7.5), 5.89 (d, *J*=2.2), 6.75 (d, *J*=8.0), 6.80 (dd, *J*=8.5, 2.0), 6.88 (d, *J*=8.5), 6.9 (d, *J*=2.0)
(-)- Epicatechin	2.72 (dd, *J*=16.8, 2.6), 2.89 (dd, *J*=16.9, 4.6), 4.26 (m),

	6.03 (d, *J*=2.0), 6.06 (d, *J*=2.0), 6.88 (brs), 6.96 (d, *J*=2.2)
Quercetin	6.27 (d, *J*= 2.0), 6.49 (d, *J*=2.0), 6.99 (d, *J*=8.6), 7.66 (dd, *J*=8.5, 2.0), 7.71 (d, *J*=2.0)
Myricetin	6.28 (d, *J*= 2.0), 6.51 (d, *J*=2.0), 7.30 (s)
Kaempferol	6.28 (d, *J*= 2.0), 6.52 (d, *J*=2.0), 6.74 (d, *J*=8.6), 8.04 (d, *J*=8.6),
trans-Caftaric acid	5.77 (s), 6.29 (d, *J*=16.0), 6.88 (d, *J*=8.4), 7.02 (dd, *J*=8.4, 2.0), 7.12 (d, *J*=2.0), 7.52 (d, *J*=16.0)
trans-*p*-Coutaric acid	5.84 (s), 6.36 (d, *J*=16.0), 6.87 (d, *J*=8.8), 7.51 (d, *J*= 8.8), 7.59 (d, *J*=16.0)
Fertaric acid	3.89 (s), 5.38 (s), 6.32 (d, *J*=16.0), 6.89 (d, *J*=8.4), 7.01 (dd, *J*=8.4, 2.0), 7.19 (d, *J*=2.0), 7.56 (d, *J*=16.0)
cis-Caftaric acid	5.34 (s), 5.92 (d, *J*=13.0), 6.71 (d, *J*=8.4), 6.81 (d, *J*=13.0), 7.03 (dd, *J*=8.4, 2.0), 7.44 (d, *J*=2.0)
cis-*p*-Coutaric acid	5.41(s), 5.94 (d, *J*=13.0), 6.73 (d, *J*=9.2), 6.86 (d, *J*=13.0), 6.93 (d, *J*=9.2), 7.61 (d, *J*=9.2),

Multivariate data analysis algorithms (supervised or unsupervised) were used to reduce the dimensionality of multivariate dataset and thus enable us to discriminate among the samples. The NMR data from the SPE fractions of all the samples were subjected to PCA to see the differences among the SPE fractions and to identify the metabolites responsible for that distinction. Figure 2 shows the score plots of PCA where samples are colored according to SPE fractions. By examining the corresponding loadings plot, metabolites responsible for this separation are revealed (Table 1).

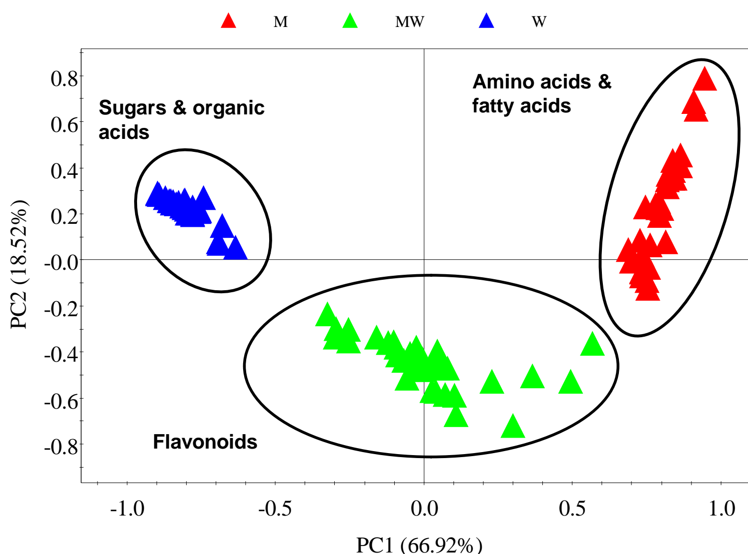

Figure 2: **Principal component analysis (PCA) score plot of SPE fractions of all the wine samples. Three fractions are clearly separated from each other. Samples in blue, green, and red indicates water, methanol:water (1:1), and methanol fractions, respectively.**

PLS-DA is a supervised multivariate data analysis method in which samples are classified into various classes on the basis of activity by creating dummy *Y*-variables. The score plot of PLS-DA (Figure 3A) shows good separation of samples with high activity from the others but no clear distinction was found among samples with medium and low activity. To see the difference among low and medium activity samples, another supervised algorithm, bidirectional orthogonal PLS-DA (O2PLS-DA), was used. Figure 3B shows very nice separation among all the three classes of samples. Metabolites responsible for the separation are identified by examining the corresponding loadings plot. Samples with high activity are found with higher levels of phenolics like quercetin, myricetin, (+)-catechin, caftaric acid, and coutaric acid while metabolites like glucose, sucrose, valine, proline, methionine, and alanine are found more concentrated in low and high activity samples.

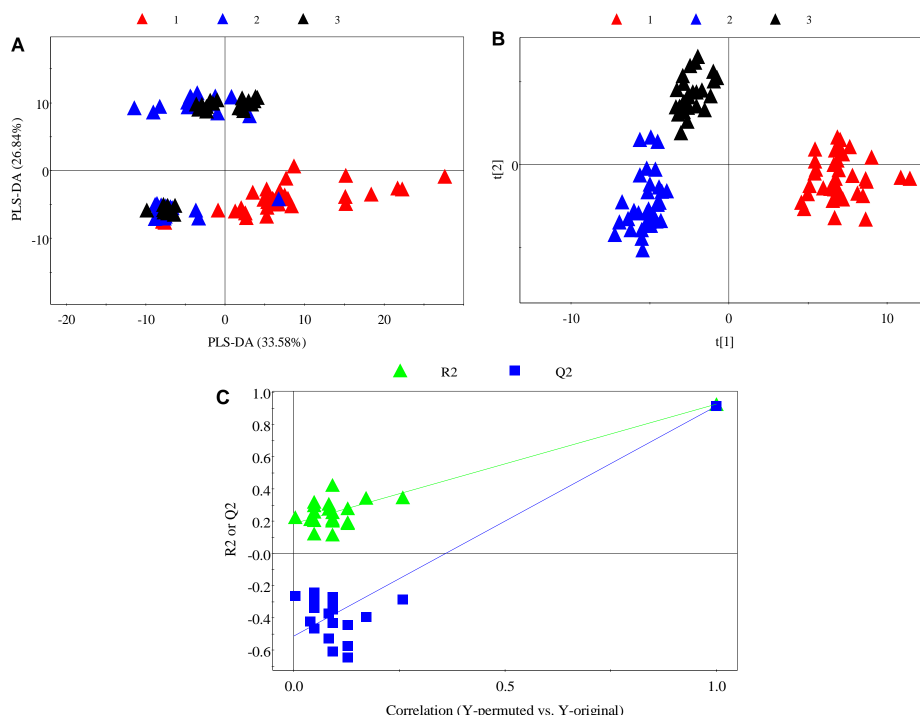

Figure 3: Score plots of PLS-DA (A) and O2PLS-DA (B) and validation plot of permutation test for PLS-DA (C). Samples with high, medium, and low activity are presented red, blue and black colors.

Projection to latent structures (PLS) is a supervised data reduction algorithm in which actual activity values were used instead of dummy *Y*-variables. The score plot of PLS analysis (Figure 4A) shows that samples with high activity are well separated, but no distinction was found among low and medium activity samples. Bidirectional orthogonal PLS was also used in order to separate all the three classes of samples but as shown by the score plot (Figure 4B), only high activity samples are clearly separated from the others. Permutation test is often used for validation of PLS and PLS-DA. The R2 and Q2 values for PLS and PLS-DA were calculated using six components for both analyses.

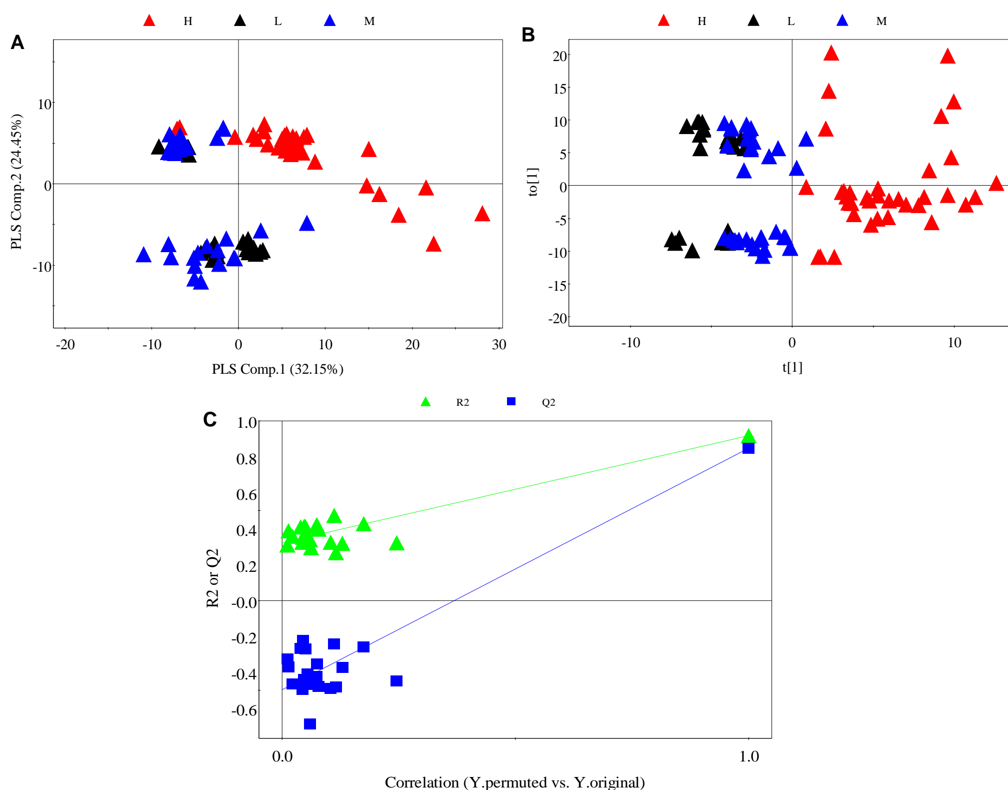

Figure 4: Score plots of PLS (A), O2PLS (B) and validation plot of permutation test for PLS (C). Samples with high, medium, and low activity are presented in red, blue and black colors.

For anti-TNF-α activity the R2 and Q2 values for PLS analysis were 0.91 and 0.84, respectively, while for PLS-DA these figures were 0.92 and 0.91 (Figure 4C and 3C). For O2PLS-DA and O2PLS analyses, VIP values for several phenolic compounds, responsible for separation on the score plot, are calculated. It has been reported that factors with VIP values more than 0.7 could be regarded influential for the separation of samples (L. Eriksson et al., 2006). VIP values of the major contributing metabolites are as follows; caftaric acid at δ 7.02: 1.91, quercetin at δ 7.81: 1.74, coutaric acid at δ 7.68: 1.42, and (+)-catechin at δ 5.92: 1.18. It is thus assumed that these compounds with high VIP scores are directly related to the inhibition of TNF-α production.

Various multivariate data analysis methods are used in combination with NMR spectroscopy in order to correlate the activity data of the extract with the spectroscopy data of the same. Several studies showed the analysis of the

extracts (Bailey et al., 2004; Cho et al., 2009; Roos et al., 2004) with NMR spectroscopy using chemometrics methods to identify the active compounds. This approach is very effective in the screening of various plant extracts in order to identify the plant compounds with an activity without laborious fractionation and chromatographic separation of the crude extract. Fractions from SPE of various red wines from Portugal were analyzed for anti-TNF-α activity and the combination of NMR spectroscopy and chemometrics was successfully applied to identify the active ingredients.

The vintage effect on the metabolic profile of grapes and ultimately on wine has been extensively studied (Lee et al., 2009; Pereira et al., 2006) and the importance of the vintage is widely accepted. The amino acids and polyphenols contents are highly affected by the climatic conditions of a grape production area. It has been shown that a hot and dry climate results in a higher proline and phenolic contents in wine (Lee et al., 2009). This study is also capable to highlight the effects of vintage on the TNF-α inhibition potential of different wines. It is evident from the results that samples from 2010 are more active than samples from the other vintages and based on this observation it can be postulated that in the 2010 vintage the berries experienced a hot and dry climate which ultimately resulted in higher phenolic contents and more potency towards TNF-α inhibition.

Our diet has beneficial effects on health and the consumption of antioxidant rich food (fruits, vegetables, tea, and wine) may have health promoting effects. The medicinal importance of moderate wine consumption has been proven by many studies. Wine metabolites, especially phenolics, are now well known to act against cardiovascular diseases (Cordova and Sumpi, 2009), renal disorders (Bertelli et al., 2005), Alzheimer's disease (Marambaud et al., 2005), cancer (Barstad et al., 2005), and also against bacteria (Murray et al., 2002) and viruses (Takkouche et al., 2002). Several health promoting activities associated to wine polyphenols were comprehensively reviewed recently (Ali et al., 2010b; Cordova et al., 2009; Halpern, 2008; Opie and Lecour, 2007). Phenolics are well known for their potency to inhibit TNF-α production as they are widely accepted to have anti-oxidative and antiinflammatory properties (Baur et al., 2006; Chuang et al., 2010). Phenolics like resveratrol (Stewart et al., 2008) and quercetin (Rivera et al., 2008) are known to reduce inflammation, while others like cinnamates, benzoates, flavonols, flavan-3-ols, and anthocyanins, are well known antioxidants (Lee et al., 2009; Meyer et al., 1997).

7.4. Conclusion

In this study, solid phase extraction integrated with NMR spectroscopy and multivariate data analysis methods were used to identify the correlation of metabolites with a TNF-α inhibition data set. Active ingredients in an extract could be identified using PLS-based regression models with ^{1}H NMR and activity data set. Phenolics like quercetin, caftaric acid, and (+)-catechin are identified as most influential in inhibiting TNF-α production among the other wine metabolites. The approach presented here, can be applied for the identification of TNF-α inhibiting compounds in crude plant extracts using NMR and multivariate data analysis. The methodology can also be applied to infer the various bioactivities associated to wine without any laborious chromatographic separation of metabolites.

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