NMR spectroscopy and chemometrics-based analysis of grapevine

Ali, K.

CHAPTER 8

Red wines attenuates TNFα production hystiocytic lymphoma cell line: An NMR spectroscopy and chemometrics-based study

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Abstract

Nuclear magnetic resonance (NMR) spectroscopy and multivariate data analysis methods are used for the metabolite profiling of different wines produced in Portugal. The water, methanol:water (1:1), and methanol fractions from solid phase extraction were subjected to *in vitro* TNFα activity assay. Principal component analysis (PCA) allowed the clear separation among the different SPE fractions. Various supervised data reduction algorithms were tested and compared to identify the signals related to the TNFα inhibition observed for the SPE fractions of wines. Projections to latent structures-discriminant analysis (PLS-DA) and orthogonal bidirectional PLS-DA were found most effective in discriminating the high activity samples from the low and medium activity samples. By calculating variable importance in the projections (VIP), the active ingredients in the high activity samples have been identified as caftaric acid, quercetin, and (+)-catechin. Among the different vintages, samples from the 2010 vintage were found to have maximum anti-TNFα activity. The present study shows the usefulness of NMR spectroscopy in combination with chemometrics to identify the possible bioactivity in the several crude extracts.

Introduction

Inflammation plays a vital role in various widely occurring diseases in the world like asthma, atherosclerosis, and rheumatoid arthritis. Mediators, such as pro-inflammatory cytokines including interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), and interferon-γ (IF-γ), are known to be released during an inflammatory response. The imbalance between pro-oxidants and antioxidants in an organism leads to a condition known as oxidative stress which is known to play a critical role in various degenerative diseases like diabetes, cancer, cardiovascular diseases, and artherosclerosis. Tumor necrosis factor-α is one of the most important pro-inflammatory cytokines and is produced mainly by macrophages but can also be formed by various cells like T-cell, neutrophils, NK cells, and synovial cells (Vilcek and Lee 1991). TNF-α is secreted during the early phase of inflammatory diseases and responsible to initiate the secretion of other cytokines like IL-1, IL-6, and IL-8 (Cho et al. 1999; 2001). Hence the local

effect of TNF-α can be considered as beneficial but its overproduction can lead to systemic toxicity and in that case anti-inflammatory therapy is becomes important.

Wine is a beverage of long tradition and high value and known to contain a complex mixture of compounds at a wide range of concentrations. These 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. 2010; 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). Red wines contains much higher concentrations of phenolic compounds as compared to white wine as skin, seeds, and stem, which are rich in phenolics, are left in contact for longer period with must in red wine making, whereas they are separated immediately from the must in the case of white wine.

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. 2006). 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 (Kim et al. 2005; 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; 2006a; 2006b). Several studies showed the analysis of the extracts from *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), for the prediction of different pharmacological activities using NMR spectroscopy with the combination of chemometrics methods.

The present study is aimed to measuring the *in vitro* anti-TNFα activity of different red wines from different vintages. Several wine phenolics and other primary metabolites were also 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.

Materials and Methods

Wine samples

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

Solid phase extraction (SPE)

A sample of 10 mL of each wine was completely dried under vacuum and then subjected to solid phase extraction (SPE) on SPE-C18 cartridges (Waters, Milford, MA, USA). Prior to its use, the SPE cartridge was prepared by elution with 10 mL of methanol followed by 10 mL of water. Then, the redissolved wine sample (in 1 mL of deionized water) was applied to the cartridge and eluted successively with 5 mL of water and then 5 mL of methanol:water (1:1) and finally with 5 mL of methanol. All three fractions were collected in round bottomed flasks and evaporated under vacuum and used for further NMR analysis. All the solvents were purchased from Biosolve B.V. (Valkenswaard, the Netherlands).

Growth of cells, lipopolysaccharides stimulation, and treatment with wine

Human monocyte-like histiocytic lymphoma cells U937 obtained from the ATCC (CRL-1593.2) were grown in RPMI-1640 medium, supplemented with 10% (v/v) fetal calf serum and 2 mM L-glutamine (Life technologies, Breda, The Netherlands) at 37 °C, 5% CO_2 in a humidified atmosphere. U937 monocytic cells ($5x10^5$ cells per well) were plated in a 96-well culture plate and then differentiated into macrophages using phorbol 12-myristate 13-acetate (PMA, 10 ng mL^{-1}, overnight, Omnilabo, Breda, The Netherlands). The PMA-differentiated macrophages were allowed to recover from PMA treatment for 48 h, during which the culture medium was replaced daily. Lipopolysaccharides stimulation of cells was performed as described by (Sajjadi et al. 1996). Immediately after stimulation cells were treated with wine samples at the concentrations of 100 μgmL^{-1} and then incubated at 37ºC for 4 hours. Only DMSO was added to control samples. Supernatants were then collected and measured for TNFα content using the Human TNFα enzyme linked immunosorbent assay (ELISA) kit (R&D systems, Europe Ltd).

Enzyme-linked immunosorbent assay for TNFα

TNFα in culture supernatants was determined by quantitative 'sandwich' enzyme-linked immunosorbent assay using paired antibodies purchased from (Biosource International, Inc., USA). In brief, all wells of high-binding Immulon-plates (96 well NUNC MaxiSorp microplates) were coated with 100 µL of the captured antibody (anti-Human TNFα) (0.250 mg 0.125 mL^{-1}). After overnight incubation at 4ºC, plates were washed with washing buffer and blocked for 1 hour with 1% bovine serum albumin in phosphate-buffered saline. Plates were aspirated and inverted on adsorbent paper to remove excess liquid. Samples and standards were diluted with assay buffer. 100 µL of diluted standards (recombinant Human TNFα protein) were filled in sixteen wells of first two columns of plates. Rests of the wells were filled with 100 µL of samples in different concentrations. Immediately after, 50 µL of working detection antibody (0.025 mg 0.125 mL^{-1}) was plated in every well and then plates were incubated for 2 hours at room temperature with continuous shaking at 700 rpm. The wells were washed again 5 times with washing buffer before addition of 100 µL of streptavidin-HRP to the wells and further incubated at room temperature for 30 minutes with continuous shaking at 700 rpm. Again wells were aspirated and washed 5 times before addition of 100 µL of TMB substrate. Plates were incubated for 30 minutes at room temperature with continuous shaking at 700 rpm. After 30 minutes the reaction was terminated by addition of 100 µL of 2M H_2SO_4, and absorbance was determined using a microtiter plate reader (Bio-Tek Instruments Inc., Winooski, VT, USA) at 450 nm. The concentration of TNFα in the samples was calculated by comparison of the absorbance of the samples to the standard curve. The ratio (%) of TNFα inhibition release was calculated by the equation, i.e. Inhibition (%) = 100 x (1 – T/C), where 'T' represents the concentration of TNFα with wine samples while 'C' was the concentration of TNFα with only DMSO.

Cell viability assay

Cell viability after treatment with different wine samples was determined by using MTT assay. Briefly, U937 cells having concentration of ($5x10^5$ cells mL^{-1}) were placed in a 96 well plate. The culture media also contain different plant extracts (100 µg mL^{-1}) in

the presence or absence of 200 µg mL^{-1} LPS at 37°C. After 2.5 hours of incubation at 37°C, the medium was discarded and the formazan blue, which is formed by reacting MTT with mitochondrial dehydrogenase in the living cells, was dissolved with 100 µL DMSO. The optical density (OD) was measured at 540 nm. The background signal inherent to the plates when no cells were present was subtracted from the absorbance obtained from each sample.

^{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 as explained in Chapter 7.

Data analysis and statistics

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 (δ 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 based on Pareto, while projections to latent structures-discriminant analysis (PLS-DA), and O2PLS-discriminant analysis (O2PLS-DA), with scaling based on Unit Variance were performed with the SIMCA-P+ software (v. 12.0, Umetrics, Umeå, Sweden). The TNFα content 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.

Results and Discussion

Visual analysis of ^{1}H NMR spectra

Solid phase extraction (SPE) in combination with NMR spectroscopy was applied for the metabolic profiling of different red wines. The ^{1}H NMR spectra of water, methanol:water (1:1), and methanol fractions are shown in Figure 1. It is quite obvious

from the figure that the metabolic contents in each SPE fraction are very different and dominated by distinct classes of metabolites. The water fraction shows mostly sugars, organic acids, with few signals related to phenolics. The methanol fraction shows high signal intensity in amino acids and fatty acids regions with relatively less sugars and no phenolics. The highest phenolic contents was observed in methanol:water fraction with relatively less sugars and amino acids contents. The distribution of specific metabolites in SPE fractions will be discussed later.

Identification of metabolites

Different metabolites have been identified using ^{1}H NMR with the help of the above mentioned 2D techniques and cover a wide diversity and including amino acids, organic acids, carbohydrates, hydroxycinnamates, hydroxybenzoates, stilbenes, flavanoids, and flavonoids. Phenolics belong to one of the major classes of wine metabolites and many characteristic wine phenolics are identified in this study. Among the flavonoids, quercetin, and myricetin 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 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. The signal of H-8 at δ 6.68 and H-7 at δ 6.76, with a coupling constant of 13.2 Hz, suggest that this compound is the *cis*- isomer of resveratrol. These olefinic protons correlate 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 characteristic doublets of 16.0 Hz in the range of δ 6.39-6.50 and δ 7.59-7.70 represent respectively the H-8′ and H-7′ (olefinic protons) of *trans*-cinnamic acids, which are 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 acid derivatives, along with *trans*-feruloyl derivative, were also identified to be 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 through up-field shifted signals for H-8′ and H-7′ along with the reduction in the coupling constant from 16.0 Hz to 13.0 Hz.

The high signal intensities in the amino acid region allowed the identification of a number of amino and organic acid signals. The amino acids 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. The signals in the carbohydrate regions were highly overlapping and showed signals of the anomeric protons of β-glucose, α-glucose, and sucrose. Other compounds, including choline, 2,3-butanediol, and γ-amino butyric acid (GABA) were also identified in this region. A number of signals were assigned to the organic acids like acetic acid, succinic acid, fumaric acid, formic acid, citric acid, lactic acid, malic acid, and tartaric acid. All of these assignments (Table 1) were done by comparing the spectra with previous reports (Ali et al. 2009; 2011) and 1D and 2D NMR spectra of common plant metabolites in our in-house library.

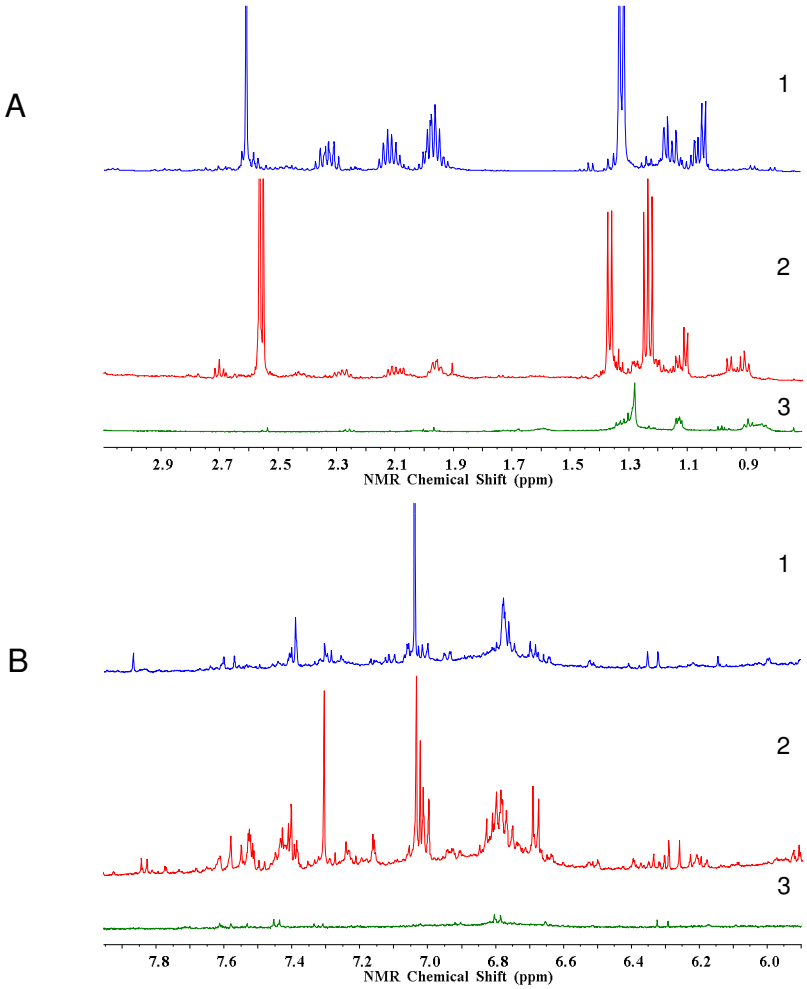


Figure 1. Comparison of ^{1}H NMR spectra of phenolics (A) and amino acids (B) regions of SPE fractions. Both (A) and (B) shows water (blue), methanol:water (1:1) (red), and methanol (green) fractions.

Wine type, vintage, and anti-TNFα activity

Anti-TNFα activities resulting from the SPE fractions of different wine samples are shown in Figure 2. Among the water fractions, the lowest activity is shown by Petit Verdot 2008 while the most active water fraction is of Aragones 2010 wine. Different vintages from the wines like Petit Verdot (2008 and 2010), Touriga Nacional (2009 and 2010), and Aragones (2007 and 2010) showed significantly different TNFα inhibition

by the water fraction. By comparing the water fraction from the 2010 vintage of all wine types, it is evident that Aragones is the most active against TNFα production.

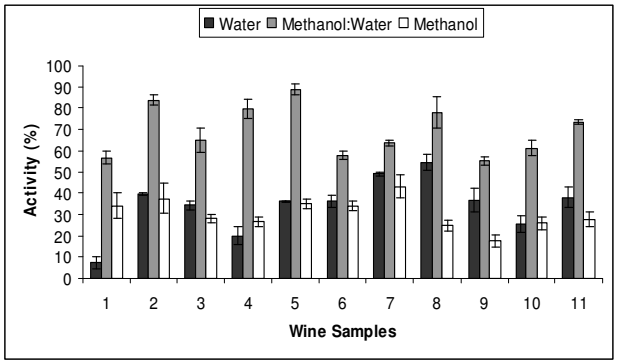
Table 1. ^{1}H NMR Chemical Shifts (δ) and coupling constants (Hz) of wine metabolites identified by using 1D and 2D NMR Spectra of the reference compounds (CD3OD-KH2PO4 in D2O, pH 6.0)

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)
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),

The methanol fraction of SPE showed nearly equal activity in the case of different vintages of the same wine except for Aragones, for which the 2009 vintage is significantly higher in activity than the 2010 vintage. The most active methanol fraction is also from the Aragones 2009 while the least active fraction is from Alicante Bouschet 2008. In most cases the activity shown by the methanol fractions are similar to their respective water fractions.

The methanol:water (1:1) SPE fractions is showed significantly higher anti-TNFα activity than the other two fractions. The vintage effect is very obvious in methanol:water fractions as Petit Verdot, Touriga Nacional, Aragones, and Alicante Bouschet from 2010 vintage are significantly higher in inhibiting TNFα production than the vintages of 2008 (Petit Verdot, Touriga Nacional, and Alicante Bouschet) and 2007 (Aragones). Among the different wine types, the Touriga Nacional (2010) showed the maximum anti-TNFα activity, but not significantly higher than the other wines from the same 2010 vintage.



1. Petit Verdot 2008
2. Petit Verdot 2010
3. Touriga Nacional 2008
4. Touriga Nacional 2009
5. Touriga Nacional 2010
6. Aragones 2007
7. Aragones 2009
8. Aragones 2010
9. Alicante Bouschet 2008
10. Alicante Bouschet 2009
11. Alicante Bouschet 2010

Figure 2. Anti-TNFα activity (%) shown by SPE extracts of different wine samples at the concentration of 100 μgmL^{-1}.

Principal component analysis (PCA)

Multivariate data analysis algorithms are an essential component of any metabolomics studies. These methods, either supervised or unsupervised, are used to reduce the dimensionality of multivariate dataset and thus enable to recognize possible differences or similarities among the samples. Principal component analysis (PCA) is a primary tool among the various multivariate data analysis methods. It is an unsupervised method and samples are clustered or separated purely due to metabolic similarities or differences, respectively. The NMR data from the SPE fractions of all the samples have been subjected to PCA in order to highlight the differences existed among the SPE fractions and to identify the metabolites responsible for that distinction. Figure 3 shows the score plot of PCA where samples are colored according to SPE fractions.

The PCA score plot shows clear separation of all three fractions of SPE with tight clustering among the samples of same fraction. It is evident that water fractions are clustered on the negative side of PC1 (61.4%) and positive side of PC2 (20.9%) while the methanol fractions are grouped on the positive side of PC1 and PC2, with few exceptions. The methanol:water fractions are assembled on the negative side of PC2 while nearly distributed on both positive and negative sides of PC1. By examining the corresponding loadings plot, metabolites responsible for this separation are revealed. As shown by the NMR spectra (Figure 1), the methanol fractions are rich in fatty acids and amino acids including alanine, threonine, valine, arginine, and glutamic acid while the water fraction contain higher levels of glucose and sucrose with major organic acids like malic acid, tartaric acids, and succinic acids. The water:methanol fractions contain

relatively higher amounts of phenolics like quercetin, caftaric acid, coutaric acid, and resveratrol, as compared to other fractions of solid phase extraction. The unsupervised PCA thus clearly separates the fractions but did not show separation on the basis of activity.

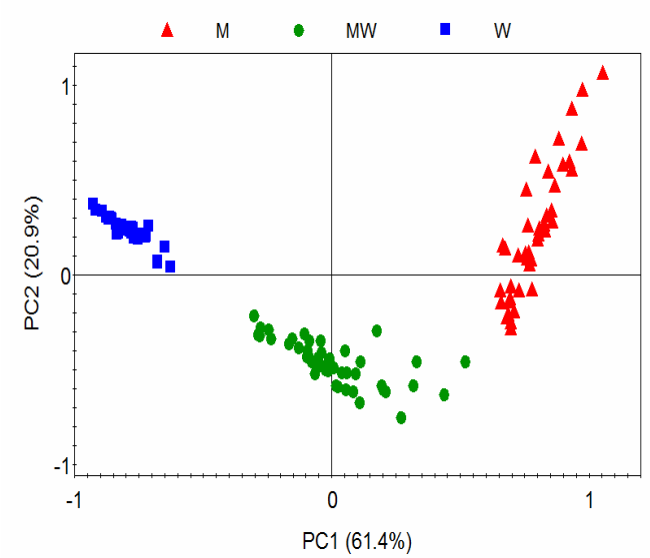


Figure 3. Principal component analysis (PCA) score plot of SPE fractions of all the wine samples. All the three fractions are clearly separated from each other. Samples in blue, green, and red represents water, methanol:water (1:1), and methanol fractions, respectively.

Projections to latent structures-discriminant analysis (PLS-DA)

To identify compounds related to activity, supervised methods are required. This aims to separate actives from lesser actives. PLS-DA is a supervised multivariate data analysis method, performed with a pre-input data regarding the analyzed samples i.e. classification of samples by creating dummy *Y*-variables. The samples were classified into high (>50%), medium (<50% and >29%), and low (≤29%) activity classes. The 3D-score plot of PLS-DA (Figure 4A) shows good separation of samples with high activity from the others. Not a clear distinction between the samples with medium and low activity is observed. To discriminate the low and medium activity samples, another supervised algorithm, bidirectional orthogonal PLS-DA (O2PLS-DA), was used. As shown by the score plot (Figure 4B), a very nice separation among all the three classes of samples is achieved. By examining the corresponding loadings plot, metabolites

responsible for the separation are identified. 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 medium activity samples.

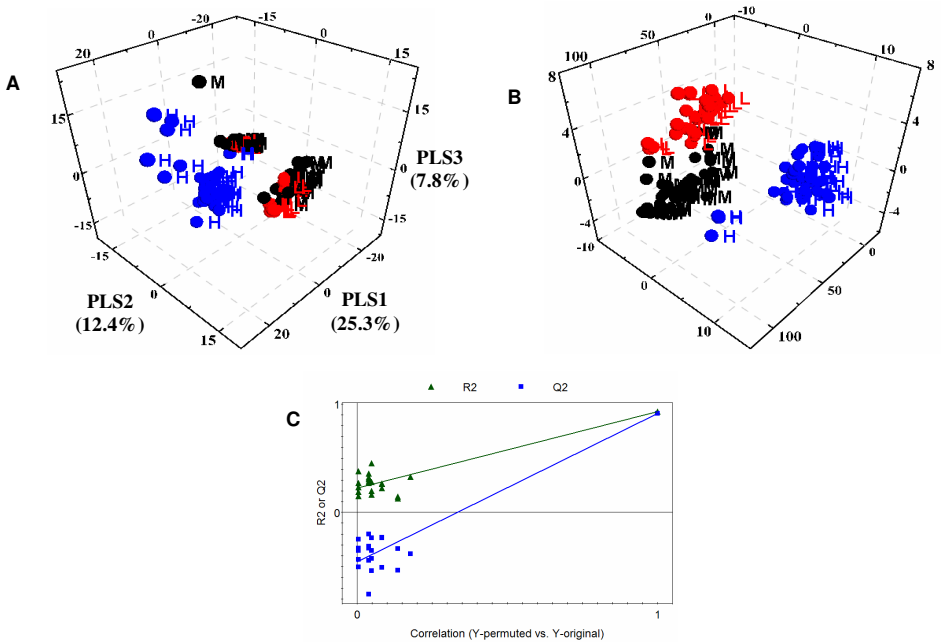


Figure 4. Score plots of PLS-DA (A) and O2PLS-DA (B). Samples with high, medium, and low anti-TNF α activity are presented with 'H', 'M', and 'L'. The validation plot of permutation test for PLS-DA (C) using ^{1}H NMR resonances and anti-TNFα based on three classes.

Validation of PLS-DA

One of the key aspects of a supervised regression algorithm is model validation. For the data reduction methods like PLS-DA, a permutation test is often used for validation. The permutation test is the calculation of goodness of fit (R2, describes how well the data is mathematically reproducible) and the predictive ability of the model (Q2). The R2 value can vary from 0 to 1, where 1 means a model with a perfect fit. If the Q2 value is more than 0.5, the model is considered to have good predictability and if it is higher than 0.9 and less than 1.0, then the model is considered to have an excellent predictability. It is suggested that if more than five PLS components are included in the

model the training set data generally reproduce excellently. The R2 and Q2 values of PLS-DA were calculated using six components. For anti-TNFα activity the R2 and Q2 values for PLS-DA analysis were 0.92 and 0.91. This PLS-DA models was validated by the permutation method through 20 applications in which all Q2 values of permuted Y vectors were lower than original ones and the regression of Q2 lines intersect at below zero (Figure 4C).

Variable importance in the projections (VIP)

Variable importance in the projections can be defined as a weighted sum of squares of the PLS weights. It has been indicated that it is directly proportional with the influence of a factor on the separation on score plot, meaning, factors have higher VIP values are more important for the sample separation. For O2PLS-DA analysis, 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 (Eriksson et al. 2006). Among the identified phenolics in wine during this study the VIP values of the major contributing metabolites are as follows; caftaric acid at δ 7.02: 1.91, quercetin at δ 7.71: 1.74, coutaric acid at δ 7.59: 1.42, and (+)-catechin at δ 5.89: 1.18. Such high VIP scores for the identified phenolics authenticate their involvement in the separation of high activity samples and suggest a role of these compounds in inhibiting TNFα production.

Discussion

Various multivariate data analysis methods were used in combination with NMR spectroscopy in order to correlate the activity data of the extracts with the spectroscopy data of the same. Such analyses of extracts from *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), were successful in linking pharmacological activities with certain compounds. This approach is very effective in the screening of plant extracts in order to identify active compounds without laborious fractionation and chromatographic separation of the crude extract. Fractions from SPE of various red wines from Portugal were analyzed for anti-TNFα activity and the

combination of NMR spectroscopy and chemometrics was successfully applied to identify the active ingredients.

The vintage effect on metabolic profile of grapes and ultimately on wine has been extensively studied (Pereira et al. 2006b; Lee et al. 2009) 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 TNFα 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 either in 2010 vintage the berries experienced hot and dry climate which ultimately resulted in higher phenolic contents and more potency towards TNFα inhibition. Another possibility is the fact that aging of wine can cause significant decrease in its phenolics content which ultimately resulted in the lower activity of old wine sample (2008 and 2009 in this case) (Waterhouse 2002).

It is a fact that diet has beneficial effects and the consumption of antioxidant rich food (fruits, vegetables, tea, and wine) has 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 prevent cardiovascular diseases (Cordova et al. 2005), renal disorders (Bretelli et al. 2005), Alzheimer's disease (Marambaud et al. 2005), cancer (Barstad et al. 2005), and also active against bacteria (Murray et al. 2002) and viruses (Takkouche et al. 2002). Several health promoting activities associated to wine polyphenols were comprehensively reviewed recently (Opie and Lecour 2007; Halpern 2008; Cordova and Sumpio 2009; Ali et al. 2010). Phenolics are well known for their potency against TNFα production and they are widely accepted to have anti-oxidative and anti-inflammatory properties (Chuang et al. 2009; Baur et al. 2006). 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). The present study is an attempt to analyze different wine types and vintages for TNFα inhibition. The proposed approach is found very effective

in discriminating the SPE fractions from different wine types and vintage based on the efficacy to reduce TNFα production, and relate the activity with active constituents.

Conclusion

This study used an NMR spectroscopy-based metabolomics approach to reveal the correlation of characteristic metabolic profiles of different wines with anti-TNFα activity. Solid phase extraction in combination with different chemometrics methods proved that the active ingredients in an extract could be identified using a PLS-based regression models with ^{1}H NMR and anti-TNFα activity data set. Phenolics like quercetin, caftaric acid, and (+)-catechin are identified as most influential in inhibiting TNFα production among the other wine metabolites. It is suggested that the similar approach can be applied for the prediction of anti-TNFα activity of crude plant extract using NMR and multivariate data analysis. The methodology proposed here can be applied to connect bioactivities associated to wine with active constituents without any laborious chromatographic separation of metabolites.