^{1}H NMR Spectroscopy with Multivariate Statistical Analysis as a Tool for a Rapid Screening of the Molecular Changes Occurring During Micro-Oxygenation of an Italian Red Wine

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Abstract: A red wine from Aglianico vine was subjected to a micro-oxygenation procedure for five weeks. The wine was weekly sampled together with a non micro-oxygenated control and extracted with di-ethyl-ether in order to simplify NMR analyses. In fact, ^{1}H NMR spectra of the whole wine samples were dominated by water and ethanol signals which prevented visibility of the resonances generated by the less concentrated wine components. The ^{1}H NMR analyses of the di-ethyl-ether extracts revealed an increase of the amount of oxygen-containing materials. Such increase was attributed to a wine ageing process which resulted, hence, fastened by application of micro-oxygenation.

Keywords: ^{1}H-NMR, wine; micro-oxygenation, principal component analysis, wine ageing.

INTRODUCTION

Red wine is a complex mixture of different compounds among which water, ethanol, glycerol, sugars, many organic acids and several ions play a major role. Conversely, aromatic alcohols, aminoacids and phenols are present at a very low concentration [1]. Notwithstanding their low concentration, phenolic compounds are the main responsible for some wine organoleptic properties such as colour and astringency [2]. In addition, they appear to affect positively human health due to their capacity to reduce coronary heart diseases when a regular and moderate consumption of wine is achieved [3].

Phenolic composition of wines depends on both the nature of the grapes used and on wine-making conditions [4]. Particularly, changes in the phenolic content can be observed when oxygen either accidentally, during processes such as filtration, or intentionally, during processes as the splash racking, is dissolved in wines [5].

Oxygen is involved not only in the changes of the phenolic composition, but also in promoting synthesis of sterols and fatty acids in yeast cell walls during fermentation, in increasing the apparent mouth-feel of wines stored in barrels, in diminishing wine herbaceous characters and in removing unpleasant sulfidic aromas [6].

In order to control the amount of oxygen dissolving in wines and to address the oxygen-requiring reactions to more desirable changes in wine texture and aroma, the very controversial and still misunderstood technique of micro-oxygenation has been developed during the nineties [5-9].

Micro-oxygenation consists in the introduction of small and measured amounts of oxygen to wines while avoiding its accumulation. The organoleptic changes during micro-oxygenation can be separated in two distinct phases [8]. The first one, also referred to as structuring phase, has been related to an increase in the aggressiveness and intensity of tannins to the palate, while a decline of the aromatic intensity and wine complexity is tasted [8]. According to Lemaire [5], the length of the structuring phase is dependent upon the initial wine phenolic content, the timing of the micro-oxygenation, the amount of the added oxygen, the temperature, the level of sulphur dioxide, and the extent of aeration.

The second phase, called harmonization, is characterized by an increase of tannin softness and general wine complexity and length [8]. The optimum end point of the micro-oxygenation process is believed to belong at the maximum complexity of wine, when the highest tannin softness and suppleness are reached [6, 8].

In the recent years a number of papers appeared in the literature reporting about chemical transformations occurring during wine production [2, 4, 10-17]. However, up to now no papers dealt with the molecular changes occurring during the use of the micro-oxygenation procedure.

The present paper does not pretend to exhaustively detail the molecular composition of a red wine during micro-oxygenation. Indeed, the intention was to show that application of fast mono-dimensional ^{1}H NMR spectroscopy coupled with multivariate statistical analysis allow a rapid screening of the changes occurring in a red wine subjected to micro-oxygenation, thereby revealing that controlled amount of oxygen in wines favours wine ageing.

MATERIALS AND METHODS

Wine Samples

An Aglianico wine was produced by using grapes from October 2004 vintage. Two different stainless steel containers were used to store the wine. One of the tanks was connected to a Parsec® SAEN 4010 micro-oxygenation equip-

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1874-7698/08

ment in order to instil into the wine 4 ml of oxygen per liter of wine per month. The wine stored in the second tank was used as a control and kept alike. Wine was weekly sampled from each stainless steel tank for five weeks. A total of 10 samples were obtained: five samples were from the micro-oxygenated tank and 5 from the non micro-oxygenated container. After sampling, the wines were immediately extracted as reported below.

Di-Ethyl-Ether Extraction

Di-ethyl-ether extraction was applied as described in Rodriguez-Delgado *et al*. [18]. Namely, 10 mL of each wine sample were added with a 0.1 M HCl solution till pH 2.0. Thereafter, a liquid-liquid extraction was performed by adding 10 mL of HPLC grade di-ethyl-ether (supplied by Sigma©) and repeated trice. The organic phase was separated, roto-evaporated (Heidolph WB 2000 rotoevaporator) at 50°C and stored for NMR analyses.

NMR Analysis

The dried di-ethyl-ether extracts were dissolved in 0.50 mL of $CDCl_3$ and analyzed by monodimensional ^1H NMR spectroscopy. A Bruker Avance 400™ operating at a proton frequency of 400.075 MHz, equipped with a 5 mm SB PABBI ^1H-BB probe and set with an acquisition time of 4 s, a recycle delay of 2 s, a 32 k time domain, and a 90° ^1H pulse of 7.60 μs with a power attenuation of -2.0 dB was used to acquire all the spectra. Topspin 1.3 software was used to acquire and elaborate all the NMR spectra.

Multivariate Statistical Analysis

A bucket table consisting of a (n x 9) matrix was generated from the NMR spectra. The n rows represented the wine samples. The nine columns were the relative areas of the NMR spectral intervals 0-1.1, 1.1-1.8, 1.8-2.1, 2.1-3.1, 3.1-3.5, 3.5-4.0, 4.0-4.6, 4.6-6.5, and 6.5-8.5 ppm. The 9th-dimentional vector for the data set was analyzed by Principal Component Analysis (PCA) after data normalization. The statistical software package XLSTAT 6.1 (Addinsoft SARL, France) was applied to run the analysis.

RESULTS AND DISCUSSION

The ^1H NMR analyses of the bulk wines (either non-micro-oxygenated, NMO, or micro-oxygenated, MO) produced a series of spectra consisting of only two sets of signals attributed to water and ethanol. In fact, water and ethanol are the most abundant components in wines and their NMR signals hide all the other resonances generated by the less abundant wine components.

In order to enhance detectability of the less abundant wine molecular systems, extraction of wines with di-ethyl-ether was conducted. Rodriguez-Delgado *et al.* [18] reported that wine di-ethyl-ether extraction produces polyphenolic mixtures. For this reason, according to these authors, ^1H-NMR spectra containing the largest signals mainly in the interval attributed to aromatic protons (around 9 to 7 ppm) should be expected. Conversely, the spectra of NMO and MO wines revealed not only signals in the interval 9-7 ppm, but also between 7 and 4.5 ppm (olephinic protons) and between 4.5 and 0 ppm (protons in O/N containing systems and aliphatic H), thereby suggesting that polyphenols were not the only molecules extracted by di-ethyl-ether (spectra not shown).

The discrepancy between the experimental NMR results and those which would have been theoretically expected was attributed to the sensitivity of the NMR spectroscopy which enabled detection of all the materials co-extracted with polyphenols. Moreover, it must be also ruled that the di-ethyl-ether extracts described in Rodriguez-Delgado *et al.* [18] were analysed by liquid chromatography with UV and fluorescence detectors, which allow only visibility of molecules with a very high degree of aromaticity.

The quantitative NMR measurements were used to generate data set for principal component analysis (PCA). PCA is an unsupervised multivariate statistical method [19] used for both recognition of similarities and/or dissimilarities among samples and reduction of the high amount of original variables into just few principal components retaining high percentage of the original information. In the present study, PCA reduced the number of variables to only 3 (PC1, PC2 and PC3) that accounted for 91% of the total variance (Fig. (**1A**)). The 3D plot reported in Fig. (**1**) A revealed that the samples can be grouped in two main sets. The first one (set 1) holds the extracts obtained from NMO and MO samples in weeks 1 to 4 (1w to 4w), whereas the second set (set 2) contains the di-ethyl-ether extracts from the non micro- and micro-oxygenated wines at the 5th week from the starting of the experimental session (5w).

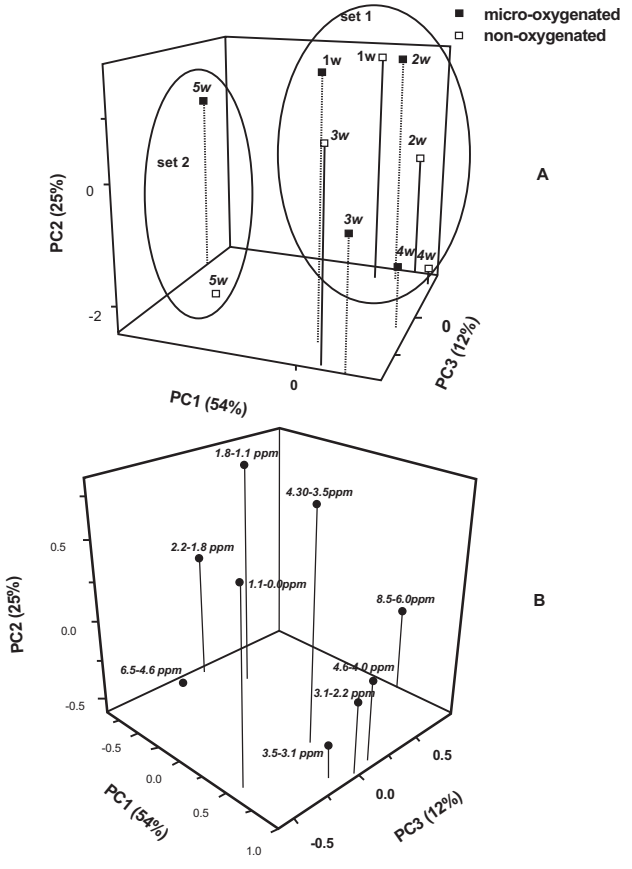

Fig. (1). 3D-score (**A**) and loading (**B**) plots obtained from the NMR spectra. set 1 and set 2 are the two different groups identified by PCA. The numbers followed by w indicate the i-th week during which wine sampling was done.

Table 1. Possible Attributions of the Signals Differentiating the Spectrum of the Wine after 5 Weeks of Micro-Oxygenation

Compounds	Signals in the 1.8-1.1 ppm interval	Signals of the spin system in the remaining spectrum
Acetoin	1.40 (CH3, d)	4.24 (CH, q); 3.65 (OH, s); 2.18 (CH3, s)
L-Alanine	1.29 (CH3, d)	3.79 (CH, q)
Diethyl oxalate	1.34 (CH3, t)	4.50 (CH2, q)
Acetaldehyde diethyl oxalate	1.30 (CH3, d); 1.21 (CH3, t)	4.68 (CH, q); 3.63 (CH2, q)
Ethyl acetate	1.26 (CH3, t)	4.20 (CH2, q); 2.03 (CH3, s)
Diethyl malonate	1.29 (CH3, t)	4.24 (CH2, q)
Diethyl succinate	1.26 (CH3, t)	4.14 (CH2, q); 2.82 (CH2, t)

The samples contained in set 1 can be differentiated due to variations along PC2 and PC3. In particular, the main NMR signals characterizing both 1w samples were those included in the regions 4.0-3.5, 1.8-1.1 and 1.1-0 ppm (Fig. (**1B**)). The three intervals can be attributed to alkenes (4.0-3.5 ppm) and to alkyl chains (1.8-1.1 and 1.1-0 ppm), respectively. After the 2nd week the extract from the micro-oxygenated sample (black 2w square in Fig. (**1A**) was similar to the 1w ones. Conversely, the NMO extract (white 2w square) was richer in acid and aromatic protons (8.5-6.0 ppm), and alcohol systems (4.6-4.0 ppm) (Fig. (**1B**)). At the 3rd week only the MO sample changed since a diminution of alkenes and alkyls was followed by an increase of acidic and aromatic protons and alcohols (Fig. (**1B**)).

When the 4th week of the experiments was reached, both NMO and MO extracts revealed similar composition (Fig. (**1A**)). However, they differed from the samples collected in weeks 1 to 3 because of a larger amount of protons resonating in the interval 3.5-3.1 ppm (Fig. (**1B**)). According to literature [20], signals resonating between 3.5 and 3.1 ppm can be attributed to N-alkyl groups such as in aminoacids.

Set 2 containing polyphenolic extracts at the 5th week were differentiated from the other samples due to changes along PC1 (Fig. (**1A**)). However, the two samples at the 5th week differed between each other along PC2. In fact, the main constituents of MO (black 5w square in set 2) were resonating in the region 1.8-1.1 ppm, whereas NMO (white 5w square) showed signals in the interval 6.5-4.6 ppm as main components of the di-ethyl-ether mixture.

Since the main difference among the samples at the 5th week were found mainly in the interval 1.8-1.1 ppm (Fig. (**1**)), an attempt to assign the signals in that interval is reported in Table **1** according to the data base at http://riodb01.ibase.aist.go.jp/sdbs/cgi-bin/cre_index.cgi?lang=eng.

The doublet at 1.40 ppm was attributed to the methyl group of acetoin. The remaining signals of acetoin were identified in the quartet at 4.24 ppm (CH), in the broad singlet at 3.65 ppm (OH), and in the singlet at 2.18 ppm (CH_3). L-alanine was recognized in the doublet at 1.29 ppm (CH_3) and in the quartet at 3.79 ppm (CH). Diethyl oxalate was assigned to the triplet at 1.34 ppm (CH_3), and the quartet at 4.50 ppm (CH_2). The doublet at 1.30 ppm (CH_3), the triplet at 1.21 ppm (CH_3), and the quartets at 4.68 (CH) and 3.63 ppm (CH_2) were all assigned to the groups of acetaldehyde diethyl oxalate. Ethyl acetate, diethyl malonate and diethyl succinate were recognized in the signals at 1.26, and 1.29 ppm (methyl groups in the 3 systems). These signals were associated to the quartet at 4.20 ppm (CH_2) and the singlet at 2.03 ppm (CH_3) typic of ethyl acetate, to the quartet at 4.24 ppm (CH_2) typic of diethyl malonate, and to the multiplets at 4.14 and 2.82 ppm (CH_2) characteristic of the diethyl succinate.

CONCLUSIONS

The application of a multivariate statistical method to compare the ^{1}H NMR spectra of different wine systems permitted a satisfying discrimination among samples. In fact, the PCA was able to extract the main similarities and dissimilarities among the molecular components identified in the spectra of wine samples stored in different conditions in stainless steel tanks. The wine in one of these tanks was subjected to micro-oxygenation, whereas the second wine in the different tank was not micro-oxygenated.

Before ^{1}H NMR measurements, the MO and NMO wines were extracted with di-ethyl-ether. The extraction was needed to eliminate the interferences of water and ethanol which were the sole visible signals in the ^{1}H NMR spectra of the whole wine samples.

The relative areas in ^{1}H NMR spectra of di-ethyl-ether wine extracts were measured and used as input parameters for the statistical comparison among the MO and NMO wines.

The statistical analyses performed with PCA revealed that the main dissimilarities between the micro- and non micro-oxygenated wines were observed at the fifth week from the starting of the micro-oxygenation process. In particular, the dissimilarities were due to the spectral region 1.8-1.1 ppm. The main signals in this region were attributed to acetoin, an aminoacid, and some esters (Table **1**). According to literature data [21-22], increase of oxygenated molecular systems can be related to wine ageing, thereby suggesting that the micro-oxygenation fasten a process that normally requires years to be achieved.

Notwithstanding the preliminary nature of this study (a more detailed NMR analysis is needed to univocally assign

all the resonances in the spectra), the results of this work revealed that datasets obtained from ^{1}H NMR spectra are useful for a rapid and satisfactory statistical discrimination among wine samples and are a valid help for quality evaluation in wine chemistry.

ACKOWLEDGEMENTS

Azienda Agricola Mustilli (Sant'Agata dei Goti, Benevento, Italy) is acknowledged for the wine samples. NMR machine time was scheduled at the NMR centre CERMANU of the University of Naples Federico II.

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Received: February 27, 2008 Revised: June 09, 2008 Accepted: June 12, 2008