Comparison of two sample preparation methods for ^{1}H-NMR wine profiling: Direct analysis and solid-phase extraction

I. Ocaña-Rios[1], F. Ruiz-Terán[2], M. E. García-Aguilera[1], K. Tovar-Osorio[1], E. Rodríguez de San Miguel[2] and N. Esturau-Escofet[1]

[1] Instituto de Química, Universidad Nacional Autónoma de México, Ciudad de México, México
[2] Facultad de Química, Universidad Nacional Autónoma de México, Ciudad de México, México

Summary

This study compares two sample preparation methods: direct analysis (DA) and solid-phase extraction (SPE) for wine samples analysis by proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy. For this purpose, the profile of Mexican commercial wines was evaluated. The selected wines were produced with different grape varieties: ('Barbera', 'Nebbiolo', 'Zinfandel', 'Merlot', 'Petite Syrah', 'Cabernet Sauvignon', 'White Zinfandel' and mixture of 'Chenin blanc' and 'Colombard') coming from the principal wine-producing region of Mexico, Baja California State. DA provided faster data acquisition in comparison to SPE and preserved the original sample composition. Using DA-NMR thirty two metabolites were identified, including organic acids, amino acids, sugars, alcohols and phenolic compounds. Furthermore, SPE-NMR using a polymeric sorbent allowed to retain phenolic compounds giving a better picture on the aromatic region and eliminating major polar compounds like water, ethanol and sugars. Fourteen compounds were identified by SPE-NMR including higher alcohols, flavanols and hydroxybenzoates. A control chart for the first principal component allowed to confirm the precision of the SPE-NMR method, while a comparison of the concentration of two metabolites found in both methods was used to evaluate their recovery (20 % for isoamyl alcohol and 78 % for phenethyl alcohol). The information obtained with both methods about the main compounds and phenolic metabolites provides new insights into the metabolomic profile of wine, which could be useful in future targeted studies.

K e y   w o r d s : wine; ^{1}H-NMR; direct analysis; solid-phase extraction; metabolomic profile.

Introduction

Wine has been described as a complex mixture of chemical compounds and its metabolomic analysis is a difficult task. The primary chemical components in this drink came from grapes during the fermentation process, the secondary compounds are produced or transformed by microorganisms, and finally wood metabolites and those formed during bottle aging are the third group of compounds (Moreno-Arribas et al. 2005).

The study of wine requires the use of a diverse range of instrumental techniques due to the matrix complexity. Techniques like high performance liquid chromatography (HPLC), gas chromatography (GC) and capillary electrophoresis (CE) coupled to mass spectrometry (MS) (Malec et al. 2017, Song et al. 2013, Kustos et al. 2020, Acunha et al. 2016), vibrational spectroscopy (e.g. Infrared) (Croce et al. 2020) and nuclear magnetic resonance (NMR) spectroscopy (Hong 2011, Spyros et al. 2012) have been used for wine analysis. Among these, NMR is non-destructive, robust, highly reproducible and it is capable of simultaneous detection of a great number of compounds. It has also short time analysis and it provides very specific structural information of the compounds, which can be used for fingerprinting (Cozzolino 2016). The use of ^{1}H-NMR spectroscopy to analyze the unaltered wine metabolic profile by direct analysis (DA) has been very useful to study wine evolution during bottle aging (Cassino et al. 2019), to differentiate wines according to variety, geographical origin, vintage and for authenticity (Fan et al. 2018, Ragone et al. 2015, Gougeon et al. 2018, Gougeon et al. 2019a and b, Son et al. 2018). For DA, wine samples are mixed with a small amount of deuterated water (D_2O) with or without a buffer, so the most intense peaks in spectra correspond to water and ethanol, which are the main wine components. Therefore, solvent suppression techniques are essential in wine ^{1}H-NMR metabolic studies, and one of the most widely used is the application of automated eightfold suppression of water and ethanol signals (Monakhova et al. 2011).

DA has mainly been used for the characterization of wines and a great amount of metabolites have been identified; however, due to the wine chemical complexity there are compounds that require to be concentrated and/or extracted from the beverage in order to get a better compound identification. Sample preparation methods like lyophilization and nitrogen-flow concentration have been used to pre-concentrate compounds (minor compounds). In both methods, a decrease in signal intensity for some metabolites was observed compared to the ^{1}H spectrum of

wine without pre-concentration, especially in the aromatic region, in addition the residual ethanol signals are still observed. Furthermore, lyophilization presented long-time consumption and precision problems (Amaral *et al.* 2005, Aru *et al.* 2018).

Another method of pre-concentration is solid-phase extraction (SPE) that can be applied to concentrate volatile and semi-volatile compounds from liquid samples. This technique is based on the selective retention and the subsequent elution by an appropriate solvent. The selectivity is achieved according to the type of sorbent and eluent employed (Castro *et al.* 2008). There are studies where sorbents were used to analyze the volatile fraction of red and white wines by gas chromatography (GC). The most reported sorbents used for wine analysis are polymers, such as styrene-divinylbenzene copolymers; they have a great load capacity, stability at extreme pH values and they can be used in normal and reversed mode (Castro *et al.* 2008, Andujar-Ortiz *et al.* 2009, Sánchez-Palomo *et al.* 2015). SPE is mainly used before chromatographic analysis and the analytical conditions need to be selected carefully (mobile phase, column type, etc) (Anastasiadi *et al.* 2009). In NMR-based metabolomics, NMR spectra can be obtained with higher reproducibility than chromatograms, and SPE has successfully been used, for example, to selectively retain phenolic compounds in grape juices (Ali *et al.* 2011, Savage *et al.* 2011). These kind of compounds are also very important for wine analysis because they can be used as a metabolic fingerprint for the classification of wines according to variety, vintage, geographical region and even production winery, as phenolic secondary metabolites are strongly affected by several factors, like grape variety, soil, climate and vinification technique (Anastasiadi *et al.* 2009, García-Esparza *et al.* 2018). Furthermore, in wine analysis SPE can provide a suitable means to obtain information to find tentative marker in 'Corvina' and 'Primitivo' wines (Kessler *et al.* 2018).

The aim of this work is to apply and evaluate SPE as pre-concentration method to improve the aromatic compounds profiling by ^{1}H-NMR. For this purpose, the comparison of the DA and the SPE methods was performed analyzing Mexican white and red wines by ^{1}H-NMR.

Material and Methods

C h e m i c a l s ,   r e a g e n t s   a n d   m a t e r i a l s : Sterile cryovials (2 mL) were acquired from Simport Scientific (Quebec, Canada). CHROMABOND HR-P SPE cartridges (6 mL, 200 and 500 mg) from MACHEREY-NAGEL (Germany). Milli-Q water (18.2 MΩ cm^{-1}resistivity) was obtained from a deionizer (Millipore Direct-Q3 UV, USA). 3-(trimethylsilyl)propionic-2,2,3,3-d$_4$ acid sodium salt (TSP, D 98 atom %), hydrochloric acid (36.5 %), sodium hydroxide (97 %) and all HPLC grade solvents (methanol, ethanol, and dichloromethane) were acquired from Sigma-Aldrich (USA). Monobasic potassium phosphate (KH$_2$PO$_4$, 99 %) was obtained from J.T. Baker (Mexico). Deuterated chloroform (CDCl$_3$, D 99.8 atom %) was acquired from Cambridge Isotope Laboratories (USA) and deuterated methanol (MeOD-d_4, D $\geq$ 99.8 atom %) from Sigma-Aldrich (Switzerland).

W i n e   s a m p l e s : All commercial wines, produced in Baja California State, Mexico, were purchased in a local store. Six red and two white wines were employed, their variety, harvest year and alcohol content were: 'Barbera' (2015, 14 % v/v), 'Nebbiolo' (2016, 13.7 % v/v), 'Zinfandel' (2016, 13.5 % v/v), 'Merlot' (2017, 13 % v/v), 'Petite Sirah' (2016, 13.8 % v/v), 'Cabernet Sauvignon' (2017, 13 % v/v), 'White Zinfandel' (2019, 11.5 % v/v), Blanc of Blancs wine made with 'Chenin Blanc' and 'Colombard' grapes (2019, 12 % v/v). It is important to mention that according to Mexican legislation varietal wine should be indicated on the label when 75 % of one variety grape was used, whereas when two or more varieties were used two of them, at least, should be mentioned on the label (NOM-199-SCFI-2017).

D i r e c t   a n a l y s i s : Nine hundred microliters were taken from the wine samples using a one milliliter automatic pipette (Eppendorf, Germany) and placed in a cryovial. pH was adjusted using a combined Titration pH Unit (BTpH, Bruker, Germany), where samples were diluted with 100 µL of a phosphate buffer (1 M, pH 3.1, in D$_2$O) with 0.1 % (w/w) of TSP and then, pH was adjusted to 3.1 using 1M HCl/1M NaOH. Thereafter, 600 µL of this solution was transferred to a 5 mm NMR tube.

S o l i d - p h a s e   e x t r a c t i o n   p r o c e d u r e : For SPE, a vacuum manifold was used (Chromabond SPE, Macherey-Nagel, Germany). Chromabond HR-P cartridges were conditioned with 5 mL of methanol, 3 mL of ethanol and 5 mL of ethanol-water solution (10:90, v/v). Then, 40 mL of sample was loaded at flow rate of about 2 mL·min^{-1}. After that, the cartridge was washed with 20 mL of water and dried under vacuum. Subsequently, the compounds were eluted with 5 mL of methanol. The extract was evaporated to dryness under nitrogen stream, and then re-dissolved with 700 µL of 0.024 % (w/w) TSP solution in MeOD-d_4. Finally, 600 µL of this solution were transferred to a 5 mm NMR tube.

The precision in terms of repeatability was assessed by triplicate determination of a 'Cabernet Sauvignon' sample. To reduce data dimensionality binning was performed from 0.002 to 9.998 ppm with a bin size of 0.004 ppm (Chenomx NMR suite v. 8.4), giving 2450 bins in total. The precision was evaluated using a control chart at 95 % confidence for the first component (PC1) using SIMCA software (Umetrics, Sartorius Stedim Biotech, v 16.0.1).

Accuracy was evaluated with the recovery obtained by comparing the metabolites concentration of those detected with both methods: SPE-NMR and DA-NMR, considering that this last methodology requires no sample pretreatment and the original composition is maintained. The concentration was obtained using the relative method with the following equation:

$$C_X = \left(\frac{I_X}{I_{IS}}\right) * \left(\frac{N_{IS}}{N_X}\right) * (C_{IS})$$

where C, I and N are concentration, integral area and number of protons giving rise to the selected signal, respectively, of the metabolite (X) and the internal standard (IS) which was TSP. The integral areas from ^{1}H-NMR spectra of each

sample were calculated with MestReNova software v. 12.0. (Mestrelab Research SL., Santiago de Compostela, Spain).

N M R   w i n e   a n a l y s i s : NMR analysis were performed at 300 K on a 700 MHz Avance III spectrometer set at 699.95 MHz ¹H frequency (Bruker, Billerica, MA, USA) with a 5-mm z-axis gradient TCI cryoprobe and a SampleJet autosampler. For temperature equilibration a 5 min delay was applied before starting the experiments. Data were recorded automatically using ICON-NMR (Bruker, Billerica, MA, USA).

For DA, two successive ¹H-NMR experiments were recorded for each sample with a time domain (TD) of 65,536 data points (64 k), a spectral width (SW) of 20.14 (14097.74 Hz), a relaxation delay (RD) of 4 s and an acquisition time (AQ) of 2.3 s, resulting in a total recycle time of 7.3 s. The first experiment was a standard water presaturation (Bruker sequence *zgpr*) to determine the exact frequencies of the ethanol and water signals in each specific sample. ¹H 90° hard pulse length (P1) was calibrated on each sample. Parameters for this experiment were 4 dummy scans (DS) and 4 scans (NS). Second experiment was a ¹H-NMR pulse sequence with suppression of water and ethanol signals using the frequencies identified in the first experiment (8-fold suppression, Bruker sequence *noesygppszgpr1d* (MONAKHOVA *et al.* 2011). For this experiment DS = 4, NS = 32, RG=16 and mixing time (D8) of 10 ms were set. FIDs were multiplied with an exponential function corresponding to LB = 0.3 Hz prior to Fourier transformation.

For the SPE extracts, standard ¹H-NMR spectra were recorded (Bruker pulse sequence *zg30*) with RD = 2 s, AQ = 2.3 s, DS = 2, NS = 32, SW = 20.14 (14097.74 Hz), TD = 64K and RG = 8. Additionally, 2D-NMR experiments were performed on representative samples to confirm chemical shift assignments, including ¹H-¹H Correlation Spectroscopy (COSY), Heteronuclear Single Quantum Coherence Spectroscopy (HSQC) and Heteronuclear Multiple Bond Correlation (HMBC) using standard Bruker pulse programs.

All NMR spectra were phased and baseline-corrected using Topspin v. 3.5.6 (Bruker, Billerica, MA, USA) and processed using MestReNova v. 12.0 (Mestrelab Research SL., Santiago de Compostela, Spain). ¹H chemical shifts

are reported in ppm (δ) relative to TSP signal (0.0 ppm) for experiments in D_2O. For experiments in MeOD-d_4 chemical shifts were referenced to the residual solvent resonance at 3.3 ppm.

Results and Discussion

D i r e c t   w i n e   a n a l y s i s : Typical ¹H-NMR spectra obtained from red and white sampled wines by DA are shown in Fig. 1. The region between δ 0.9 and 4.0 ppm gives information about amino acids (alanine, valine, proline, arginine and 4-aminobutyrate) and organic acids (succinic, acetic, lactic, citric, pyruvic and malic acids). Other compounds including choline, 2,3-butanediol and acetoin were also identified in this region. The δ 4.0-5.5 ppm region was shown to be the place where sugars anomeric protons appeared; this region showed signals of fructose, glucose, arabinose and xylose. The aromatic region, δ 6.0-9.0 ppm, showed less intense peaks than other regions, signals from different kind of metabolites such as tyrosine, shikimic acid, gallic acid and phenolic compounds as trigonelline, epicatechin, phenethyl alcohol and caffeic acid were observed. In total the analysis of ¹H-NMR spectra allowed the identification of thirty two metabolites (Tab. 1). Assignments of these compounds were done by comparing published data (GOUGEON *et al.* 2018, ARU *et al.* 2018) and using Chenomx NMR suite v. 8.4 (Chenomx Inc, Edmonton, Canada).

As expected, signals of organic acids like citric and malic, as well as sugars were more intense in white wines than those observed in red wines. On the other hand, the aromatic region signals were more intense on spectra coming from red wines. Proline and arginine were the most predominant amino acids and those have also been reported as the most abundant in grape musts (MORENO-ARRIBAS *et al.* 2005). The higher alcohols 2,3-butanediol and phenethyl alcohol were also found in all wine samples, but their signals were observed more intense in red wines than in white wines.

S o l i d - p h a s e   e x t r a c t i o n : 'Cabernet Sauvignon' wine was used to test the sorbent amount (200, 500 mg), sample volume (20, 30, 40 and 100 mL); eluting
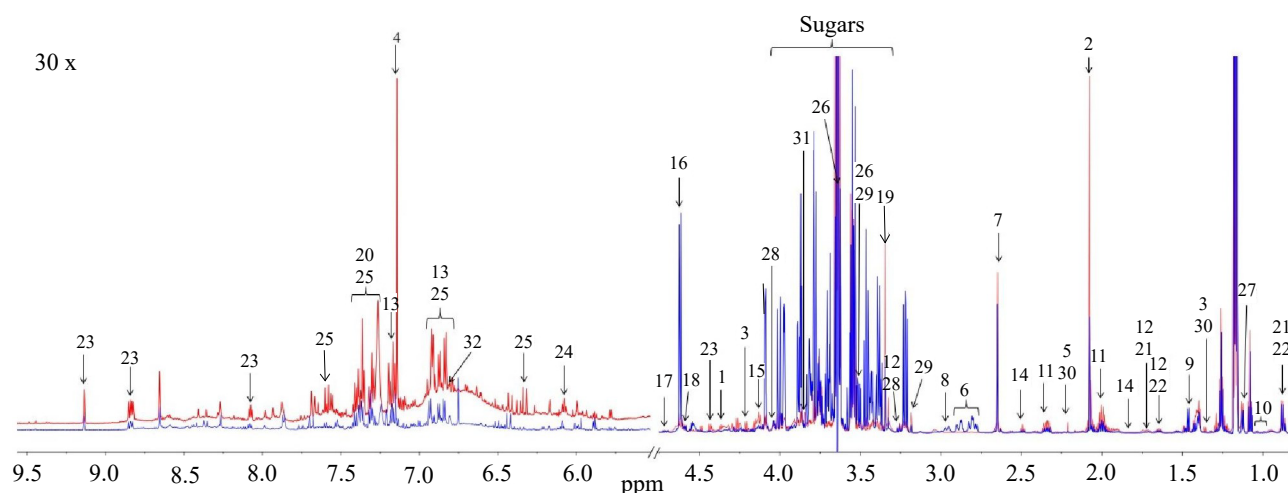

Fig. 1: ¹H-NMR spectrum (700 MHz, D_2O, 25 °C) of 'Barbera' (red) and 'White Zinfandel' (blue) wine samples by DA-NMR. The aromatic region was vertical multiplied by factor of thirty. Compounds are listed in Tab. 1

Table 1

Metabolites identified by DA-NMR in red and white wine samples. Chemical shifts in D_2O (δ), proton multiplicity, coupling constants (J) and assignment used for identification are presented

	Peak	Compound	δ ^1H in ppm (Multiplicity, J in Hz, assignment)
Organic Acids	1	Tartaric acid	4.38 (s, CH)
	2	Acetic acid	2.08 (s, CH_3)
	3	Lactic acid	1.39 (d, $J = 7.0$, CH_3), 4.37 (q, $J = 7.0$, CH)
	4	Gallic acid	7.15 (s, CH)
	5	Pyruvic acid	2.21 (s, CH_3)
	6	Malic acid	2.81 (dd, CH_2), 2.89 (dd, $J = 16.3, 4.4$, CH_2)
	7	Succinic acid	2.65 (s, CH_2)
	8	Citric acid	2.96 (d, $J = 15.6$, CH)
Amino acids	9	Alanine	1.49 (d, $J = 7.2$, CH_3)
	10	Valine	0.99 (d, $J = 7.2$, CH_3), 1.04 (d, $J = 7.0$, CH_3)
	11	Proline	2.00 (m, CH_2), 2.34 (m, CH_2),
	12	Arginine	1.65 (m CH_2), 1.73 (m, CH_2), 3.26 (t, $J = 6.7$, CH_2)
	13	Tyrosine	6.84 (m, CH), 7.17 (m, CH)
	14	4-Aminobutyrate	1.89 (m, CH_2) 2.50 (t, $J = 7.4$, CH_2)
Sugars	15	Fructose	4.13 (m, CH)
	16	Glucose	4.62 (d, $J = 7.9$, CH), 5.21 (d, $J = 3.7$, CH)
	17	Arabinose	5.24 (d, $J = 4.6$, CH)
	18	Xylose	4.58 (d, $J = 7.8$, CH), 5.17 (d, $J = 3.8$, CH)
Alcohols	19	Methanol	3.35 (s, CH_3)
	20	Phenethyl alcohol	7.28 (m, CH), 7.34 (m, CH)
	21	Isobutanol	0.88 (d, $J = 6.7$, CH_3); 1.73 (m, CH)
	22	Isoamyl alcohol	0.88 (d, $J = 6.8$, CH_3), 1.65 (m, CH)
Phenolic compounds	23	Trigonelline	4.43 (s, CH_3), 8.08 (m, CH), 8.84 (m, CH) 9.14 (s, CH)
	24	Epicatechin	6.07 (d, $J = 2.3$, CH), 6.09 (d, $J = 2.3$, CH)
	25	Caffeic acid	6.33 (d, $J = 15.9$ Hz, CH), 6.83 (d, $J = 8.6$ Hz, CH), 6.92 (m, CH), 7.20 (d, $J = 2.2$ Hz, CH), 7.59 (d, $J = 16.0$ Hz, CH)
Polyols	26	Glycerol	3.55 (dd, $J = 11.7$ and 6.5, CH_2), 3.64 (m, CH)
	27	2,3-Butanediol	1.13 (d, $J = 6.2$, CH_3)
	28	Myo-inositol	3.26 (t, $J = 9.4$, CH), 4.04 (t, $J = 2.7$, CH)
Others	29	Choline	3.18 (s, CH_3), 3.51 (dd, CH_2)
	30	Acetoin	1.36 (d, $J = 7.0$, CH_3), 2.22 (s, CH_3)
	31	Mannitol	3.85 (dd, $J = 11.8, 2.9$, CH_2)
	32	Shikimic acid	6.81 (m, CH)

solvent (MeOH and DCM) as well as reconstituting solvent (CDCl$_3$ and MeOD-d_4). The best conditions were 200 mg of sorbent; 40 mL of wine sample; methanol as eluting and MeOD-d_4 as reconstituting solvents. These conditions allowed to reduce sample volume, solvent waste and analysis time without losing sensitivity. Extracts reconstituted with CDCl$_3$ were not stable, after five days the solution originally pale pink turns dark brown and a precipitated appears, therefore MeOD-d_4 was used. Solvent evaporation was implemented as an additional concentration step; this also allowed dissolving the extract in MeOD-d_4 avoiding presaturation in NMR analysis. Fig. 2 shows the typical ^1H-NMR spectra obtained for SPE extracts of wines. It was observed that alcohols and most of the carbohydrate signals were removed while more aromatic signals were detected. Fourteen compounds were detected, among them isoamyl alcohol, phenethyl alcohol, tyrosol and aromatic compounds (Tab. 2). Structural assignments of main extracted wine compounds were done comparing published data (Anastasiadi *et al.* 2009, Ali *et al.* 2011), Chenomx NMR suite v. 8.4 (Chenomx Inc, Edmonton, Canada) and 2D-NMR experiments. An example of the work flow of how 2D spectra were used to assign a compound was included in suppl. work flow S11 and a complete set of 1D and 2D NMR spectra were shown in suppl. Figs S12 -S16. The higher extracts concentration obtained using SPE as sample preparation method allowed
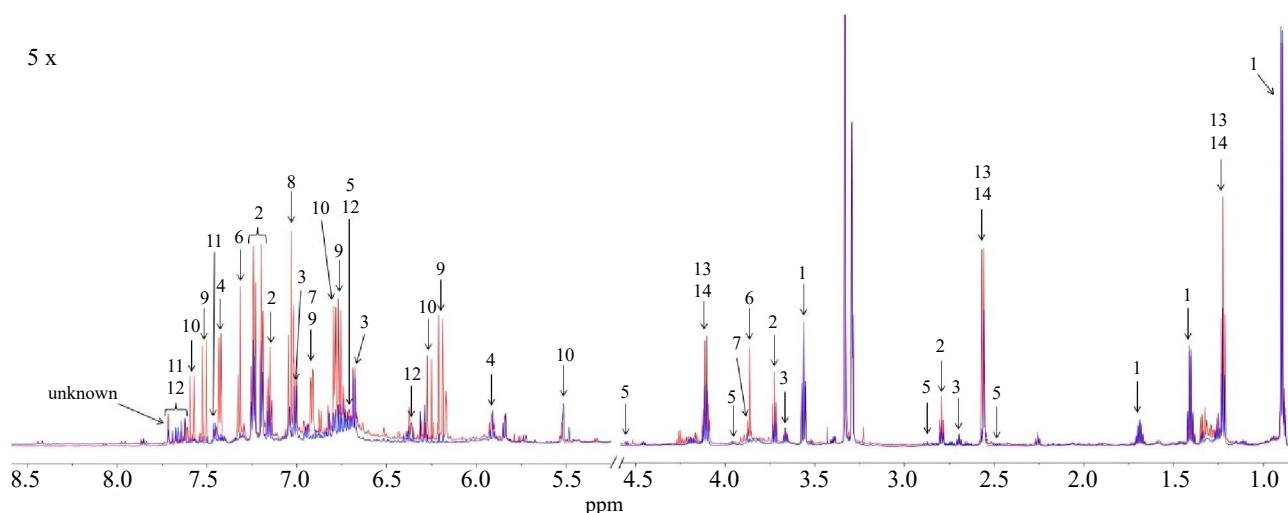
Fig. 2: ^{1}H-NMR spectrum (700 MHz, MeOD-d_4, 25 °C) of 'Barbera' (red) and 'White Zinfandel' (blue) wine samples extracted using Chromabond HR-P SPE cartridges. The aromatic region was vertically multiplied by factor of five. Compounds are listed in Tab. 2.

Table 2

Metabolites identified by SPE-NMR in red and white wine samples extracted using Chromabond HR-P SPE cartridges. Chemical shifts in MeOD-d_4 (δ), proton multiplicity, coupling constants (J) and assignment used for identification are presented

	Peak	Compound	δ ^{1}H in ppm (Multiplicity, J in Hz, assignment)
Higher alcohols	1	Isoamyl alcohol	0.90 (d, $J = 6.7$, CH₃), 1.41 (q, $J = 6.9$, CH₂), 1.68 (m, CH), 3.56 (t, $J = 6.9$, CH₂)
	2	Phenethyl alcohol	2.79 (t, $J = 7.1$, CH₂), 3.72 (t, $J = 7.1$, CH₂), 4.11 (q, $J = 7.1$, CH₂), 7.15 (t, $J = 7.3$, CH), 7.20 (d, $J = 8.3$, CH), 7.24 (t, $J = 7.6$, CH)
	3	Tyrosol	2.70 (t, $J = 7.2$, CH₂), 3.66 (t, $J = 7.2$, CH₂), 6.68 (d, $J = 8.5$ Hz, CH), 7.01 (d, $J = 8.5$, CH)
Phenolic compounds	4	Epicatechin	5.92 (d, $J = 2.3$, CH), 5.90 (d, $J = 2.3$ Hz, CH), 7.43 (d, $J = 8.6$ Hz, CH)
	5	Catechin	2.49 (dd, $J = 16.1, 8.1$, CH), 2.83 (dd, $J = 16.1, 5.4$, CH), 3.96 (m, $J = 7.9, 5.4$, CH), 4.55 (d, $J = 7.5$, CH), 6.68 (d, $J = 8.5$, CH)
	6	Syringic acid	3.86 (s, CH₃), 7.32 (s, CH)
	7	Vanillic acid	3.87 (s, CH₃), 6.92 (dd, $J = 8.3, 2.2$, CH)
	8	Gallic acid	7.03 (s, CH)
	9	Caffeic acid	6.20 (d, $J = 16.0$, CH), 6.76 (d, $J = 8.2$, CH), 6.91 (d, $J = 2.1$, CH), 7.5 (d, $J = 16.0$, CH)
	10	*Trans*-Caftaric acid	5.53 (m, CH), 6.26 (d, $J = 16.0$, CH), 6.76 (d, $J = 8.6$, CH), 7.63 (d, $J = 16.0$, CH)
	11	*Trans*-Coutaric acid	7.46 (d, $J = 8.7$, CH), 7.67 (d, $J = 8.7$, CH), 7.70 (d, $J = 15.8$, CH)
	12	p-Coumaric acid	6.36 (d, $J = 15.9$), 6.71 (d, $J = 8.8$), 7.67 (d, $J = 8.8$), 7.70 (d, $J = 15.9$)
Others	13	Diethyl succinate	1.23 (t, $J = 7.1$, CH₃), 2.57 (s, CH₂), 4.11 (q, $J = 7.1$, CH₂)
	14	Monoethyl succinate	1.23 (t, $J = 7.1$, CH₃), 2.56 (m, CH₂), 4.11 (q, $J = 7.1$, CH₂)

an easier analysis and assignment of the minor metabolites signals than DA. Metabolites like isoamyl and phenethyl alcohols were found in both methods but with high signal intensities when SPE was used, other metabolites like epicatechin, caffeic and gallic acids were clearly detected with SPE but only at noise level with DA. On the other hand, catechin, succinates and syringic, vanillic, coutaric, caftaric, coumaric acids were only detected when SPE was used to concentrate the compounds (suppl. Figs S1-S6).

Regarding to SPE repeatability, the visual inspection of the ^{1}H-NMR spectra of triplicate extracts showed no difference among them (suppl. Fig. S7), which was confirmed by statistical analysis. The control chart at 95 % confidence of

the first component (PC1) was used to evaluate precision for SPE-NMR, it explained 94 % of data variability with R^2 of 0.94 and Q^2 of 0.84 (suppl. Fig. S8). Results indicated that the method used was precise as in the control chart the samples lay within ± 3 s (standard deviation) from the mean indicating that the residual variability can be attributed to random error and there is no significant difference between samples. The increase in the aromatic signal intensities observed in SPE is in agreement with a previous ^{1}H-NMR report in which in the analysis of 150 mL of wine, using column chromatography with 15 g of XAD-4 resin, 60 mL of ethanol as elution solvent, evaporation to dryness (vacuum, 40 °C) and reconstitution in 700 μL of MeOD-d_4, ten

phenolic compounds were reported (Anastasiadi *et al.* 2009). However, the methodology here reported had several advantages since it is miniaturized, consumes less time and has a lower sample and solvent consumption. Recoveries were obtained for the two metabolites identified in both methods, they were around 20 % and 78 % for isoamyl and phenethyl alcohol, respectively. As expected, recoveries for phenethyl alcohol were higher than isoamyl alcohol, because the HR-P sorbent (polystyrene-divinylbenzene copolymer) has a better retention for aromatic compounds. In general, classification of wine is feasible through qualitative analysis, however if the method was desired for quantitative purposes, to increase recoveries the sample can be diluted with water to diminish its eluent strength.

An advantage of using SPE is that few compounds were found in the extract due to elimination of polar wine components. The elimination of water and ethanol avoid the need of solvent suppression leading to less disturbances of some signals and allow the observation of other signals of metabolites that appear in water and ethanol frequencies. The sugars elimination allows the observation of signals of minor metabolites around 3.1 and 4.7 ppm like the triplets of phenethyl and isoamyl alcohol at 3.72 ppm and 3.56 ppm respectively (suppl. Figs S9-S10), or the doublet of catechin at 4.55 ppm, the quartets of monoethyl and diethyl succinates in 4.11 ppm and other non identified signals (suppl. Fig. S5).

In general white wines have a significantly lower phenolic content than red ones, so the aromatic region had less intense signals (Fig. 2) and SPE-NMR could be very useful for white wines analysis.

Conclusions

In this study the application SPE-NMR method enable us to identify more aromatic metabolites. Using DA-NMR analysis gives a broad picture of the wine metabolome, thirty-two metabolites were identified, mainly amino acids, sugars and organic acids. In SPE-NMR fourteen metabolites were identified, mainly higher alcohols and aromatic compounds, like *trans* caftaric and coutaric acids, gallic, vanillic and syringic acids, catechin and succinates, demonstrating it is a suitable complementary method to DA-NMR, specially to improve the aromatic compounds profiling. SPE procedure is simple, it has the disadvantage of being time-consuming, however it was found to be very effective for the extraction of phenolics, even for white wines which have lower phenolic content than red wines, which indicates that SPE could be a better option for targeted studies.

References

Acunha, T., Simó, C.; Ibáñez, C.; Gallardo, A.; Cifuentes, A.; 2016: Anionic metabolite profiling by capillary electrophoresis-mass spectrometry using a noncovalent polymeric coating. Orange juice and wine as case studies. J. Chromatogr. A **1428**, 326-335. DOI: https://doi.org/10.1016/j.chroma.2015.08.001

Ali, K.; Maltese, F.; Fortes, A. M.; Pais, M. S.; Verpoorte, R.; Choi, Y. H.; 2011: Pre-analytical method for NMR-based grape metabolic fingerprinting and chemometrics. Anal. Chim. Acta **703**, 179-186. DOI: https://doi.org/10.1016/j.aca.2011.07.036

Amaral, F. A.; Caro, M. S. B.; 2005: Investigation of different pre-concentration methods for NMR analyses of Brazilian white wine. Food Chem. **93**, 507-510. DOI: https://doi.org/10.1016/j.foodchem.2004.09.039

Anastasiadi, M.; Zira, A.; Magiatis, P.; Haroutounian, S. A.; Skaltsounis, A. L.; Mirkos, E.; 2009: ¹H NMR-based metabonomics for the classification of greek wines according to variety, region, and vintage. Comparison with HPLC data. J. Agric. Food Chem. **57**, 11067-11074. DOI: https://doi.org/10.1021/jf902137e

Andujar-Ortiz, I.; Moreno-Arribas, M. V.; Martín-Álvarez, P. J.; Pozo-Bayón, M. A.; 2009: Analytical performance of three commonly used extraction methods for the gas chromatography–mass spectrometry analysis of wine volatile compounds. J. Chromatogr. A **1216**, 7351-7357. DOI: https://doi.org/10.1016/j.chroma.2009.08.055

Aru, V.; Sørensen, K. M.; Khakimov, B.; Toldam-Andersen, T. B.; Engelsen, S. B.; 2018: Cool-climate red wines-chemical composition and comparison of two protocols for ¹H-NMR analysis. Molecules **23**, 160. DOI: https://doi.org/10.3390/molecules23010160

Cassino, C.; Tsolakis, C.; Bonello, F.; Gianotti, V.; Osella, D.; 2019: Wine evolution during bottle aging, studied by ¹H NMR spectroscopy and multivariate statistical analysis. Food Res. Int. **116**, 566-577. DOI: https://doi.org/10.1016/j.foodres.2018.08.075

Castro, R.; Natera, R.; Durán, E.; García-Barroso, C.; 2008: Application of solid phase extraction techniques to analyse volatile compounds in wines and other enological products. Eur. Food Res. Technol. **228**, 1-18. DOI: https://doi.org/10.1007/s00217-008-0900-4

Cozzolino, D.; 2016: Metabolomics in grape and wine: definition, current status and future prospects. Food Anal. Methods **9**, 2986-2997. DOI: https://doi.org/10.1007/s12161-016-0502-x

Croce, R.; Malegori, C.; Oliveri, P.; Medici, I.; Cavaglioni, A.; Rossi, C.; 2020: Prediction of quality parameters in straw wine by means of FT-IR spectroscopy combined with multivariate data processing. Food Chem. **305**, 125512. DOI: https://doi.org/10.1016/j.foodchem.2019.125512

Fan, S.; Zhong, Q.; Fauhl-Hassek, C.; Pfister, M. K. H.; Horn, B.; Huang, Z.; 2018: Classification of Chinese wine varieties using ¹H NMR spectroscopy combined with multivariate statistical analysis. Food Control **88**, 113-122. DOI: https://doi.org/10.1016/j.foodcont.2017.11.002

García-Esparza, M. J.; Abrisqueta, I.; Escriche, I.; Intrigliolo, D. S.; Álvarez, I.; Lizama, V.; 2018: Volatile compounds and phenolic composition of skins and seeds of 'Cabernet Sauvignon' grapes under different deficit irrigation regimes. Vitis **57**, 83-91. DOI: https://doi.org/10.5073/vitis.2018.57.83-91

Gougeon, L.; Da Costa, G.; Le Mao, I.; Ma, W.; Teissedre, P. L.; Guyon, F.; Richard, T.; 2018 Wine analysis and authenticity using ¹H-NMR metabolomics data: application to Chinese wines. Food Anal. Methods **11**, 3425-3434. DOI: https://doi.org/10.1007/s12161-018-1310-2

Gougeon, L.; Da Costa, G.; Guyon, F.; Richard, T.; 2019a: ¹H NMR metabolomics applied to Bordeaux red wines. Food Chem. **301**, 125257. DOI: https://doi.org/10.1016/j.foodchem.2019.125257

Gougeon, L.; Da Costa, G.; Richard, T.; Guyon, F.; 2019b: Wine Authenticity by quantitative ¹H NMR *versus* multitechnique analysis: a case study. Food Anal. Methods **2**, 956-965. DOI: https://doi.org/10.1007/s12161-018-01425-z

Hong, Y. S.; 2011: NMR-based metabolomics in wine science. Magn. Reson. Chem. 49, S13-S21. DOI: https://doi.org/10.1002/mrc.2832

Kessler, P.; Godejohann, M.; 2018: Identification of tentative marker in Corvina and Primitivo wines with CMC-se. Magn. Reson. Chem. **56**, 480-492. DOI: https://doi.org/10.1002/mrc.4712

Kustos, M.; Gambetta, J. M.; Jeffery, D. W.; Heymann, H.; Goodman, S.; Bastian, S. E. P.; 2020: A matter of place: sensory and chemical characterisation of fine australian chardonnay and shiraz wines of provenance. Food Res. Int. **130**, 108903. DOI: https://doi.org/10.1016/j.foodres.2019.108903

Malec, P. A.; Oteri, M.; Inferrera, V.; Cacciola, F.; Mondello, L.; Kennedy, R. T.; 2017: Determination of amines and phenolic acids in wine with benzoyl chloride derivatization and liquid chromatography-mass spectrometry. J. Chromatogr. A **1523**, 248-256. DOI: https://doi.org/10.1016/j.chroma.2017.07.061

Monakhova, Y. B.; Schäfer, H.; Humpfer, E.; Spraul, M.; Kuballa, T.; Lachenmeier, D. W.; 2011: Application of automated eightfold suppression of water and ethanol signals in ^{1}H NMR to provide sensitivity for analyzing alcoholic beverages. Magn. Reson. Chem. **49**, 734-739. DOI: https://doi.org/10.1002/mrc.2823

Moreno-Arribas, M. V.; Polo, M. C.; 2005: Winemaking biochemistry and microbiology: current knowledge and future trends. Crit. Rev. Food Sci. Nutr. **45**, 265-286. DOI: https://doi.org/10.1080/10408690490478118

Norma Oficial Mexicana; 2017: NOM-199-SCFI-2017. Bebidas Alcohólicas-Denominación, Especificaciones Fisicoquímicas, Información Comercial y Métodos de Prueba. Accessed from: http://www.dof.gob.mx/nota_detalle.php?codigo=5502882&fecha=30/10/2017. Diario Oficial de la Federación, México.

Ragone, R.; Crupi, P.; Piccinonna, S.; Bergamini, C.; Mazzone, F.; Fanizzi, F. P.; Schena, F. P.; Antonacci, D.; 2015: Classification and chemometric study of southern Italy monovarietal wines based on NMR and HPLC-DAD-MS. Food Sci. Biotechnol. **24**, 817-826. DOI: https://doi.org/10.1007/s10068-015-0106-z

Sánchez-Palomo, E.; Alonso-Villegas, R.; González-Viñas, M. A.; 2015: Characterisation of free and glycosidically bound aroma compounds of La Mancha Verdejo white wines. Food Chem. **173**, 1195-1202. DOI: https://doi.org/10.1016/j.foodchem.2014.10.148

Savage, A. K.; van Duynhoven, J. P. M.; Tucker, G.; Daykina, C. A.; 2011: Enhanced NMR-based profiling of polyphenols in commercially available grape juices using solid-phase extraction. Magn. Reson. Chem. **49**, S27-S36. DOI: https://doi.org/10.1002/mrc.2846

Son, H. S.; Kim, K. M.; van den Berg, F.; Hwang, G. S.; Park, W. M.; Lee, C. H.; Hong, Y. S.; 2008: [1]H nuclear magnetic resonance-based metabolomic characterization of wines by grape varieties and production areas. J. Agric. Food Chem. **56**, 8007-8016. DOI: https://doi.org/10.1021/jf801424u

Song, J. Q.; Li, H.; Liang, Y. Y.; Tao, Y. S.; Mi, C. Q.; Qian, M. C.; Wang, H.; 2013: Characterisation of volatile components of red and sparkling wines from a new wine grape cultivar 'Meili' (*Vitis vinifera* L.). Vitis **52**, 41-48. DOI: https://doi.org/10.5073/vitis.2013.52.41-48

Spyros, A.; Dais, P.; 2012: Wine and Beverages, 202-240. In: NMR Spectroscopy in Food Analysis. Royal Society of Chemistry, England. DOI: https://doi.org/10.1039/9781849735339-00202