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P09: The potential of NMR spectroscopy vs separation techniques: the case of balsamic vinegar analysis

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Abstract – The official control of balsamic vinegars (BV) is limited to basic physicochemical properties such as total acidity and total reducing sugar content. High performance liquid chromatography (HPLC) is the recommended method for the determination of sugars, organic acids and 5-hydroxy-methyl-2-furfuraldehyde, a potential quality criterion. Nuclear magnetic resonance spectroscopy (NMR) is a powerful tool for monitoring food quality due to its high reproducibility and sensitivity. In the present study, the potential of NMR in BV analysis was investigated for future applications in the official control of BV.

Keywords: balsamic vinegar, NMR, HPLC, HMF, anaysis

1. INTRODUCTION

The last decade, balsamic vinegars have evolved to one of the most popular type of vinegar worldwide. In the USA, within four years, 134 new balsamic vinegar products were launched while for the next category (red vinegar) only 52 new ones. Moreover, its net value in the USA for 2016 recorded an increase of 76 million dollars [1]. Globally, balsamic vinegar market share comprises approximately 34% of the vinegar market. Balsamic Family is a heterogeneous group of vinegars and condiments based on concentrated grape must (CGM) with complex sensory profiles. Among these products, industrial balsamic vinegars (BVs) are elaborated by blending concentrated and/or boiled grape must, wine vinegar and permitted additives. They gained rapidly popularity due to their competitive price compared to the traditional ones (TBVM and TBVRE) and their high diversity in gustatory traits.

The major constituents of BVs are sugars (mainly glucose and fructose), organic acids (mainly acetic acid) and 5-hydroxy-methyl-2-furfuraldehyde (HMF). Their official control though is limited to basic physicochemical properties such as their specific gravity at 25 °C, the total acidity and total reducing sugar content [2]. The relative concentration of both sugars and acids is expected to alter significantly their physicochemical stability as well as their sensory profile. For example, products with high values of glucose to fructose ratio are likely to form glucose crystals that deteriorate their quality [3]. Accordingly, prevalence of organic acids over sugars can result in an unbalanced sour and astringent mouth sensation, which is not appreciated by the consumers [2,4]. HMF, the major product of caramelization and Maillard reactions in foods rich in reducing sugars, is an undesirable constituent due to its cytotoxic, genotoxic and tumorigenic activities. Recently, its concentration has been highlighted as a quality criterion for BVs though is not required by legislation yet [2]. In literature, HMF content has also been proposed as marker for GM concentration and age estimation of TBV and BV vinegars [5].

According to the official methods of OIV, high

performance liquid chromatography (HPLC) is the recommended method for the separation and simultaneous determination of the sugars (glucose and fructose, OIV-MA-AS311-03) and organic acids (OIV-MA-AS313-04) [6]. An HPLC method (OIV-MA-F1-02) is also recommended for the determination of HMF (5-hydroxy-methyl-2-furfuraldehyde) content. In wine analysis, HPLC methods are widely used due to their high separation efficiency, versatility, precision and relatively low cost [7]. During the last decades, nuclear magnetic resonance spectroscopy (NMR) has gained general acceptance as a powerful tool for monitoring food quality. Its high reproducibility and sensitivity in combination with non destructive and high throughput characteristics compared to other techniques are highly recognized in the discipline of food analysis [5,7,8].

The ability of NMR to discriminate between BVs of different age has been studied [8] but data about its potential to be employed in the analysis of BVs are still limited. In the present study, the potential of NMR in BV analysis was investigated in comparison to data obtained with other techniques. Proposals for its future applications in the official control of balsamic vinegar are made.

2. EXPERIMENTAL

2.1 Samples and reagents.

A total of 11 representative samples of BVs were purchased from a supermarket in Thessaloniki (Greece) and a delicatessen and a supermarket in Milan in Spring and Summer of 2013 and were stored in a dark dry place at 20 ^{o}C until the first opening.

a-D-glucose was purchased from Duchefa Biochemie (Haarlem, The Netherlands). D-fructose and glycerol were from Panreac Quimica S.A. (Barcelona, Spain). 5- hydroxymethyl-furfural (>99%) was purchased from Sigma Aldrich (Milan, Italy). Methanol (Chem-Lab., Zedelgen, Belgium) was HPLC grade and ultrahigh purity water was produced using a SG 2002 v.1.01 system (Barsbuttel, Germany). D2O (99.98%) was purchased from Deutero (Kastellaun. Germany). 3-Trimethylsilyl- 3,3,2,2-tetradeuteriopropionic acid sodium salt (TSP-d4) was obtained from Cambridge Isotope Laboratories Inc. (Cambridge, MA, USA).

2.2 High performance liquid chromatography analysis

Glucose, fructose and organic acids were quantified according to [2]. Briefly, the separation was conducted on cation exchange resin-based column Agilent HI-plex H 7.7 × 300 mm, 8 µm (Agilent Technologies, Santa Clara, CA, USA) by isocratic elution with a 5 mM sulfuric acid solution at 65 ^{o}C. The flow rate was 0.5 mL/min. and the injection volume 10 µL. The HPLC system was composed of an LC-10Advp pump (Shimadzu, Kyoto, Japan), and a refractive index detector (RID-6A, Shimadzu). Sample preparation included proper dilution in the mobile phase and filtration through 0.45 mm pore size regenerated cellulose membrane filters (Schleicher & Schuell, Dassel, Germany). Quantification was made using calibration curves for standard glucose and fructose l in the range 2.5–60 mg/kg ($y = 569x + 162.5$, $R^2 = 0.999$ and $y = 634.6x + 201.5$, $R^2 = 0.999$, respectively).

The determination of 5-hydroxymethyl-furfural (HMF) was carried out according to [2]. The separation was conducted on a Nucleosil 100 C18 (250 x 4.6 mm; 5 mm) chromatographic column MZ-Analysentechnik GmbH (Mainz, Germany) under isocratic elution with methanol/water (20:80, v/v) at 0.5 mL/min. The oven temperature was 40 ^{o}C and the injected volume 10 µL. The solvent delivery system consisted of a LC-20AD pump (Shimadzu, Kyoto, Japan) and a Rheodyne 7125 injection valve with a 20 mL fixed loop (Rheodyne 7125 LP, Cotati, CA, USA). Prior to injection, the samples were diluted in the mobile phase and filtered through membrane filters (0.45 mm pore-size). The chromatograph was coupled with an SPD-10AV UV/VIS detector (Shimadzu, Kyoto, Japan). The identification of HMF was conducted by comparing its retention time with this of the standard compound and its concentration was determined by comparison with an external calibration curve in the range of 0.25– 75 mg/kg at 283 nm ($y = 101.8x + 11.92$, $R^2 = 0.998$). The results were expressed as mg HMF/kg of sample. All of the samples were analyzed in triplicate.

2.2 Nuclear magnetic resonance experiments

Nuclear magnetic resonance (NMR) experiments were performed using a Bruker AV-500

spectrometer equipped with a TXI cryoprobe (Bruker BioSpin, Rheinstetten, Germany) and a Bruker AV-400 spectrometer. A small amount of the vinegar sample (10–25 μL) was dissolved in D_2O to a final volume of 600 μL after adjusting the pH value to 2.00 and transferred to 5 mm NMR tubes. Before sample analysis the necessary optimization of the effect of pH and temperature in chemical shifts and signal overlapping was performed. As reference for chemical shifts and concentration values was used the internal standard TSP-d_4 ($Me_3SiCD_2CD_2COONa$, dH = 0.00 ppm). Data were collected at 300 K without sample rotation; 64 scans were acquired with a spectral width of 14 ppm using acquisition time of 4.334 s, relaxation delay of 5 s and 308 pulse length. The interpulse delay ($t_{acquitition}$ + relaxation delay) was set to $4xT_1$, where T_1 was the longest relaxation time (T_1) of analytes used for quantification. The only exception was formic acid for which T_1 was determined to be 8.9 s. In this case, due to the use of the 308 pulse, the error in formic acid quantification using 9 s interpulse delay was found to be less than 5% compared to an interpulse delay of 38 s. The spectra were Fourier transformed with FT size of 64k and 0.3 Hz line-broadening factor. Phase correction was performed manually for each sample and a polynomial baseline correction was applied over the entire spectral range. In some cases a manual baseline correction was necessary. The manual integration of the selected signals and the comparison with TSP-d_4 (internal standard) area allowed the quantitative determination of the corresponding compounds in the vinegar samples. The 2D 1H–13C HSQC and HMBC experiments were performed using standard software, and parameters were optimized for coupling constants of 145.0 and 8.0 Hz, respectively. The complete assignment of the identified compounds and their subsequent quantification was based on data obtained from 2D NMR spectra (2D 1H–13C HSQC and HMBC), in comparison with spectra of available standard compounds and with literature data [5, 8, 9, 10, 11].

3. RESULTS AND DISCUSSION

3.1 Optimization of experimental conditions-effects of pH and temperature

In Fig. 1 a selected region of 1H-NMR spectrum of freeze dried balsamic vinegar in D2O in different pH values is presented at 298 K. From a first exploratory 1H NMR analysis of the different samples, significant variations in the chemical shifts of several metabolites were observed mainly due to large pH variations between 2.83 and 3.50. In order to find the pH value with the best resolution in the spectrum, the pH value of a balsamic vinegar (Sample 1) was therefore adjusted to 2.00, 3.00, 4.00 & 6.50 by the dropwise addition of 1 M HCl or 1 M NaOH. This approach aimed to standardize the difference in acidity of the samples and minimize the chemical shift variations of pH-dependent signals. Concerning peaks at 2.6-3.0 ppm, better resolution was observed in the spectrum with pH value 2.00. Similar results were obtained for the rest of the regions in the spectrum.

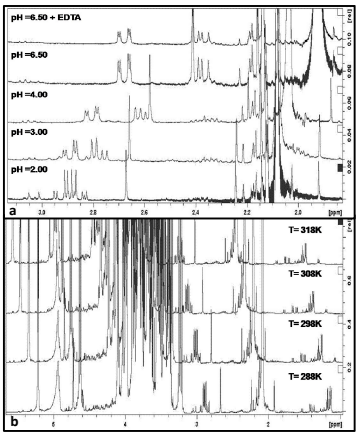

Figure 1. Selected regions of the 1H-NMR spectrum with presaturation of freeze dried balsamic vinegar (Sample 1) in D_2O (a) adjusted to different pH values at 298K (400MHz) and (b) adjusted to pH 2.00 at different temperatures (400MHz), (ns= 128, expt.=20min).

In Fig. 1b, selected region of 1H-NMR spectrum of this sample in D_2O at different temperature values is presented; no differences were observed in the acquisition of the spectrum at different temperatures. The temperature of 298 K selected was in agreement with literature data [8].

3.2. Comparison of NMR and HPLC methods.

A linear regression analysis of glucose, fructose,

acetic acid and HMF contents determined by the NMR and HPLC methods showed satisfactory correlations of 0.806, 0.816, 0.803 and 0.803 respectively (Fig. 2). Thus, the measured values with the two methods are in good agreement according to literature [12]. However, a closer inspection revealed that as far as HMF content is concerned, the values obtained by NMR are up to 3 times lower compared to those by HPLC and thus more research is needed for this particular compound.

In any case, beyond the information about the content of BVs in major components, NMR provided a useful insight to the sugar stereochemistry by its ability to discriminate a- and b-anomers of glucose and fructose providing additional information about the sweetness of BV. The latter has an important sensory aspect since the different anomers of fructose posses different sweetness with b- being sweeter than a-, thus, the different ratios can affect the intensity of the elicited sweetness.

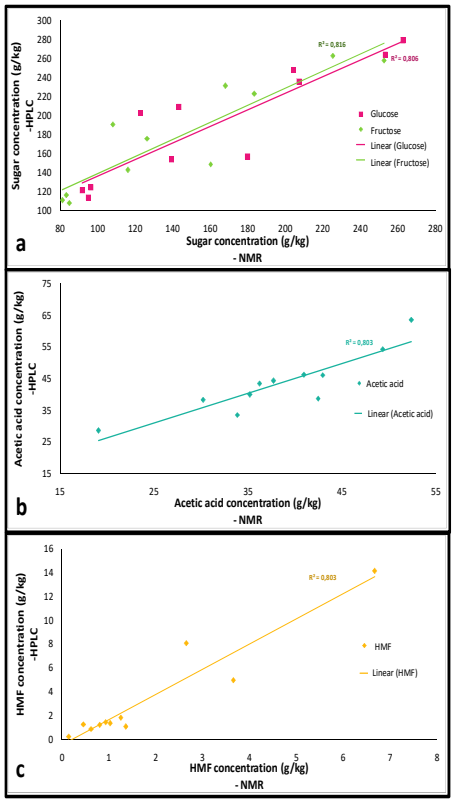

Figure 2. Correlations observed between the results obtained from independent NMR- and HPLC-based analysis of different balsamic vinegar samples (a) glucose and fructose (b) acetic acid and (c) HMF contents.

Conclusively, NMR can be a valuable tool in the analysis of BV products. It provides useful information with a single run with a small anount of sampe but further research is required to benefit from its full potential.

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