Grape Juice Quality Control by Means of ^{1}H NMR Spectroscopy and Chemometric Analysis

C. W. P. da Silva, F. R. Campos, F. Simonelli, A. Barison*

Laboratório de Ressonância Magnética Nuclear, Departamento de Química, Centro Politécnico - Universidade Federal do Paraná, C.P.19081, CEP 81530-900, Curitiba/PR, Brazil,
ander@quimica.ufpr.br

Keywords: grape juice; ^{1}H NMR; quality control; chemometric analysis.

Abstract: *This study shows the potential of ^{1}H NMR and chemometrics for quality control of grape juices. A wide range of quality assurance parameters were accessible on a single ^{1}H NMR experiment obtained directly from the samples, such as high amounts of ethanol. The results demonstrated the importance of storing the grape juices under refrigeration after the bottles were opened, in order to minimize fermentation, and that recommendation for storage time should be revised and should be indicated on all labels. The sterilization process of homemade samples was very efficient, making it possible to store them for long periods without the need of additives. Besides, chemometric analysis of the ^{1}H NMR spectra classified the best commercial grape juices closest to homemade samples, indicating that this approach can be used to determine the authenticity or adulteration of grape juice.*

Introduction

Grape juice is a healthy beverage, and is an important natural source of flavonoids and phenolic compounds,[1] which are associated with the prevention of several chronic diseases, cancer and arthritis, as well as the formation of free radicals.[1,2] Moreover, similarly to red wine, grape juice has a vasodilator effect that acts to protect against coronary disease, but has the advantage of lacking the side effects of alcohol, which is important for people with hypertension or diabetes, as well as for children.[3] Therefore, grape juice can be a good alternative source of polyphenols for the abstemious population. Furthermore, it has been demonstrated that *trans*-resveratrol can be absorbed from grape juice in effective amounts to reduce the risk of atherosclerosis, making an additional source of this substance unnecessary.[4]

Grape juice consumption and sales in Brazil, as well as its exportation, are increasing significantly,[5,6] and it is considered an important product of Brazilian agribusiness. Considering that international consumers are increasingly demanding, the continuous development of analytical methods to be employed in the quality control of food and beverages is essential in order to guarantee their healthfulness and authenticity. However, for grape juice, most of the studies described in the literature have been dedicated to determining the chemical composition.[7-11] Although such studies are essential for development of quality-control methods as the work described by Gil and co-workers that shown the good potential of NMR techniques on the quality control of grape juice.[7,8] Only a few investigations have been directed toward quality control, such as evaluation of cultivar effects on flavor characteristics of homemade Brazilian grape juice;[12] and determinations of organic acids because of their influence on organoleptic properties; and of stability and microbiological control; as well as determinations of the maturation process of grapes and the alcoholic and malolactic

fermentation processes.[13] Contamination with ochratoxin A has also been studied.[14,15]

Moreover, in most cases the methods employed for quality control are quite tedious, time and chemical consuming, rely on several steps of sample pre-treatment in order to separate and concentrate the compounds under investigation, and require the isolation or purchase of chemical standards. Therefore, increased effort has been directed toward developing more-rapid and informative analytical methods, as well as methods that permit the direct investigation of food and beverages without the need for a purification process, which may alter the nature of the sample and result in loss or dilution of some compounds. In response to these concerns, NMR spectroscopy is increasingly recognized as a versatile and powerful analytical tool for quality control of food and beverages, with the advantage of simultaneous detection of all compounds in a single NMR experiment acquired directly from the sample. For example, a simple ^{1}H NMR spectrum can provide both qualitative and quantitative information about the system under investigation. In recent years, high-resolution NMR spectroscopy has been applied to direct characterization of food and beverages, including olive oil,[16-18] fruit juices,[19-22] coffee,[23,24] wine,[25,26] beer,[27-29] monitoring malic and lactic acid levels during fermentation of grape must,[30] changes in chemical composition of mango juices[19,31] and the sorghum fermentation process,[32] and the identification and quantification of chemical components of vinegars.[33] Moreover, NMR in association with multivariate analysis (chemometrics) has been successfully employed in the quality control of food and beverages, including determination of authenticity, such as for coffee,[23] orange juice[20,34,35] and olive oil,[36,37] and determination of the geographical origin, such as for red wines,[25] olive oil,[38-40] coffee[23] and wheat flours,[41,42] as well as discrimination between apple varieties used in apple-juice production[21] and characterization of beer.[28]

The aim of this work was the development of a new analytical approach for quality control of commercial and homemade grape juices directly by ^{1}H NMR and ^{1}H NMR allied to chemometric analysis, including the time and environmental conditions of storage, detection of inconsistencies between chemical composition and the composition described on labels, follow the alcoholic and acetic fermentation, and verify the efficiency of the sterilization process.

Experimental

Samples

Samples of commercial grape juices were purchased from local supermarkets, proceeding from different regions of Brazil, and were divided in four groups according to whether they contained added sugar (sweetened) and/or preservatives.

Samples of homemade grape juice were obtained directly from local rural residents and small producers in Curitiba, Brazil. According to the producers, the bottles and stoppers for grape-juice storage were sterilized in boiling water, and then the freshly juice was immediately added to the still-hot bottles, which were then closed and stored at room temperature. This entire process was performed over a stove, in order to avoid the introduction of microorganisms due to the ascendant hot-air flow. Once the still-hot

bottles were closed, the cooling reduced the internal pressure and the stoppers were sealed. Homemade juices were prepared without any addition of sugar or preservatives.

For high-resolution NMR spectra measurements, aliquots of 0.6 mL of these samples were filtered through cotton in Pasteur pipettes, directly into NMR tubes. For the homemade juices, vacuum filtration was required prior to addition to NMR tubes. Next, three drops of a 0.1% 2,2,3,3-tetradeuterio-3-(trimethylsilyl)propionic acid, sodium salt, (TMSP-d_4) solution in D_2O were added in order to provide a NMR chemical shift reference and the lock signal. The ^{1}H NMR spectra were obtained from all commercial and homemade grape-juice samples just after the bottles were opened, and every three days until 15 days under refrigeration or at room temperature, as well as for the homemade samples stored at room temperature without opening the bottles, every month for five months.

NMR Analysis

^{1}H NMR spectra of grape juice were recorded at 295 K on a Bruker AVANCE 400 spectrometer, operating at 9.4 Tesla observing ^{1}H at 400.13 MHz using a 5 mm direct-detection multinuclear probe. The ^{1}H NMR spectra were obtained with water signal suppression by selective low-power irradiation at H_2O resonance frequency during relaxation delay using the *zgpr* pulse sequence. Each spectrum was acquired with a 90° pulse, 128 scans, 64K data points, 3634 Hz (~ 9 ppm) spectral width, acquisition time of 7.0 s and 1.0 s relaxation delay. The free induction decays (FIDs) were submitted to an exponential multiplication by a factor of 0.3 Hz prior to Fourier transform in TOPSPIN software. All ^{1}H NMR chemical shifts are given in ppm (δ) related to TMSP-d_4 signal at 0.00 ppm. The assignment was performed by comparison with published data[7,19,31] as well as analysis of ^{1}H-^{1}H correlation obtained by COSY experiment, acquired using the *dqfcosy* pulse sequence and spectral width 4400 Hz (~11 ppm) in both dimensions, which resulted in 4K data points and 16 transients in t_2 per each of 256 increments in t_1. All pulse sequences were supplied by Bruker.

Chemometric Analysis

For Principal Components Analysis (PCA), initially the ^{1}H NMR spectra were segmented into a number of continuous buckets with a width of 0.05 ppm between 0.5 and 9.00 ppm, resulting in 170 NMR variables, and the area under each segment was calculated by the special integration mode of AMIX software (Bruker Biospin, Karlsruhe, Germany). However, only the segments between 1.5-3.0 ppm were used in the PCA analysis. Therefore, the final number of NMR buckets (variables) considered was 30. The bucketing method permits data reduction in order to generate a manageable data set. For example, a ^{1}H NMR spectrum processed with 65536 data points over a spectral width of 10 ppm can be reduced to 200 buckets of 0.05 ppm width. Moreover, this approach has the advantage of eliminating the need for phase and baseline corrections, as well as minimizing drifts in the ^{1}H NMR chemical shifts between the spectra caused by differences in pH, concentrations and other factors.[29] The areas of the segments were then normalized and scaled to the total area of the spectrum.

Results and Discussion

The presence of ethanol in grape-juice samples indicates the occurrence of a fermentation process caused by microorganisms, which is a consequence of either an inefficient sterilization process in the initial raw production, or of later contamination. Moreover, high amounts of ethanol can be harmful to the health of abstemious persons. Therefore, commercial and homemade grape juices were initialy evaluted for the presence of ethanol by means of ^{1}H NMR spectroscopy direcly from the samples, without any sample pre-treatment.

The commercial samples were subdivided into four groups according to the addition of sugar (sweetened) and/or preservatives, in order to evalute the influence of these compounds on the fermentation process. The homemade samples were all without added sugar or preservatives.

Most of the ^{1}H NMR spectra of the commercial samples analyzed showed the presence of ethanol, evidenced by the triplet at 1.18 ppm (Figure 1, just after opening) assigned to the methyl group of ethanol. Mainly those samples with added sugar syrup and without preservatives showed high concentrations of ethanol. This is the first indication that addition of sugar (sucrose) from sugarcane promotes the fermentation process in grape juices. On the other hand, the sweetened samples containing preservatives showed lower ethanol concentrations, while for unsweetened samples with preservatives the presence of ethanol was minimal or even undetectable, indicating that introduction of preservatives can minimize the fermentation process. This observation indicates that the fermentation process may be starting in the raw production stage, as a consequence of contamination by microorganisms before the bottles are sealed, or else that the sterilization process was inefficient. This supposition is supported by the results for one of the grape-juice brands (sweetened without preservatives), which showed a higher concentration of ethanol in all lots analyzed.

Most Brazilian grape juices are made in the same equipment used for wine production by wineries, which may explain the finding of high ethanol concentrations in some grape juices, because for wines the fermentation process is stimulated by microorganisms. These results indicate that the addition of a preservative should be standard practice, at least for sweetened juices. Inspection of the labels of commercial grape juice bottles distributed in Brazil revealed a lack of standardization regarding the best method and time for storage of grape juice after the bottles are first opened. The information about the storage time provided on the labels varied widely, from 5 to 15 days. Moreover, 40% of commercial brands did not even mention storage time, and only a few brands recommended refrigeration after the bottles were opened. Therefore, ethanol production was evaluated according to storage time over a total period of 15 days, by means of ^{1}H NMR spectroscopy. The samples were kept either at room temperature or under refrigeration after the bottles were first opened, because high temperatures speed fermentation.

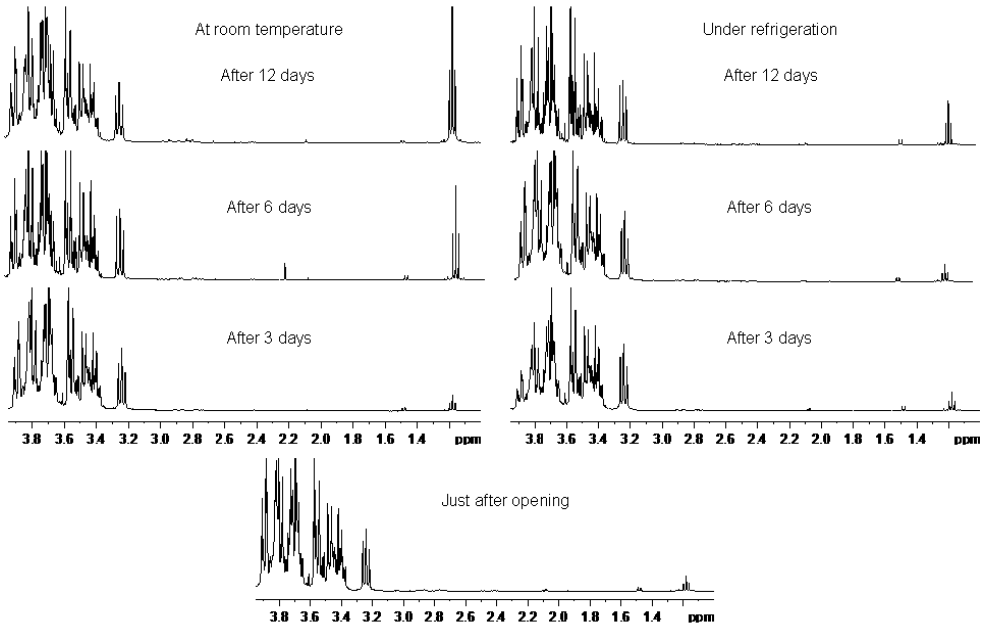

Figure 1. Representative ^{1}H NMR spectra (3.85-1.0 ppm) showing ethanol production in refrigerated and unrefrigerated samples of sweetened grape juice without preservatives during a 12-day storage period.

A significant ethanol concentration was noted after six days in commercial samples kept at room temperature, whereas for those kept under refrigeration only a small increase was observed after 12 days (Figure 1). This finding supports the assumption that warm temperatures enhance fermentation in grape juices. The largest increases in ethanol concentrations were observed for sweetened samples without preservatives, indicating that the addition of sugar enhances fermentation in grape juice. This information is supported by the observed decreases in the sucrose signals (doublets at 4.21 and 5.41 ppm)[19] with simultaneous increases in the ethanol signal at 1.18 ppm during storage (Figure 2). On the other hand, the grape-juice samples with no added sugar cane showed no notable ethanol production during storage, either at room temperature or under refrigeration. Furthermore, the sweetened and unsweetened samples with preservatives showed less production of ethanol than those without preservatives. A reduction of the fructose signal (doublet at 4.11 ppm), a natural sugar from grapes, was also observed, demonstrating that fermentation is slower even without the addition of sugar (Figure 2). These results indicated that the storage time of grape juices after the bottles are first opened needs to be revised; and also the recommendation to store grape juice under refrigeration should be consistent, at least for the sweetened varieties.

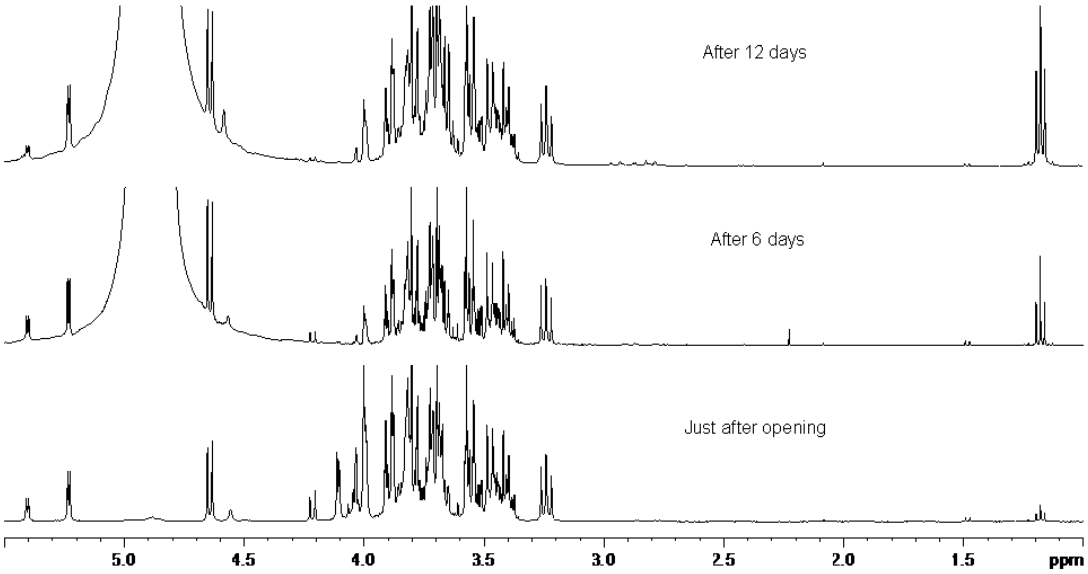

Figure 2. Representative ^{1}H NMR spectra showing ethanol production (triplet at 1.18 ppm) as a consequent consumption of added sucrose (doublets at 4.21 and 5.41 ppm) in a sample of sweetened commercial grape juice. In the bottom spectrum, the H_2O signal was eliminated to allow better visualization of the sucrose signals.

In southern Brazil the consumption of grape juice is very high, and many families traditionally make their own grape juice during the harvest season and then store it over the year. Therefore, the homemade grape juices were evaluated for ethanol concentration through ^{1}H NMR spectra during the storage time prior to opening the bottles at room temperature, and under refrigeration after the bottles were first opened.

The ^{1}H NMR spectra of homemade grape juices stored for five months at room temperature revealed no increase in ethanol concentration during the storage period prior to opening the bottles (Figure 3). After the bottles were first opened, the homemade juice samples were stored under refrigeration for 15 days, similarly to the commercial samples. Again, the ^{1}H NMR analysis showed no increase in their ethanol concentration during storage. Comparison with a similar commercial sample (without addition of sugar and preservatives) showed intensification of the ethanol signal in the ^{1}H NMR spectra only in the commercial sample (Figure 4). Taken together these results demonstrated that the sterilization process of homemade grape juice as described in the Experimental section is very efficient, even better than the industrial process. Therefore, it is possible to store homemade grape juice for long periods prior to consumption, both before and after the bottles are opened. These observations also support the hypothesis of contamination by alcoholic

yeasts during production of commercial grape juices, or else failure of the sterilization process.

The ^{1}H NMR analyses also allowed us to verify the occurrence of acetic fermentation in commercial and homemade grape juices. Monitoring of the acetate signal (singlet at 2.07 ppm) revealed the presence of this substance in all the commercial and homemade samples, in very low concentrations, when the bottles were first opened. No intensification of the acetate signal was noted during storage, independently of the environmental conditions. Only in one sample that was manipulated several times (the bottle opened and closed) it was observed a small increase in the acetate signal after several months of storage, probably because of contamination with acetic bacteria present in the air.

The amino acid alanine was present in all grape juice samples investigated, as evidenced in the ^{1}H NMR spectra by the doublet at 1.48 ppm. However, a high alanine concentration was found in one brand of commercial grape juice. According to Gil and coworkers,[19] the ripening process is accompanied by a significant increase in alanine relative to total sugars, which indicates that this specific juice was probably produced by grapes in a more advanced stage of ripening.

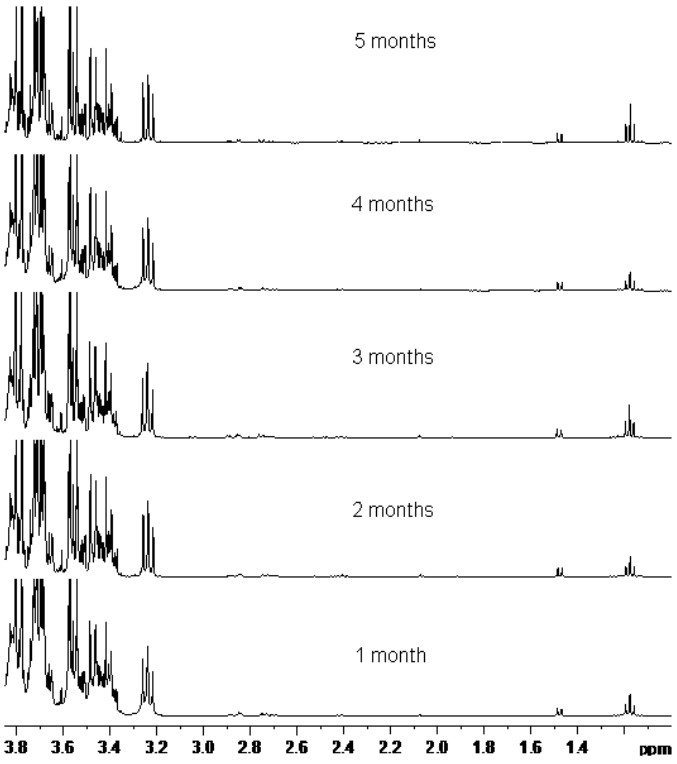

Figure 3. Representative ^{1}H NMR spectra (3.85-1.00 ppm) showing ethanol production in a sample of homemade grape juice during a five-month storage period at room temperature.

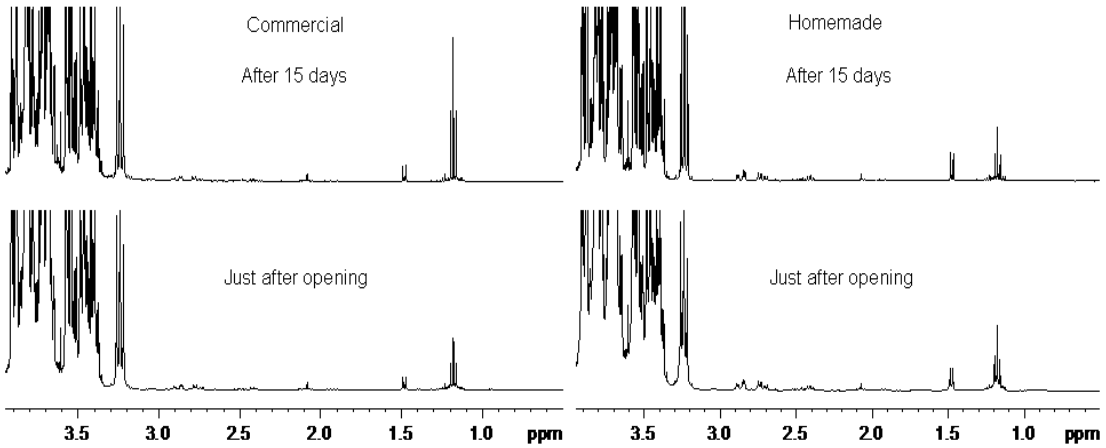

Figure 4. ^{1}H NMR spectra of commercial and homemade grape juices, just after opening and after a 15-day storage period.

The ^{1}H NMR spectra also made it possible to identify some inconsistencies between the chemical composition of some commercial grape juices and the information provided on their labels. The first inconsistency was in relation to the addition of citric acid as an acidifier. Only one of the commercial brands investigated mentioned the presence of citric acid on the label (Figure 5, A). However, this substance was identified in the ^{1}H NMR spectra by doublets at 2.72 and 2.87 ppm in two other brands (Figure 5, B and C). This is clearly observed by comparing with a spectrum obtained from a homemade sample (Figure 5, D), in which malic-acid signals (double doublets at 2.77 and 2.96 ppm) a natural compound from grapes are not overlapped by citric-acid signals.

The second inconsistency was observed in relation to the addition of sugar from sugarcane (sucrose) in some grape juices, which carried the specification “unsweetened” on their labels. However, the ^{1}H NMR spectra of these samples (Figure 5, F and G) showed the sucrose signal (doublets at 4.21 and 5.41 ppm), which was not present in homemade juice (Figure 5, H).

A third inconsistency was in the presence of preservatives. The presence of sodium benzoate was detected by multiplets at 7.51-7.55, 7.66-7.70 and 8.01-8.04 ppm on the ^{1}H NMR spectra in some commercial brands that stated on their labels that they contained no preservatives.

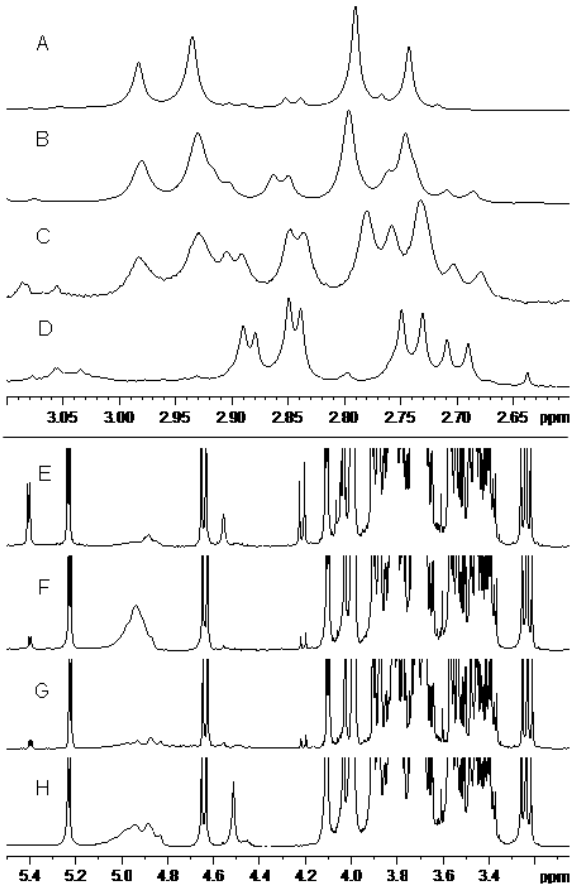

Figure 5. Representative ^{1}H NMR spectra showing the inconsistency detected between the chemical composition and the contents described on the labels of commercial grape juices. A-D, detection of citric acid in samples B and C (with no specification of citric-acid addition on the labels) in comparison with an authentic commercial acidified (A) and a homemade (D) sample. E-H, detection of sucrose in samples F and G (specified as unsweetened on the labels) in comparison with sweetened commercial (E) and homemade (H) samples.

Chemometric analysis

The chemometric approach has the advantage of considering all variables together, and shows the high-correlation variables in few new components. Therefore, principal components analysis (PCA) was applied to ^{1}H NMR spectra in order to reduce the number of variables (data points) without losing information, and to establish which samples had similar properties (characteristics), as well as to visualize patterns more clearly because the results can be represented graphically. In this way, PCA analysis was performed under the region of 1.5-3.0 ppm of the ^{1}H NMR spectra, corresponding to the signals of amino acids and acetate (Figure 6).

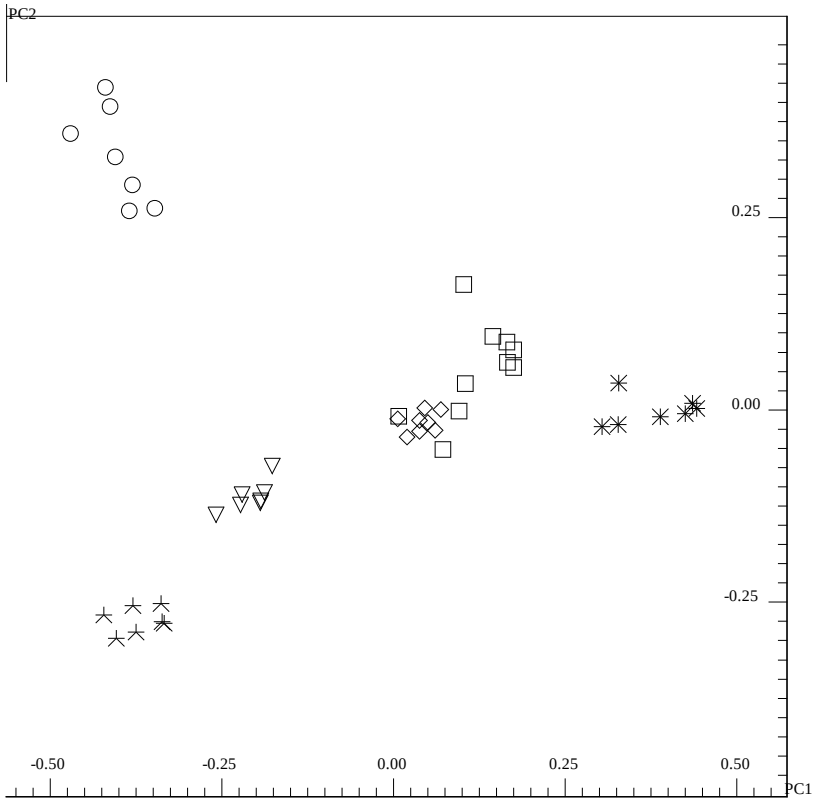

Figure 6. PC1 (64.61%) *versus* PC2 (20.45%) scores plot of chemometric analysis of the 1.5-3.0 ppm region, showing in the PC1 the similarity between the commercial (∇, ○, ✳, □ and◇) and the homemade (☆) grape-juice samples.

That region was chosen because the amino acids can best represent the authenticity of the grape juices, as adulteration would introduce changes in the ^{1}H NMR profile. Moreover, this region is free of introduced alterations such as added sugar, acidifiers, preservatives and others as well as the ethanol concentration, which results only from the fermentation process and has no relationship to the authenticity of the grape juice. The PCA analysis allowed us to classify the two best commercial grape juices, which were considered by consumers to be closer to homemade (natural) samples than the others. This finding is important, because it shows that ^{1}H NMR and chemometric analysis can be a useful tool to verify the authenticity of commercial grape juice, as well as to detect adulterations such as dilution and other factors. In other words, a classification model can be developed to recognize authentic grape-juice samples, as has been done for orange juice.[35]

Conclusions

The results presented demonstrate the good potential of the application of ^{1}H NMR spectroscopy for quality control of grape juices. The main advantage is that the ^{1}H NMR experiments can be carried out rapidly and directly on the samples without the need for sample pre-treatment, and make it possible to examine the entire spectrum and search for patterns emerging from the data. In this way, by collecting simple ^{1}H NMR spectra it was

possible to follow the ethanol production as a consequence of the occurrence of the fermentative process during storage. These analyses revealed the importance of keeping grape juices under refrigeration after the bottles are first opened, in order to minimize fermentation. A further important conclusion was that the storage time after the bottles are opened should be revised, and must be indicated on all labels. Moreover, the methodology was able to identify possible contamination by alcoholic yeasts during processing, or alternatively, failure of the sterilization process of commercial grape juices. On the other hand, it was demonstrated that the sterilization process of homemade grape juices is very efficient, even better than the industrial process, allowing this juice to be stored for long periods without the need for preservatives. This methodology also allowed the identification of some inconsistencies between the chemical composition of the grape juices and the labels, such as the addition of sugar, acidifiers and preservatives. Additionally, chemometric analysis of the ^{1}H NMR spectra allowed us to classify the best commercial grape juices (closest to homemade samples) which indicates that the methodology can be employed to determine authenticity as well as to detect adulteration of commercial grape juice.

In summary, all findings described in this work demonstrated that ^{1}H NMR, allied to chemometric techniques, is a valuable tool to be employed in the quality control of grape juices, and probably could be a high-throughput push-button NMR tool in a similar way as for orange juice.[35] Therefore, this study is important as much for the producer as for the consumer, in order to guarantee better quality of grape juices.

Acknowledgments

The authors are grateful to the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Financiadora de Estudos e Projetos (FINEP) and the Universidade Federal do Paraná (UFPR) for financial support and fellowships. We also thank Edison Perevalo Wendler and Alexandra Macedo de Oliveira for supplying the homemade grape juices, and Bruker Biospin for the Amix software.

References

1. O'Byrne, D. J.; Devaraj, S.; Grundy, S. M.; Jialal, I. *Am. J. Clin. Nutr*. **76** (2002) 1367.

2. Rice-Evans, C. A.; Miller, N. J.; Paganda, G. *Free Radic. Biol. Med.* **20** (1996) 933.

3. Coimbra, S. R.; Lage, S. H; Brandizzi, L.; Yoshida, V.; Luz, P. L. *Braz. J. Med. Biol. Res*. **2005**, *38*, 1339-1347.

4. Pace-Asciak, C. R.; Rounova, O; Hahn, S. E.; Diamandis, E. P. Goldberg, D. M. *Clin. Chim. Acta*. **1996**, *246*, 163-182.

5. Mello, L. M. R. *Vitivinicultura brasileira: Panorama 2007*. Technical article, available at: http://www.cnpuc.embrapa.br/publica/artigos on 02/27/09.

6. Mello, L. M. R. *Atuação do Brasil no mercado vitivinícola mundial – Panorama 2007*. Technical article, available at: http://www.cnpuc.embrapa.br/publica/artigos on 02/27/09.

7. Gil, A. M.; Duarte, I.; Cabrita, E.; Goodfellow, B. J.; Spraul, M.;

Kerssebaum, R. *Anal. Chim. Acta* **2004**, *506*, 215-223.

8. Gil, A. M.; Duarte, I. F.; Godejohann, M.; Braumann, U.; Maraschin, M.; Spraul, M. *Anal. Chim. Acta* **2003**, *488*, 35-51.

9. Morgano, M. A.; Queiróz, S. C. N.; Ferreira, M. M. C. *Ciênc. Tecnol. Aliment*. **1999**, *19(3)*, 344-348.

10. Malacrida, C. R.; Motta, S. *Ciênc. Tecnol. Aliment*. **2005**, *25(4)*, 659-664.

11. Sautter, C. K.; Denardin, S.; Alve, A. O.; Mallmann, C. A.; Penna N. G.; Hecktheuer, L. H. Determination of resveratrol in grape juice produced in Brazil. *Ciênc. Tecnol. Aliment*. **2005**, *25(3)*, 437-442.

12. Rizzon, L. A.; Link, M. *Ciênc. Rural* **2006**, *36(2)*, 689-692.

13. Mato, I.; Suárez-Luque, S.; Huidobro, J. F. *Food Res. Int*. **2005**, *38*, 1175-1188.

14. Shundo, L.; Almeida, A. P.; Alaburda, J.; Ruvieri, V.; Navas, S. A.; Lamardo, L. C. A.; Sabino, M. *Braz. J. Microbiol*. **2006**, *37(4)*, 533-537.

15. Varga, J.; Kozakiewicz, Z. *Trends Food Sci. Tech.* **2006**, *17(2)*, 72-81.

16. Sacchi, R.; Addeo, F.; Paolillo, L. *Magn. Reson. Chem*. **1997**, *35*, S133-S145.

17. Sacchi, R.; Mannina, L.; Fiordoponti, P.; Barone, P.; Paolillo, L.; Patumi, M.; Segre, A. *J. Agric. Food Chem*. **1998**, *46*, 3947-3951.

18. Mannina, L.; Patumi, M.; Proietti, N.; Bassi, D.; Segre, A. *J. Agric. Food Chem*. **2001**, *49*, 2687-2696.

19. Gil, A.M.; Duarte, I. F.; Delgadillo, I.; Colquhoun, I. J.; Casuscelli, F.; Humpfer, E.; Spraul, M. *J. Agric. Food Chem*. **2000**, *48*, 1524-1536.

20. Le Gall, G.; Puaud, M.; Colquhoun, I. J. *J. Agric. Food Chem*. **2001**, *49*, 580-588.

21. Belton, P. S.; Colquhoun, I. J.; Kemsley, K.; Delgadillo, I.; Roma, P.; Dennis, M. J.; Sharman, M.; Holmes, E.; Nicholson, J. K.; Spraul, M. *Food Chem*. **1998**, *61*, 207-213.

22. Belton, P. S.; Delgadillo, I.; Gil, A. M.; Roma, F.; Colquhoun, M.; Dennis, M.; Spraul, M. *Magn. Reson. Chem*. **1997**, *35*, S52-S60.

23. Charlton, A. J.; Farrington, W. H. H.; Brereton, P. *J. Agric. Food Chem*. **2002**, *50*, 3098-3103.

24. Bosco, M.; Toffanin R., Palo, D.; Zatti, L.; Segre, A. *Sci. Food Agric.* **1999**, *79*, 869-878.

25. Brescia, M. A.; Caldarola, V.; De Giglio, A.; Benedetti, D.; Fanizzi, F. P.; Sacco, A. *Anal. Chim. Acta*. **2002**, *458*, 177-186.

26. Kosir, I. J.; Kidric, J. *J. Agric. Food Chem*. **2001**, *49*, 50-56.

27. Duarte, I.; Barros, A.; Belton, P. S.; Righelato, R.; Spraul, M.; Humpfer, E.; Gil, A. M. *J. Agric. Food Chem*. **2002**, *50*, 2475-2481.

28. Duarte, I.; Barros, A.; Almeida, C.; Spraul, A. M. *J. Agric. Food Chem*. **2004**, *52*, 1031-1038.

29. Spraul, M.; Humpfer, E.; Keller, S.; Schafer, H. *Spin Reports* **2004**, *154/155*, 26-30.

30. Avenoza, A.; Busto, J. H.; Canal, N.; Peregrina, J. P. *J. Agric. Food Chem*. **2006**, *54*, 4715-4720.

31. Duarte, I. F.; Delgadillo, I.; Gil, A.M. *Food Chem*. **2006**, *96*, 313-324.

32. Correia, I.; Nunes, A.; Duarte, I. F.; Barros, A.; Delgadillo, I. *Food Chem*. **2005**, *90*, 853-859.

33. Caligiani, A.; Acquotti, D.; Palla, G.; Bocchi, V. *Anal. Chim. Acta* **2007**, *585*, 110-119.

34. Vogels, J. T. W. E.; Terwel, L.; Tas, A. C.; Van Den Berg, F.; Dukel, F.; Van Der Greef, J. *J. Agric. Food Chem.* **1996**, *44*, 175-180.

35. Spraul, M.; Thiel, T.; Rinke, P. *Spectroscopy: The application notebook* **2008**, *March*, 16.

36. Fauhl, C.; Reniero, F.; Guillou, C. *Magn. Reson. Chem*. **2000**, *38*, 436-443.

37. Vigli, G.; Philippidis, A.; Spyros, A.; Dais, P. *J. Agric. Food. Chem.* **2003**, *51*, 5715-5722.

38. Vlahov, G.; Del Re, P.; Simone, N. *J. Agric. Food Chem.* **2003**, *51*, 5612-5615.

39. Zamora, R.; Gómez, G.; Dobargenes, C.; Hidalgo, F. J. *J. Am. Oil Chem. Soc.* **2002**, *79(3)*, 261-266.

40. Zamora, R.; Gómez, G.; Hidalgo, F. J. *J. Am. Oil Chem. Soc.* **2002**, *79(3)*, 267-272.

41. Brescia, M. A.; Di Martino, G.; Fares, C.; Di Fonzo, N.; Platini, C.; Ghelli, S.; Reniero, F.; Sacco, A. *Cereal Chem.* **2002**, *79*, 238-242.

42. Sacco, A.; Bolsi, I. N.; Massini, R.; Spraul, M.; Humpfer, E.; Ghelli, S. *J. Agric. Food Chem.* **1998**, *46*, 4242-4249.