SNIF-NMR AND CHEMOMETRIC METHODS APPLIED TO ^{1}H NMR IN THE STUDY OF BRAZILIAN BRANDY AUTHENTICITY

Elisangela Fabiana Boffo[1], Márcia Miguel Castro Ferreira[2], Antonio Gilberto Ferreira[1]

[1] Departamento de Química, Universidade Federal de São Carlos, São Carlos - SP, Brazil.
[2] Instituto de Química, Universidade Estadual de Campinas, Campinas - SP, Brazil.

1 INTRODUCTION

The terminology *aguardente,* used for Brazilian brandy, designate the alcoholic beverage obtained by fermentation and distillation of sugars from several sources as sugar-cane, honey, pineapple, banana and grape, among others[1]. The sugar source characterises the produced beverage type and shows peculiar chemical composition and sensory profile. The difficulty is to characterise the correct source of material fermentation, since the ethanol produced is chemically the same, and besides, a huge difference in the price and in the products quality is observed.

Site Specific Natural Isotopic Fractionation studied by Nuclear Magnetic Resonance (SNIF-NMR)[2] is proving to be an efficient technique to control the food authenticity and adulteration when the biosynthetic origin of product is in question[3]. This technique provides isotopic criteria to characterise a biochemical transformation such as fermentation and enables measuring the isotopic ratios for the end products which can be correlated with the precursors[4,5]. Therefore, the biosynthetic origin of *aguardente* can be correlated with the sugar, from which it is originated, through the biosynthetic mechanisms C_3, C_4 and CAM that are used for the plants to fix the CO_2.

However, no information about other components in *aguardente* is obtained via this method. ^{1}H NMR spectroscopy is a strong tool for assessing additional constituents, such as aromatics, carbohydrates and acids. However, ^{1}H NMR spectra of simple foods are often complex. For this reason, it is advantageous to analyze the spectra by multivariate methods[6,7]. Chemometric methods, as Principal Component Analysis (PCA)[8] and Hierarchical Clusters Analysis (HCA)[9], allow describing samples clustering and detecting the biochemical compounds responsible for the samples separation[10]. The main use of PCA is to reduce the dimensionality of a data set while retaining as much information as is possible. It generates a compact and optimal description of the data set[11]. The aim of HCA is to reduce the complex data to a minimum and to highlight the natural groupings in the data. The graphical output of HCA is a dendrogram, a tree-like chart which allows visualization of clustering[12].

The aim of this work is demonstrate the application of SNIF-NMR technique and chemometric methods applied to ^{1}H NMR to determine Brazilian *aguardente* authenticity.

2 MATERIALS AND METHODS

2.1 Samples

Forty *aguardentes* obtained from different sources were studied. Some samples were provided by the manufacturer and others were bought in the Brazilian stores, particularly in the S. Paulo state. All samples were collected in the years 2005 and 2006.

2.2 SNIF-NMR analysis

^{2}H and ^{1}H NMR spectra were acquired at room temperature on a Bruker DRX400 9.4 Tesla spectrometer, using a 5 mm direct-detection probe head without fluorine lock device.

The *aguardentes* were distilled and the ethanol was collected with the boiling point in the range of 76 - 78 °C. Samples were prepared, in triplicate, using 600 μL of ethanol and 100 μL of *N,N,N',N'*-tetramethylurea (TMU), 99.0 %, used as an internal standard. Tetramethylsilane (TMS) was used as internal reference (δ 0.0).

^{2}H NMR spectra (61.4 MHz) were acquired using broadband ^{1}H decoupling; spectral width, 983 Hz; 10240 data points; acquisition time, 5.2 s; relaxation delay, 3.0 s; pulse (90°), 17.5 μs and 1024 FIDs were accumulated. Spectra were processed with zero-filling using an exponential multiplication associated to a line broadening (LB) of 1.0 Hz.

^{1}H NMR spectra (400.2 MHz) were acquired using 65563 data points; spectral width, 4664 Hz; acquisition time, 5.2 s; relaxation delay, 3.0 s; pulse (90°), 10.5 μs and 16 FIDs were accumulated. Spectra were processed with zero-filling using a LB of 0.3 Hz. The phase and baseline were corrected in both spectra.

The isotopic ratios were determined to methyl $(^2H/^1H)_I$ and methylene $(^2H/^1H)_{II}$ sites of ethanol using ^{2}H and ^{1}H NMR. Quantitative data were obtained by manual integration of sample and internal standard peaks and using equation 1 [13,14]

$$\left(\frac{^2H}{^1H}\right)_i^A = \frac{I_i^A}{I^P} * \frac{P^P}{P_i^A} * \frac{m^P}{m^A} * \frac{M^A}{M^P}\left(\frac{^2H}{^1H}\right)^P \quad (1)$$

where I_i^A and I^P are the areas of signal i of A and TMU methyl in the ^{2}H NMR spectrum. P_i^A and P^P are the stoichiometric numbers of hydrogens in site i and in the TMU. M^A, m^A and M^P, m^P are the molecular weight and mass of the A and the TMU, respectively.

2.3 Chemometric analysis applied to ^{1}H NMR spectra

Samples were prepared, in triplicate, using 600 μL of *aguardente* and three drops of D_2O. Sodium-3-trimethylsilylpropionate (TMSP-2,2,3,3-d_4) was used as internal reference (δ 0.0).

All ^{1}H NMR spectra were obtained with three signals suppression, i. e., water, methyl and methylene of ethanol signals in a 5 mm inverse-detection probe head. Eight FIDs were collected as 65536 data points using a 8.5 μs pulse (90°); spectral width, 8013 Hz; acquisition time, 4.1 s and relaxation delay, 6.4 s. Spectra were processed using 32768 data points by applying an exponential line broadening of 0.3 Hz for sensitivity enhancement before Fourier transformation and were accurately phased, baseline adjusted and converted into JCAMP format to build the data matrix.

All calculations were carried out using the Pirouette® software (v. 3.11, InfoMetrix, Woodinville, Washington, USA). The data matrix was built with 3815 variables (columns)

and 78 spectra (lines – 26 samples in triplicate). PCA was applied for exploring the data and for feature selection. Each NMR spectrum was normalized to norm one (the area under the sample profile is set equal to one) and first derivative was taken. The data were also autoscaled, i. e., meancentered and scaled to unit variance, in order to give equal weight to each variable and so, large and small peaks gained the same importance. In HCA, the Euclidean distances among samples are calculated and transformed into similarity indices ranging from 0 to 1 by using the incremental linkage method.

3 RESULTS AND DISCUSSION

3.1 SNIF-NMR analysis

In Figure 1, the ^1H and ^2H NMR spectra of ethanol from a grape *aguardente* are shown, wherein the ethanol, TMU and water signals can be visualised. The results of SNIF-NMR study are shown in the Table 1.

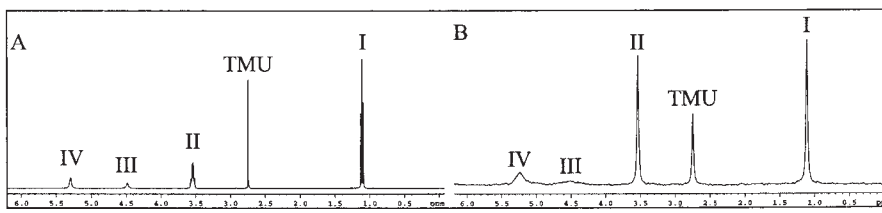

Figure 1 *Natural abundance NMR spectra of A) ^1H and B) ^2H of ethanol from a grape aguardente, I methyl, II methylene, IV hydroxyl of ethanol and III water signals*

Analysing the isotopic relations (Table 1), it can be observed that the $(^2H/^1H)_{II}$ values are in the range from 126.0 to 129.7 ppm. However, the $(^2H/^1H)_I$ values are useful for the discrimination of sugars produced by the C_3 and the C_4 metabolic pathways[2]. Sugars from C_4 plants have a higher content of heavy isotope than C_3 sugars.

For the banana and grape standard *aguardentes* and two commercial grape *aguardentes* the $(^2H/^1H)_I$ values were observed between 99.7 and 102.8 ppm. These values are characteristic of plants that fix CO_2 using the C_3 mechanism.

$(^2H/^1H)_I$ values of honey *aguardentes* point out that the honeys were probably made from C_3 plants nectars, because they are similar to those of C_3 plants and to those found in honey authenticity studies[15,16].

The cassava *aguardente* (C_3 plant) had a $(^2H/^1H)_I$ value of 99.7 ppm, characteristic of this plant type and confirming its authenticity.

For the sugar-cane and maize *aguardentes* (C_4 plants), $(^2H/^1H)_I$ values are in the range from 111.7 to 113.6 ppm. Therefore, a satisfactory discrimination (approximately 10 ppm) between C_3 and C_4 *aguardentes* has been observed.

For all banana, peach, coconut and pineapple commercial *aguardentes* (C_3 plants), $(^2H/^1H)_I$ values were similar to those of sugar-cane *aguardentes*. For one commercial grape *aguardente* (Grape3), a $(^2H/^1H)_I$ value was found superior to those of standard samples. These results suggest the predominance of C_4 plant in the origin of sugars used in their production.

Table 1 *$^2H/^1H$ relations for methyl (I) and methylene (II) sites of ethanol (in ppm)*

	Isotopic Relation (ppm)** $(^2H/^1H)_I$	$(^2H/^1H)_{II}$	Biosynthetic pathway
Grape1*	101.3 (± 1.2)	127.5 (± 0.6)	C_3
Grape2*	102.8 (± 0.5)	128. 2 (± 1.6)	C_3
Grape3	106.6 (± 0.2)	128.4 (± 0.2)	C_3
Grape4	101.3 (± 0.2)	128.0 (± 0.1)	C_3
Grape5	102.6 (± 0.1)	127.7 (± 0.4)	C_3
Honey1*	99.7 (± 0.8)	128.8 (± 0.7)	--
Honey2*	100.4 (± 0.7)	129.1 (± 0.7)	--
Honey3*	101.3 (± 0.9)	128.0 (± 0.5)	--
Honey4	103.6 (± 0.2)	126.9 (± 1.1)	--
Honey5	102.1 (± 0.2)	126.9 (± 0.2)	--
Banana1*	101.9 (± 0.3)	127.7 (± 0.3)	C_3
Banana2*	102.2 (± 0.2)	127.4 (± 0.4)	C_3
Banana3*	102.3 (± 0.2)	127.2 (± 0.3)	C_3
Banana4	112.8 (± 0.2)	126.6 (± 0.3)	C_3
Banana5	113.9 (± 0.1)	128.3 (± 0.0)	C_3
Banana6	113.2 (± 0.2)	128.1 (± 0.2)	C_3
Banana7	111.4 (± 0.2)	127.5 (± 0.3)	C_3
Cassava1	99.7 (± 0.3)	128.3 (± 0.4)	C_3
Peach1	113.0 (± 0.3)	126.9 (± 0.1)	C_3
Coconut1	112.2 (± 0.1)	127.2 (± 0.3)	C_3
Pineapple1*	107.8 (± 0.6)	129.0 (± 0.3)	CAM
Pineapple2*	107.9 (± 0.4)	128.0 (± 0.3)	CAM
Pineapple3*	107.1 (± 0.2)	128.6 (± 0.3)	CAM
Pineapple4	113.3 (± 0.2)	128.5 (± 0.4)	CAM
Sugar-cane1	111.7 (± 0.2)	127.8 (± 0.2)	C_4
Sugar-cane2	111.7 (± 0.6)	129.7 (± 0.1)	C_4
Sugar-cane3	112.3 (± 0.4)	126.0 (± 0.5)	C_4
Sugar-cane4	112.3 (± 0.2)	127.5 (± 0.2)	C_4
Sugar-cane5	112.5 (± 0.3)	125.4 (± 0.4)	C_4
Sugar-cane6	112.6 (± 0.2)	128.1 (± 0.2)	C_4
Sugar-cane7	112.7 (± 0.2)	128.2 (± 0.2)	C_4
Sugar-cane8	112.8 (± 0.1)	127.7 (± 0.2)	C_4
Sugar-cane9	112.8 (± 0.4)	125.5 (± 0.4)	C_4
Sugar-cane10	112.8 (± 0.6)	128.5 (± 0.9)	C_4
Sugar-cane11	113.4 (± 1.1)	127.1 (± 1.3)	C_4
Sugar-cane12	113.4 (± 0.4)	127.2 (± 1.0)	C_4
Sugar-cane13	113.4 (± 0.3)	128.4 (± 0.4)	C_4
Sugar-cane14	113.3 (± 0.8)	126.0 (± 0.7)	C_4
Sugar-cane15	113.6 (± 0.7)	127.7 (± 1.1)	C_4
Maize1	113.9 (± 0.3)	127.4 (± 0.3)	C_4

* standard samples ** triplicate medium values

Pineapple *aguardentes* (CAM plant) has shown $(^2H/^1H)_I$ values between those of C_3 and C_4 plants, because CAM plants fix CO_2 in the photosynthesis using an intermediary mechanism to those used by C_3 and C_4 plants.

The results have shown that a great number of commercial *aguardentes* have undergone for some adulteration process, possibly by using the sugar-cane *aguardentes*, which have a low cost involved in its manufacture process.

3.1 Chemometric analysis applied to ^1H NMR spectra

The ^1H NMR spectra with three signals suppression from *aguardentes* were compared (Figure 2), with the purpose to verify the existence of differences that could be responsible for the samples discrimination. In the Grape4 *aguardente* spectrum the signals more easily

identified were the doublets in δ 4.60 and 5.20, with coupling constant *J* of 8.0 and 3.7 Hz, with respect to anomeric hydrogens of β-glucose (H1 – B) and of α-glucose (H1 – **A**), respectively. In the Sugar-cane1 *aguardente* spectrum, H1 – B and H1 – A signals were also observed, but those of sucrose presented higher intensity, H1 – C was observed as one doublet in δ 5.40 (*J* = 3.8 Hz). In the banana and honey *aguardentes* spectra, the carbohydrates signals were not visualised.

Moreover, acetic acid, ethyl acetate and acetone were identified in Grape4 *aguardente* spectrum, presenting the methyl singlets in δ 2.02, 2.07 and 2.22, respectively. In this sample it was still possible to identify the methanol presence: its methyl was observed as one singlet in δ 3.35. This identification was made by standard addition.

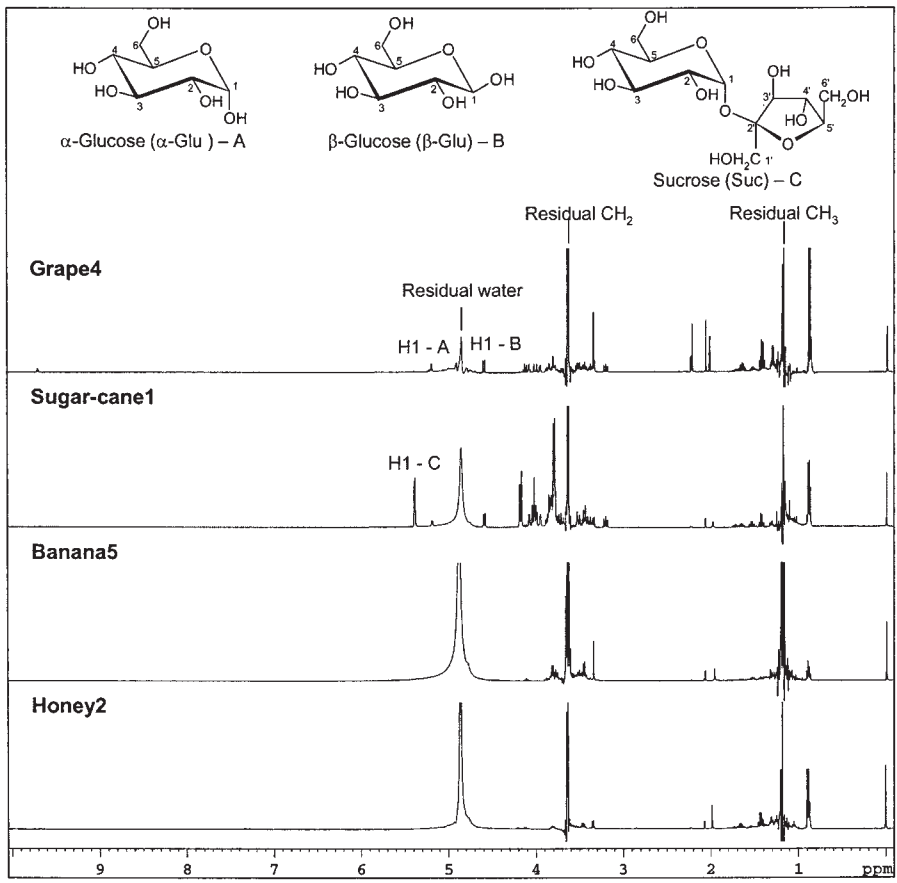

Figure 2 *^{1}H NMR spectra with three signals suppression of aguardentes (D_2O) and ^{13}C satellites decoupling*

Principal component analysis (PCA), applied to ^{1}H NMR spectra data of *aguardentes* allowed the discrimination of different types of samples and pointed out the compounds related to it. PC1 x PC2 scores plot is shown in the Figure 3. PC1 describes 25.3% of the total variance, while PC2 14.8%, the two PCs together express 40.1% of the original

information. In this graph, it is verified that two samples presented different behaviour which can be explained by analysing the loadings graph. Cassava *aguardente* is positioned at positive values of PC1 and negative of PC2 because it contains the largest amount of acetic acid and ethyl acetate. Sugar-cane10 sample is located at negative values in PC1 and PC2 because of two doublets in δ 2.72 and 2.81 (J = 15.5 Hz), not present in other samples from the same group. These two peaks were attributed to the citric acid. Maize, banana and grape samples were grouped near to the sugar-cane *aguardentes* group. A distinct group was obtained for honey *aguardentes*. Adulterated commercial *aguardentes* from banana, peach, coconut and pineapple has shown ^{1}H NMR spectra quite similar, what suggested that they were made from the same raw material.

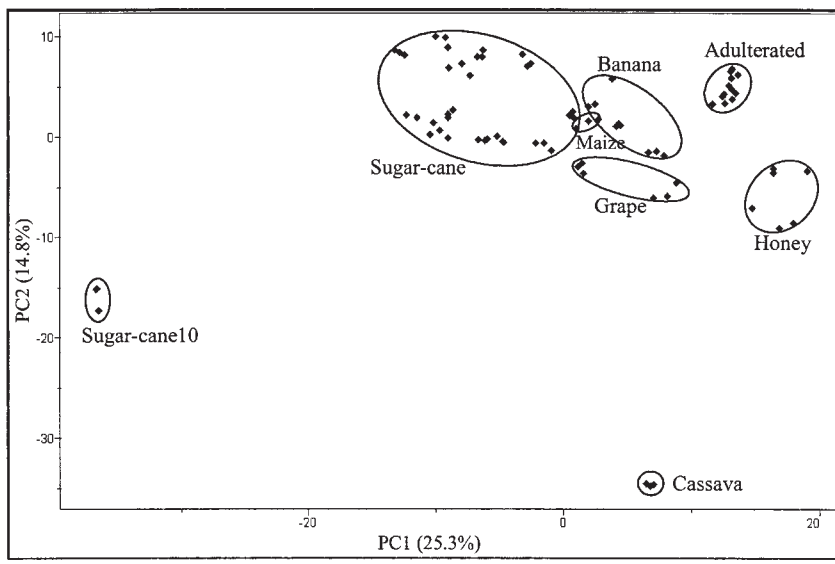

Figure 3 *PC1 x PC2 scores plot of the ^{1}H NMR data from aguardentes (40.1%)*

The dendrogram obtained from HCA analysis is shown in Figure 4. Using a similarity index of 0.504, eight subgroups can be identified. This result is similar to that obtained in PCA, however, maize *aguardente* is grouped to banana samples and the Grape3 grouped to the sugar-cane samples.

These results have shown that Sugar-cane10 and Cassava *aguardentes* are well distinguished since they have distinct chemical compositions. Therefore, they were excluded from the data set and a new PCA analysis was carried out, with a purpose to obtain a better discrimination among the other *aguardentes*.

The PC1 x PC2 scores plot (Figure 5) represents 39.1 % of the information of the data. PC1 describes 26.6 % of the total variance while PC2 12.5 %. Sugar-cane and maize *aguardentes* form groups at negative values of PC1 because they have the higher quantity of sucrose. On the other hand, distinct groups were observed for honey and adulterated commercial *aguardentes* at positive values of PC1 because of absent carbohydrates signals in the ^{1}H NMR spectra. Grape4 placed on the inferior right side of the plot for having only α- and β-glucose signals. Grape3 sample is grouped near to the sugar-cane group because

of higher sucrose content than Grape4 sample. Moreover, it is observed that banana *aguardentes* are not grouped because they have different carbohydrates concentrations.

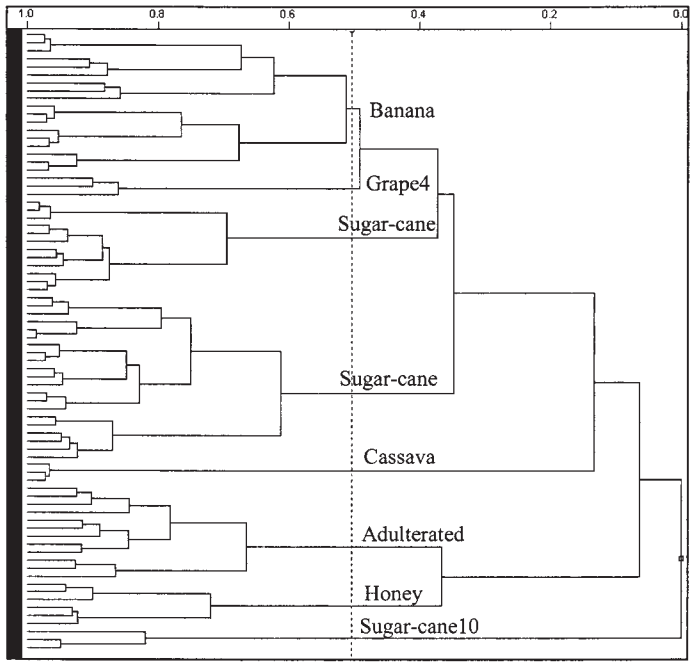

Figure 4 *HCA dendrogram obtained from ^{1}H NMR spectra from different aguardentes types (similarity index: 0.504)*

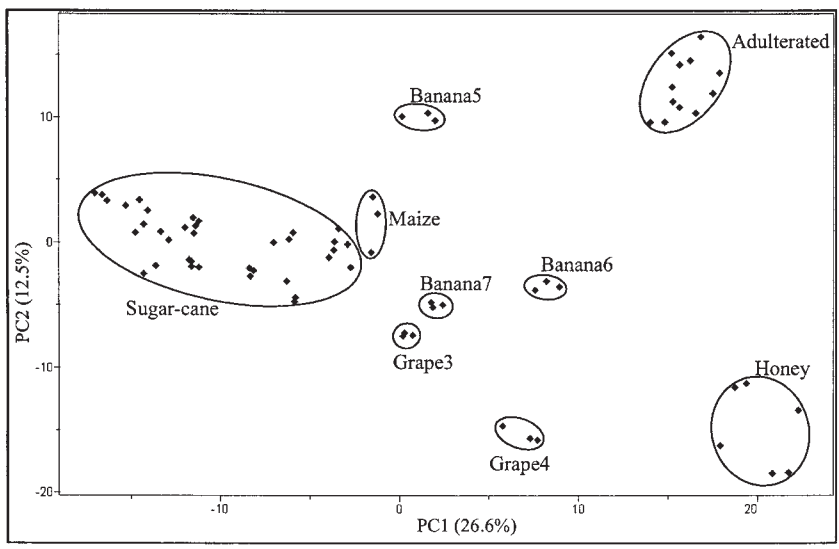

Figure 5 *PC1 x PC2 scores plot of the ^{1}H NMR data from aguardentes (39.1%)*

4 CONCLUSIONS

SNIF-NMR method and chemometric analysis applied to ^{1}H NMR spectra can distinguish *aguardentes* produced from plants with different biosynthetic origin as C_3 (grape, banana, cassava and honey), C_4 (sugar-cane and maize) and CAM (pineapple).

Acknowledgements

We are grateful to CAPES, CNPq, FAPESP and FINEP for financial support.

References

1 BRASIL, Decreto Presidencial nº 4.851, 02/10/2003. Altera Decreto nº 2.314, de 04/09/1997. Diário Oficial da União, Brasília, DF, 03 out 2003.
2 J.F. Cotte, H. Casabianca, J. Lhéritier, C. Perrucchietti, C. Sanglar, H. Waton and M.F. Grenier-Loustalot, *Anal. Chim. Acta*, **582**: 125 – 136, 2007.
3 G.J. Martin, B.L. Zhang, N. Naulet and M.L. Martin, *J. Am. Chem. Soc.,* 1986, **108**, 5116.
4 G.J. Martin, C. Guillou, M.L. Martin, M.T. Cabanis, Y. Tep and J. Aerny, *J. Agric. Food Chem.,* 1988, **36**, 316.
5 S. Pionnier, R.J. Robins and B.-L. Zhang, *J. Agric. Food Chem.* 2003, **51**, 2076.
6 F.H. Larsen, F. van den Berg and S.B. Engelsen, *J. Chemom*., 2006, **20**, 198.
7 R. Consonni, L.R. Cagliani, F. Benevelli, M. Spraul, E. Humpfer and M. Stocchero, *Anal. Chim. Acta* 2008, **611**, 31.
8 H. Winning, F.H. Larsen, R. Bro and S. B. Engelsen, *J. Magn. Reson.,* 2008, **190**, 26.
9 O. Beckonert, M.E. Bollard, T.M.D. Ebbels, H.C. Keun, H. Antti, E. Holmes, J.C. Lindon, J.K. Nicholson, *Anal. Chim. Acta,* 2003, **490**, 3.
10 B. Lavine and J. Workman, *Anal. Chem*., 2006, **78**, 4137.
11 M. Jalali-Heravi, S. Masoum and P. Shahbazikhah, *J. Magn. Reson.*, 2004, **171**, 176.
12 J.C. Lindon, E. Holmes and J. K. Nicholson, *Prog. Nucl. Magn. Reson. Spectrosc.* 2001, **39**, 1.
13 G.J. Martin, X.Y. Sun, C. Guillou and M.L. Martin, *Tetrahedron*, 1985, **41**, 3285.
14 M.L. Martin, G.J. Martin and C. Guillou, *Mikrochim. Acta*, 1991, **II**, 81.
15 P. Lindner, E. Bermann, and B. Gamarnik, *J. Agric. Food Chem.,* 1996, **44**, 139.
16 E.F. Boffo, L.A. Tavares, A.G. Ferreira, M.M.C. Ferreira and A.C.T. Tobias, in *Magnetic Resonance in Food Science. From Molecules to Man.* eds. I.A. Farhat, P.S. Belton and G.A. Webb, Royal Society of Chemistry, Cambridge, 2007, p. 105.