ADULTERATION STUDY IN BRAZILIAN HONEY BY SNIF AND ^{1}H NMR

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1 INTRODUCTION

The apiculture in Brazil are increasing in the last years and will be possible to ingress in the set of the honey exportation country selling to Europe, United States and Japan[1]. However, it is very important to have a good quality control to attend the exigency of the consuming market. Therefore, a more rigorous quality control must be carried to increase its competitively.

The food products authentication is a primary importance and from commercial rules point of view, quality standards have been established through the requirement of quality labels that specify the product chemical composition, validate/expire data, origin country and in some case the specific place that they are produced and/or collected in a particular country. From the economic point of view, product authentication is essential to avoid unfair competition[2]. Besides this, different countries have they property legislation about the quality control which difficult the standardisation. For instead in Brazil the legislation, according to resolution no.11/2000, forbidden the use of any additive in the honey which is characterised like a natural, sweet substance produced by *Apis mellifera* bees from nectar of plants or from secretions of living parts of plants or excretions of plant-sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in honeycombs to ripen and mature[3].

Site-specific Natural Isotope Fractionation Nuclear Magnetic Resonance (SNIF-NMR) is one of the best answer for the question of adulterations when are involved the product biosynthetic origin. Stemming from the work of Gérard J. Martin and colleagues at Nantes University, France, SNIF-NMR is probably the most applied and specific method for food product authenticity. After the technique was developed in the early 1980s, the original application of it was the detection of the chaptalization or enrichment, in wines[4]. Since then, SNIF-NMR has been extended to other products than wine to show the addition of undeclared sugars[4-7]. Martin et al. applied SNIF-NMR alone[8] or SNIF-NMR and stable isotope ratio analysis mass spectrometry (SIRA-MS)[9] to detect added sugar and to evaluate authentication in fruit juices.

SNIF-NMR techniques, which determine the $^{2}H/^{1}H$ relationship, provides an isotopic criteria to characterise a biochemical transformation such as fermentation and it is enable

to measure the isotopic ratios for the end products which can be correlated with the precursors[10]. Therefore, it is possible to verified the type of plant where the nectar was collected by the bees, if they use the cycle of carbon fixation in their biosynthetic mechanisms C_3 and C_4[11, 12] which result in a different isotopic ratios. It also allows to inquire if the honey was adulterated with some another type of sugar.

With this objective, ethanol produced from fermentation of honey from different source were analysed. The ethanol molecule possess three mono-deuterated isotopomers at natural abundance[13] $CH_2\mathbf{D}CH_2OH$ (I), $CH_3CH\mathbf{D}OH$ (II) and $CH_3CH_2O\mathbf{D}$ (III), therefore, the deuterium can be located either on the methyl (I), the methylene (II) or the hydroxyl (III) sites. Because of the low natural abundance of deuterium relative to hydrogen, the probability to find a bi-deuterated species is very low and can be ignored[14].

Honey adulteration is easily carried out by several ways like a simple sucrose addition (of a C_4 plant like sugar cane), a mixture of honey from different source and more recent, to feeding the bees with a sucrose solution or sucrose syrup with vegetal extract when they are harvesting the nectar. The deuterium/hydrogen ratios measured at the methyl site of ethanol $(^2H/^1H)_I$ in honeys differ significantly according to the nectar origin. By feeding the bees with sucrose solutions from sugar-cane, for instance, will increase the $(^2H/^1H)_I$ value if compared with that produced from C_3 plant, like nectar from eucalyptus or citrus[12].

This work describe the use of SNIF-NMR techniques to classified honey commercial samples using the $^2H/^1H$ isotopic relation of the methyl and methylene groups in ethanol by NMR. In this context, we can confirm the honey precedence and we can investigate the product adulteration. ^1H NMR was also used together with chemometric methods, Hierarchical Cluster Analysis (HCA), to complement this study. The HCA method examines the distances between all of the samples and represents the information in the form of a two-dimensional plot called a dendrogram[15].

2 MATERIALS AND METHODS

2.1 Samples

Forty honey samples obtained from the flowers of different plants like: citrus (*Citrus sp.*), eucalyptus (*Eucaliptus sp.*), assa-peixe (*Vernonia sp.*), wildflowers and feeding the bees with a solution of sucrose (sugar-cane - *Saccharum sp.*) were studied. Some of these samples were provided by the beekeepers and the others were bought in the Brazilian shopping particularly in S. Paulo state. All the sample are collected in the years 2004 and 2005.

2.2 Fermentation

The sample (10 g) was introduced into a 500 mL glass flask at 24 °C. 5 g of baker's yeast, *Saccharomyces cerevisiae*, were added with a continuous stirring during 48 hours. At the end of fermentation the ethanol was distilled and the liquid collected with the boiling point in the range of 76-78 °C.

Samples for NMR measurements were prepared using 600 μL of distillate ethanol and 100 μL of tetramethylurea (TMU), 99.0 %, which was used as an internal pattern.

2.3 ^{2}H and ^{1}H NMR Spectra

All the ^{2}H and ^{1}H NMR spectra of ethanol were carried out on a Bruker DRX400 - 9.4 Tesla spectrometer without fluorine lock device and all measurements were made in triplicate in a 5 mm direct probe, maintaining the temperature constant at 298K and with the same data acquisition and processing.

The ^{2}H NMR data (61.4 MHz) were acquired using broadband proton decoupling, spectral width 983 Hz, memory size 10k, acquisition time 5.2 s, relaxation delay 2 s, rf pulse (90°) 17.5 μs and 1024 FIDs were accumulated. The FIDs were processed with zero-filling using an exponential multiplication associated with a line broadening 1.0 Hz and was made the automatic correction of the baseline.

The ^{1}H NMR data were acquired using spectral width 6410 Hz, memory size 64k, acquisition time 5.2 s, relaxation delay 2 s, rf pulse (90°) 10.5 μs and 16 FIDs were accumulated. The FIDs were processed with zero-filling using an exponential multiplication associated with a line broadening 0.3 Hz. and was made the automatic correction of the baseline. The internal reference for ^{1}H NMR spectra was TMS (tetramethylsilane).

2.4 Calculations

The isotopic ratios were determined from the methyl $(^{2}H/^{1}H)_{I}$ and methylene $(^{2}H/^{1}H)_{II}$ sites from ethanol molecule using deuterium/hydrogen NMR. The quantitative data were obtained by the automatic integration of the peaks from the sample and internal pattern (TMU).

The isotopic ratios at the two ethanol sites were determined according to the equation 1[16,17]

$$\left(\frac{^{2}H}{^{1}H}\right)_{i}^{A} = \frac{I_{i}^{A}}{I^{P}} * \frac{P^{P}}{P_{i}^{A}} * \frac{m^{P}}{m^{A}} * \frac{M^{A}}{M^{P}} \left(\frac{^{2}H}{^{1}H}\right)^{P} \quad (1)$$

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

2.5 ^{1}H NMR Spectra for chemometric analysis

The samples for ^{1}H NMR were obtained dissolving 150 mg of honey in 450μL of $D_{2}O$. All the ^{1}H NMR spectra were obtained using a 5 mm probe with inverse detection, in triplicate and in the same parameter conditions of acquiring and processing data, using zgcppr pulse program, acquisition time 7.02 s, memory size 64K, spectral width 4664 Hz, relaxation delay 1.5 s, rf pulse 8.50 μs with 64 FIDs accumulated. In the processing were used 32k points and was made baseline automatic correction.

2.6 Chemometrics treatment

The software used was the Pirouette® version 2.02. The data matrix was built with 4666 variables (columns) and 120 spectra (lines – 40 samples in triplicate). The variable pre-processing was autoscaling, and samples transformations was normalisation and apply the first derivative. The method used was incremental linking with Euclidian distance.

3 RESULTS AND DISCUSSION

In figure 1, we can observe the ^{1}H and ^{2}H NMR spectra of ethanol obtained from fermentation of a wildflower honey, where can be visualised the signals of ethanol, TMU and water.

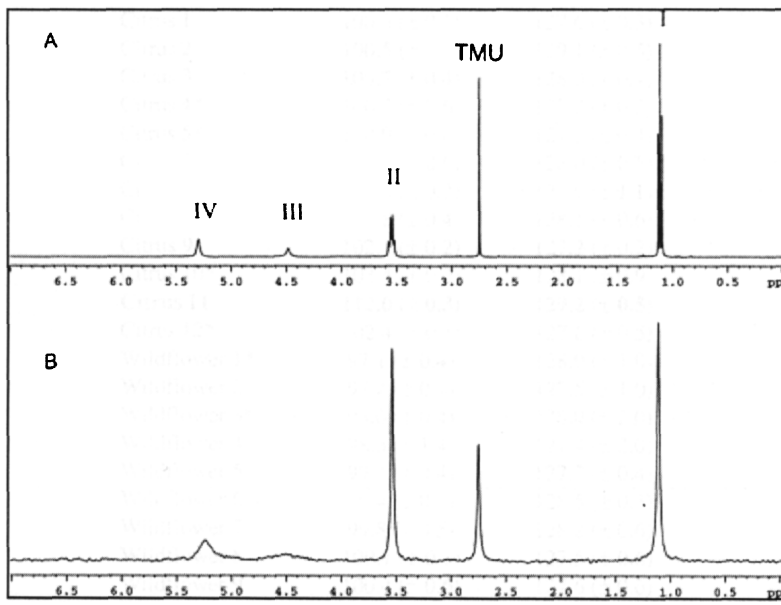

Figure 1 *Natural abundance NMR spectra of A) ^{1}H and B) ^{2}H of ethanol from a honey sample fermentation where I methyl, II methylene and IV hydroxyl from ethanol signals and III water*

The isotopic ratios $(^{2}H/^{1}H)_{I}$ and $(^{2}H/^{1}H)_{II}$ of ethanol samples obtained from honey fermentation are shown in table 1.

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

Honey	Isotopic Relation (ppm)** $(^2H/^1H)_I$	$(^2H/^1H)_{II}$
Eucalyptus 1*	96.9 (± 0.1)	128.3 (± 1.1)
Eucalyptus 2	96.9 (± 1.0)	128.4 (± 0.2)
Eucalyptus 3*	97.3 (± 0.8)	129.6 (± 0.5)
Eucalyptus 4	97.9 (± 0.2)	128.2 (± 0.1)
Eucalyptus 5	99.2 (± 0.7)	127.5 (± 0.6)
Eucalyptus 6	99.4 (± 0.2)	128.8 (± 0.2)
Eucalyptus 7*	99.7 (± 0.8)	128.8 (± 0.7)
Eucalyptus 8	99.7 (± 1.0)	129.1 (± 0.5)
Eucalyptus 9	98.7 (± 0.5)	128.1 (± 0.5)
Eucalyptus 10	**112.2 (± 0.5)**	**128.0 (± 0.3)**
Citrus 1	100.3 (± 0.1)	127.6 (± 0.3)
Citrus 2	100.5 (± 0.3)	129.1 (± 0.5)
Citrus 3	100.7 (± 0.4)	128.0 (± 0.4)
Citrus 4*	100.7 (± 0.6)	128.2 (± 0.2)
Citrus 5*	100.9 (± 0.6)	127.3 (± 0.4)
Citrus 6	101.3 (± 0.9)	128.0 (± 0.5)
Citrus 7*	101.8 (± 0.3)	127.9 (± 1.1)
Citrus 8	101.8 (± 0.4)	128.1 (± 0.6)
Citrus 9	102.1 (± 0.2)	127.2 (± 0.2)
Citrus 10*	103.4 (± 0.2)	128.1 (± 0.9)
Citrus 11	**112.0 (± 0.3)**	**129.2 (± 0.5)**
Citrus 12*	102.4 (± 0.1)	127.6 (± 0.5)
Wildflower 1*	97.1 (± 0.4)	128.9 (± 1.0)
Wildflower 2	97.4 (± 0.4)	127.5 (± 1.0)
Wildflower 3*	98.0 (± 0.4)	128.0 (± 2.0)
Wildflower 4	98.5 (± 1.4)	127.4 (± 2.0)
Wildflower 5	99.4 (± 0.4)	127.7 (± 0.4)
Wildflower 6	99.4 (± 0.7)	128.5 (± 0.3)
Wildflower 7	99.8 (± 0.5)	128.2 (± 0.6)
Wildflower 8	100.1 (± 0.1)	127.5 (± 0.6)
Wildflower 9	100.4 (± 0.6)	127.6 (± 0.6)
Wildflower 10*	100.4 (± 0.7)	129.1 (± 0.7)
Wildflower 11	100.5 (± 0.4)	128.4 (± 0.4)
Wildflower 12	101.1 (± 0.6)	127.7 (± 0.1)
Wildflower 13	102.0 (± 0.1)	128.4 (± 0.6)
Wildflower 14	102.5 (± 0.4)	127.7 (± 0.4)
Wildflower 15*	103.5 (± 0.2)	129.3 (± 0.2)
Assa-peixe 1*	101.2 (± 0.1)	127.8 (± 0.3)
Assa-peixe 2*	101.7 (± 0.1)	127.9 (± 0.4)
Sugar-cane*	**111.8 (± 0.2)**	**128.0 (± 0.5)**

* standard samples.

** triplicate medium values

Analysing the isotopic relation values presented in the table 1 we can see that the value for methyl $(^2H/^1H)_I$ and for methylene $(^2H/^1H)_{II}$ from ethanol were kept in the range of 98-102 ppm and 127-129 respectively.

The isotopic relation values obtained for honey which were feeding the bees with sugar, from sugar cane, was higher showing the predominance of the influence of C_4 plant in the origin of the sugars produced for the bees.

For two samples which pretend to be honeys collected from eucalyptus (sample 10) and citrus (sample 11) we found the $(^2H/^1H)_I$ relationship values 112.2 and 112.0 ppm, respectively. These are very similar to that found for honey produced when the bees are feeding with sucrose solution (111.8 ppm). Probably this sample was adulterated.

Besides the SNIF-NMR could separate sample adulterated when we feed bees with sugar cane, it is not able to distinguish honey sample with different origin like eucalyptus, citrus and wildflower. Train to solve this we use the ^1H NMR spectroscopy.

In figure 2 is showed a honey ^1H NMR spectrum, where can be observed that the majority compounds have been identified as being the glucose and fructose. Moreover, small amounts of oligosaccharides were verified, predominantly sucrose and maltose, and other minority compounds that give proper characteristics to each honey type[18].

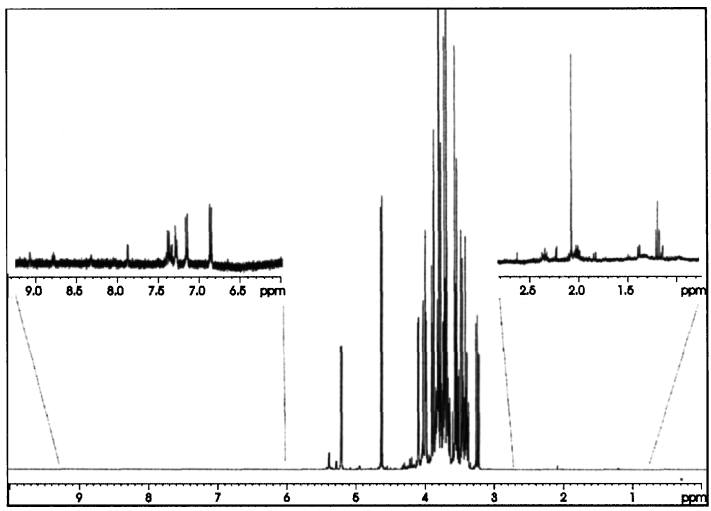

Figure 2 *^1H NMR spectrum of a honey sample*

The samples eucalyptus 10 and citrus 11, were bought as like of eucalyptus and citrus honeys but they presented the ^1H NMR spectra very similar. This is an indication that this samples have unlike characteristics to others, therefore after comparison of all the ^1H NMR spectra for all honeys we do not observed spectra so similar for honeys of different kinds. In figure 3 is showed a comparison between ^1H NMR spectra from citrus (citrus 11 and 7) and eucalyptus (eucalyptus 9 and 10) honeys. The difference observed in this spectra were attributed to three substances, that were identified by gCOSY and gHMBC NMR experiments. Ethanol, a triplet in 1.1 ppm referring to its methyl group; citric acid, two doublets in 2.7 and 2.9 ppm and 5-hydroxymethylfurfuraldehyde (HMF) with signals in the region of 4.5 to 9.7 ppm.

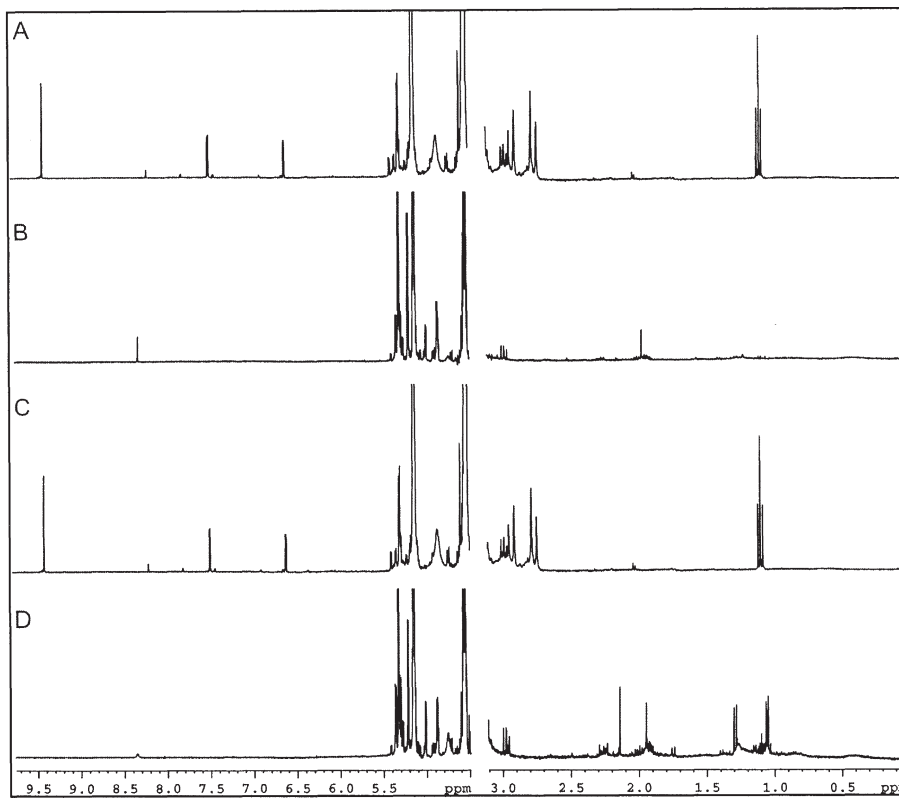

Figure 3 *^{1}H NMR spectra showing the region of 0.0 to 3.1 and 4.5 to 9.7 ppm from honeys (A) citrus 11, (B) citrus 2, (C) eucalyptus 10 and (D) eucalyptus 8*

The presence of ethanol in these honeys (eucalyptus 10 and citrus 11) is an indicative that the fermentation was beginning probably during the storage. Citric acid probably was intentionally added to act as antioxidant, since it was not observed in the ^{1}H NMR spectra for the other citrus honeys; and 5-hydroxymethylfurfuraldehyde (HMF) has been very used as marker in adulteration of the honeys by addition of sucrose, fructose, and other sugars. However, it is also formed by heating the honey when we have a long period product storage and/or it are storage in an inadequate conditions; variations in pH and others factor[19-23]. Therefore the HMF as an indicative of adulteration has been questioned. Honey processing frequently requires heating for both, to reduce viscosity and to prevent crystallisation or fermentation. This results in HMF production, which is formed during acid-catalysed dehydration of hexoses. The presence in honey of simple sugars like glucose and fructose and many acids is a favourable condition for production of this substance[20]. Moreover, in recent years the presence of HMF in foods has been increased a toxicological concerns if the compound and its similar derivatives (5-chloromethyl and 5-sulphidemethylfurfural) have been show to have cytotoxic, genotoxic and tumoral (colon-rectum, hepatic and skin cancers) effects. However, further studies suggest that HMF not offer serious risks to the heath, but the subject still a matter of debate[24].

The application of Hierarchical Cluster Analysis (HCA) in the ^{1}H NMR spectra permitted a discrimination of the citrus and eucalyptus honeys, and moreover separated

other honey kinds. The dendrogram obtained is shown in figure 4. The line dotted with a similarity index of 0,452 discriminates the wildflowers, citrus, eucalyptus honey kinds and those that presented anomalous behaviour eucalyptus 10 and citrus 11 samples.

In an analysis more detailed of each group we can observe that the wildflowers, citrus and eucalyptus honeys showed distinct groups, with few exceptions for some wildflowers honeys that were grouped to citrus (wildflowers 2, 19 and 21), probably because they have predominance of nectar collected from citrus plant. We also observe two other groups that are classified like wildflower and citrus. They are assa-peixe (*Vernonia sp*) and sugar-cane (sugar-cane syrupy used to the feed bees). The probably reason for this is that the native assa-peixe area are very small and the bees need to collect nectar from the others kinds of flowers around the area. In the case of sugar-cane, probably it inclusion in the wildflower group should be because it is a more heterogeneous group.

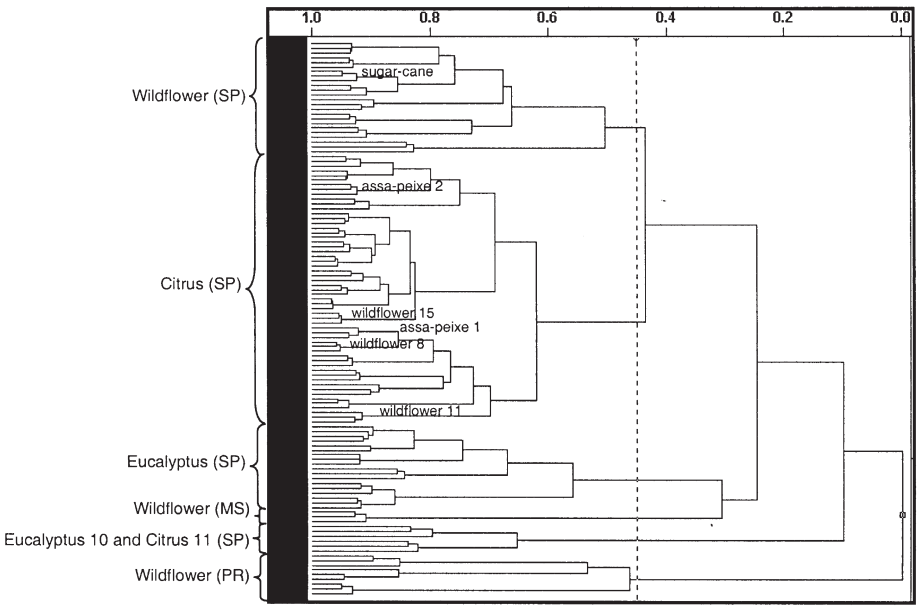

Figure 4 *HCA dendrogram obtained from ^{1}H NMR spectra from different kinds of honeys*

Another tendency observed was the discrimination regarding the geographical origin, where the three groups located in superior part of dendrogram are the samples originated from São Paulo state (SP), in sequence one sample from Mato Grosso do Sul state (MS) and three from Paraná state (PR).

4 CONCLUSIONS

The SNIF-NMR method can distinguish honeys which were produced from flowers with different biosynthetic origin as C_3 (eucalyptus and citrus) and C_4 (sugar-cane) and

chemometric analysis applied to ^{1}H NMR spectra, for the same samples, can discriminate honeys from eucalyptus and citrus.

Acknowledgements

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