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Dedicated to Prof. Bogdan C. Simionescu, on the occasion of his 65th anniversary. The authors acknowledge the long and fruitful collaboration with Prof. Simionescu, as well as his contribution in developing and opening the "Petru Poni" Institute from a dedicated macromolecular research institute to one involved in almost all braches of earth and life sciences.

FAST NMR JUICE IDENTIFICATION BASED ON SUGARS AND OTHER PLANT METABOLITES FROM FRUITS

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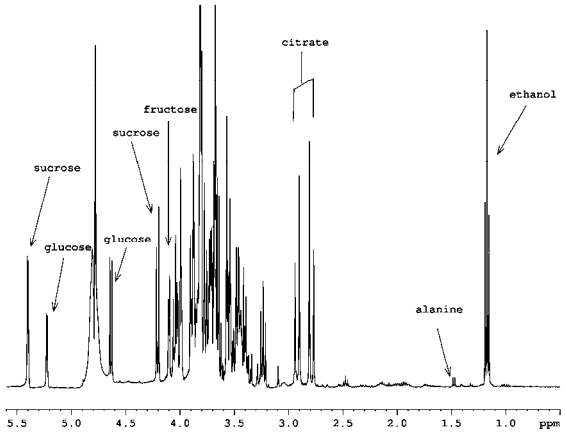
Fast discrimination of three types of fruits (apples, grapefruits and oranges) was achieved based on ^{1}H NMR spectra. When applying statistical processing on three different spectral regions, sugars' region discriminates well between fruits. The best discrimination is achieved based on the aromatic region, whereas the aliphatic region is the less discriminating one. The ^{1}H NMR spectroscopy can easily provide quantitative results for 13 metabolites from fruits.



INTRODUCTION

NMR spectroscopy is already established as a powerful analytical tool in food sciences.[1-10] We have previously used NMR spectroscopy to characterize various foodstuff including, edible oils,[11-16] fruits and vegetables,[17-19] and wines.[20-22] As it concerns sugars, we have been previously exploring analytical protocols for their analysis in biofluids,[23-25] as well as their chemistry [26-34] and biochemistry.[35-39]

The composition of fruit juices (freshly squeezed or industrially manufactured) is influenced by many factors of natural or

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"artificial" origin. From the degree of ripping, type of cultivar, country of origin to storage conditions or the method used to obtain the juice, all these factors influence the composition of fruit juices.

There are several NMR studies on fruits, dealing with various issues, including seeds characterization, [14-40] metabolic fingerprinting (like type of cultivar, degree of ripening, geographic origin, or sample preparation and chemometric protocols)[41-45] and adulteration.[46-49] One of the fruit juices used as illegal mixture with other more expensive juices is the apple juice. For this reason apples have been included in the present study in addition to two citrus.

The present paper reports on a feasibility study on applying ^{1}H NMR spectroscopy in combination with chemometric techniques on both fresh and freeze dried fruit juices. The freeze drying process is often used in food sciences for preserving food stuff without degrading thermally labile compounds. For NMR spectroscopy the technique has the potential for allowing access to the less abundant ^{13}C nuclei by re-dissolving the freeze dried sample in a smaller quantity of water than it was initially present in the juice.

The present study was performed on both fresh and commercial juices from oranges, grapefruits and apples. The study was performed on a total of 18 juices, *i.e.* 6 juices from each type of fruit, 4 fresh and 2 commercial. From each fruit there have been prepared and analyzed a total number of 216 fresh samples and 360 freeze dried samples. In order to asses the possibility of using the chemometric model for fast discrimination in quality control laboratories, we have involved a number of 8 operators, out of which 4 operators have been members of the NMR laboratory and 4 operators have been students in a 3 months internship in the NMR laboratory. The 576 fresh and freeze dried samples have been divided between the 8 operators.

RESULTS AND DISCUSSIONS

Typical ^{1}H NMR spectra of juices from apple, orange and grapefruit are presented in Fig. 1. The spectra are similar, containing the signals from almost the same metabolites, but in different concentrations depending on the fruit type.

The ^{1}H NMR spectra of fruit juices can be rationalized in three regions: the high field region between 0.5-3.1 ppm with signals from aliphatic metabolites, the intermediate region between 3.1-6.0 ppm dominated by the sugars signals and the low field region between 6.0-9.0 ppm with aromatic derivatives, heterocycles, organic acids and polyphenols.

Based on known chemical shifts,[41,47] for the purposes of this research we assigned and quantified signals of the following metabolites: citric acid, malic acid, citramalic acid, quinic acid, dimethylproline, glucose, sucrose, fructose and galactose. Some of these compounds are present in all the analyzed juices while others (ex. dimethylproline or citramalic acid) depend on the fruit type. The compounds mentioned above had intense and resolved signals and we were able to safely assign them. For all the analyzed juices, the most intense signals belong to three main sugars: sucrose, fructose and glucose. Galactose has also been identified in apple juices, but its concentration is significantly lower as compared to the other sugars.

Although the NMR spectra of fruit juices are crowded, there are enough isolated signals to allow direct quantification of 9 metabolites. Table 1 presents absolute concentrations for four sugars and other five low molecular metabolites and Table 2 presents concentrations relative to alanine for the same metabolites. Using values relative to an internal standard (alanine in our case) make the comparison between various samples easier and more informative, particularly in cases when there are large differences in the dilution factor of juices. In both tables, the first set of data on each row refers to natural juices, while the second set of data refers to commercial juices.

For some of the quantified compounds we were unable to identify the corresponding signals in all the analyzed juices, although they are known to be present. For example citric acid is present in apples, malic acid in grapefruits and quinic acid has been reported in oranges. But they are present either in low concentrations or the corresponding signals are overlapped by other signals.

Based on the data from Table 1, we obtained different values for the ratio glucose:fructose: sucrose, depending on the fruit type. Thus, for natural juices, the ratios were roughly 1:2:1 for grapefruits, 1:1:1 for oranges and 4:15:1 for apples. From these values we can conclude that citrus have a similar content of sugars while apples have a lower content of sucrose as compared to glucose and fructose. Fructose is the predominant sugar in apple juices.

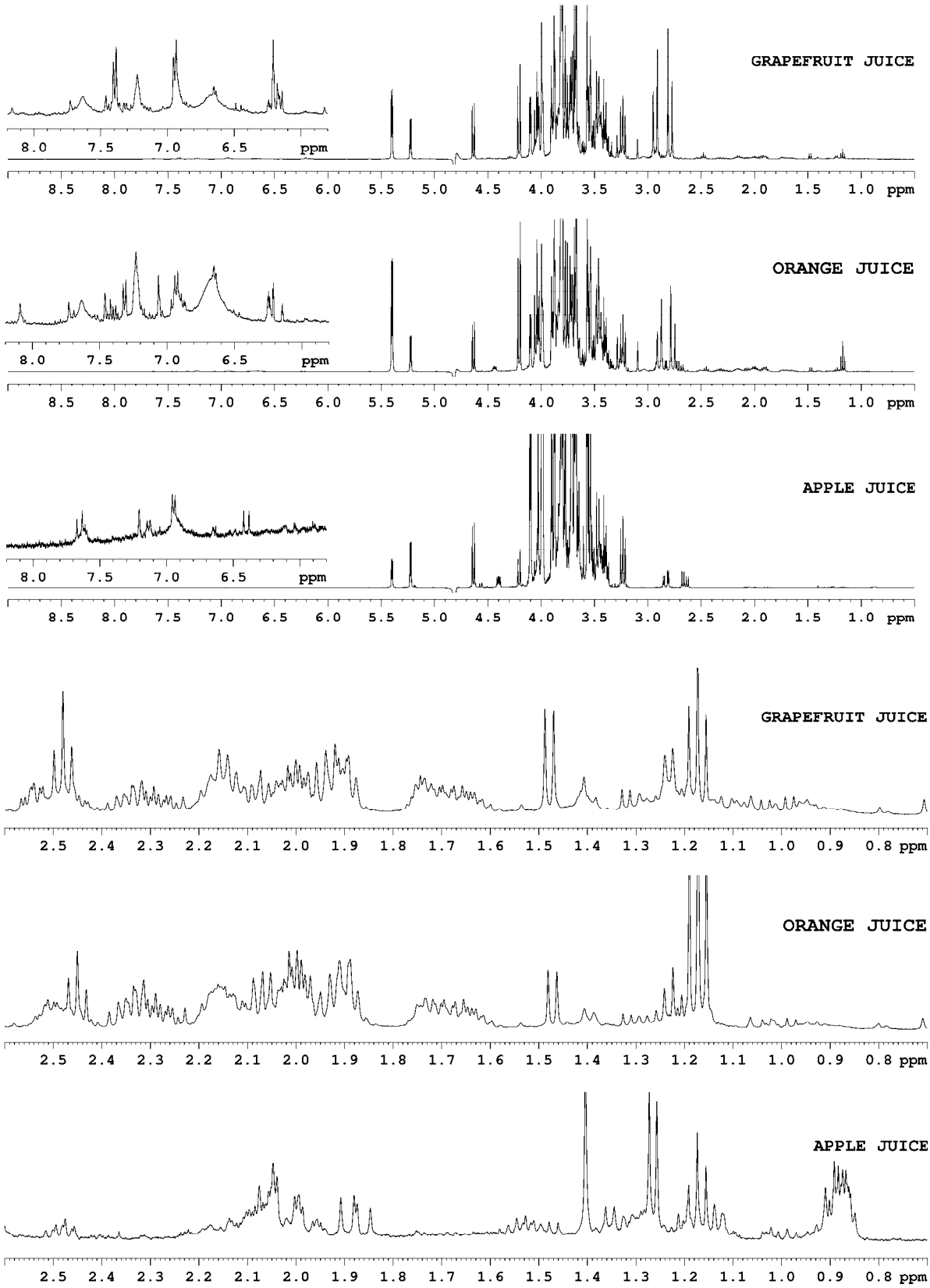

Fig. 1 – Typical ^{1}H NMR spectra of juices from apple, orange and grapefruit.
The whole spectrum (left) and the aliphatic region expanded (right).

Table 1

Averaged absolute concentrations (mmol/L) and ranges for individual values (in brackets) for the selected metabolites as measured by ^{1}H NMR spectroscopy

Metabolite	Grapefruit juices (mmol/L)	Orange juices (mmol/L)	Apple juices (mmol/L)
Citric acid	309.95 (155.24-606.21) 204.94 (27.48-390.72)	56.85 (35.23-87.49) 139.85 (116.01-162.69)	0
Malic acid	0	54.92 (36.09-97.05) 131.39 (106.24-155.78)	26.04 (20.71-44.16) 40.25 (32.69-49.61)
Citramalic acid	0	0	0.26 (0.14-0.50) 0.13 (0.11-0.16)
Quinic acid	0	0	0.88 (0.57-1.49) 2.08 (1.54-2.75)
Dimethylproline	24.04 (15.38-36.73) 17.53 (0.95-34.99)	15.38 (9.46-31.34) 59.20 (47.21-72.76)	0
Glucose	824.08 (490.79-1665.78) 833.12 (216.06-1483.97)	288.59 (183.66-605.62) 1262.29 (1044.32-1481.64)	126.03 (102.96-200.60) 109.50 (88.23-132.68)
Sucrose	592.01 (293.69-927.85) 180.32 (7.47-361.70)	335.91 (240.68-657.01) 872.57 (641.95-1143.32)	30.41 (19.46-48.70) 21.05 (14.66-27.93)
Fructose	1019.55 (632.56-2095.70) 992.49 (205.85-1824.41)	372.19 (260.80-652.09) 1585.61 (1229.75-1901.27)	459.12 (328.58-794.22) 355.73 (303.37-423.23)
Galactose	0	0	8.34 (6.43-12.75) 3.17 (2.68-3.74)

Table 2

Averaged relative concentrations (mmol/mmol alanine) and ranges for individual values (in brackets) for the selected metabolites as measured by ^{1}H NMR spectroscopy

Metabolite	Grapefruit juices (mmol/mmol Ala)	Orange juices (mmol/mmol Ala)	Apple juices (mmol/mmol Ala)
Citric acid	19.77 (8.90-34.94) 63.95 (35.10-94.18)	16.58 (10.28-20.89) 9.94 (8.85-10.52)	0
Malic acid	0	15.65 (12.77-18.89) 9.34 (8.66-10.28)	254.14 (184.50-372.75) 129.25 (120.78-138.00)
Citramalic acid	0	0	2.32 (1.38-4.25) 0.46 (0.31-0.62)
Quinic acid	0	0	7.10 (5.44-11.25) 6.57 (6.35-6.98)
Dimethylproline	1.52 (1.19-2.01) 3.20 (3.13-3.30)	4.20 (3.59-5.33) 4.20 (3.98-4.55)	0
Glucose	51.50 (30.52-84.82) 425.95 (134.75-733.84)	81.44 (67.94-99.52) 89.74 (82.83-96.04)	1225.27 (933.98-1706.25) 352.42 (326.63-378.00)
Sucrose	36.40 (31.48-47.57) 29.12 (24.51-33.56)	93.11 (80.80-102.25) 62.34 (54.61-83.69)	318.58 (208.13-619.50) 73.99 (40.09-108.50)
Fructose	64.18 (36.39-109.69) 431.98 (163.48-716.56)	105.34 (90.39-131.17) 112.65 (103.12-130.02)	4392.00 (3136.84-6757.17) 1156.86 (995.81-1310.61)
Galactose	0	0	77.99 (58.16-111.38) 10.29 (9.14-11.31)

For commercial juice the ratios of the same sugars were roughly 5:5:1 for grapefruits, 2:2:1 for oranges and 5:17:1 for apples. The values suggest a higher content of glucose and fructose in commercial citrus juices, especially grapefruits. This can be explained by the addition of glucose-fructose syrup.

In addition to sugars, citric acid and dimethylproline can be used to differentiate between citrus and apples. The grapefruit and orange natural juices analyzed by us had similar content of citric acid, while the orange juices had twice the amount of dimethylproline. For the commercial orange juices the content of citric acid and dimethylproline were three and four times higher than for natural orange juices. We can speculate that these findings are due to the addition of citric acid (as acidity corrector) and the use of pulp wash juice. It has been shown that the amount of dimethylproline is higher in the case of pulp wash adulteration of orange juice.[47]

Malic acid is present in both apples and oranges juices. For the natural juices analyzed we found that the content of malic acid was two times higher in oranges as compared with apples juices.

By looking to the compounds which are present only in significant amounts in some types of juices, one may conclude that citramalic acid, quinic acid and galactose are markers for apple juice (with potential use in proving adulteration of citrus juices) whereas citric acid is a marker for citrus juices.

Although knowing individual concentrations is a valuable information, this approach is time consuming, both as instrument time and operator involvement and is restricted only to some metabolites. The major advantage of the NMR spectroscopy is that it offers the global "profile" of the sample in just one experiment. The untargeted statistical processing of the NMR fingerprint of all metabolites should be a more powerful approach for juice screening.

Chemometric techniques are currently used to classify different types of samples. For food analyses, one would like to differentiate between authentic and falsified foods / juices or to classify different foods according to their type.

For the above mentioned untargeted approach, we conducted PCA analyses on the "global profile" of the samples, excluding the ethanol and water regions (1.15-1.20, 3.60-3.70 and 4.69-5.00 ppm). The obtained scores plots and the corresponding loadings plots of the first two principal components (PCs), are presented in *Figure 2*. Briefly, in the scores plots each point represents a sample (NMR spectrum). Clustering of points in scores plots indicates similarities for those samples. In the loading plots, each point represents a compound (signal in the NMR spectrum). Spreading of signals (points) on a particular directions in the loading plots indicate compounds which are responsible for separation of samples in the corresponding scores plots.[50,51]

As it can be seen in Fig. 2A we obtained a clear clustering according to the three types of fruits and, even more, a clear separation according to the six sub-types of juices for each fruit. Based on the corresponding loadings plots (Fig. 2B), sugars and, very likely, citrate seem to be the main contributors to this clustering. In order to verify which compounds are responsible for the clustering, we further performed separate PCA analysis on the three main spectral regions: aliphatic, sugars and aromatic. Again, in all the plots we obtained a clear clustering according to the three types of fruits.

If one uses only the aliphatic region (Fig. 2C), the classification is different. The three types of fruits are well differentiated, but in this case the two types of commercial apple juices are closer to the orange juices rather than to the natural apple juices. From the loadings plot (Fig. 2D), the compounds responsible for this separation are malic and citric acids and dimethylproline. Malic acid seems to be responsible for the separation of natural apple juices from the grapefruit and orange juices and for the separation between natural and commercial apple juices. Surprisingly, quinic and citramalic acids, specific to apple juices, seem not to have a significant contribution to the separation. The PCA analysis was based on the signals in the region 0.50-3.15 ppm, with the exclusion of the ethanol signal (1.14-1.20 ppm). We decided to exclude the ethanol signal because its amount can be "artificially" enhanced by microbiological activity, especially in the case of samples stored for a long time.

If only the sugars signals are to be used, apple juices are well separated from the orange and grapefruit juices (see Fig. 2E). From the loadings plots (Fig. 2F) the sugar responsible for this separation is fructose and not galactose, as we expected since galactose is present only in apple juices. Glucose seems to be responsible for the separation of commercial grapefruit juices from the rest of the juices. The rest of grapefruit juices and the orange juices are separated from each other, but remain quite close to each other, indicating that the sugars contribution to their separation is not very significant. These results correlate very well with the conclusions we drawn based on individual concentrations.

The PCA analysis was based on the sugars signals in the regions 4.08-4.12 ppm and 5.14-5.80 ppm. In these spectral regions only the sugars have signals (fructose at 4.10 ppm, galactose at 5.19 ppm, glucose at 5.23 ppm and sucrose at 5.40 ppm). It is well known that most of the signals corresponding to different sugars appear in the region 3.00-4.08 ppm. We excluded this region from the PCA analysis due to the presence of the signals from other compounds like ethanol, alanine, arginine, GABA, naringin or dimethylproline.

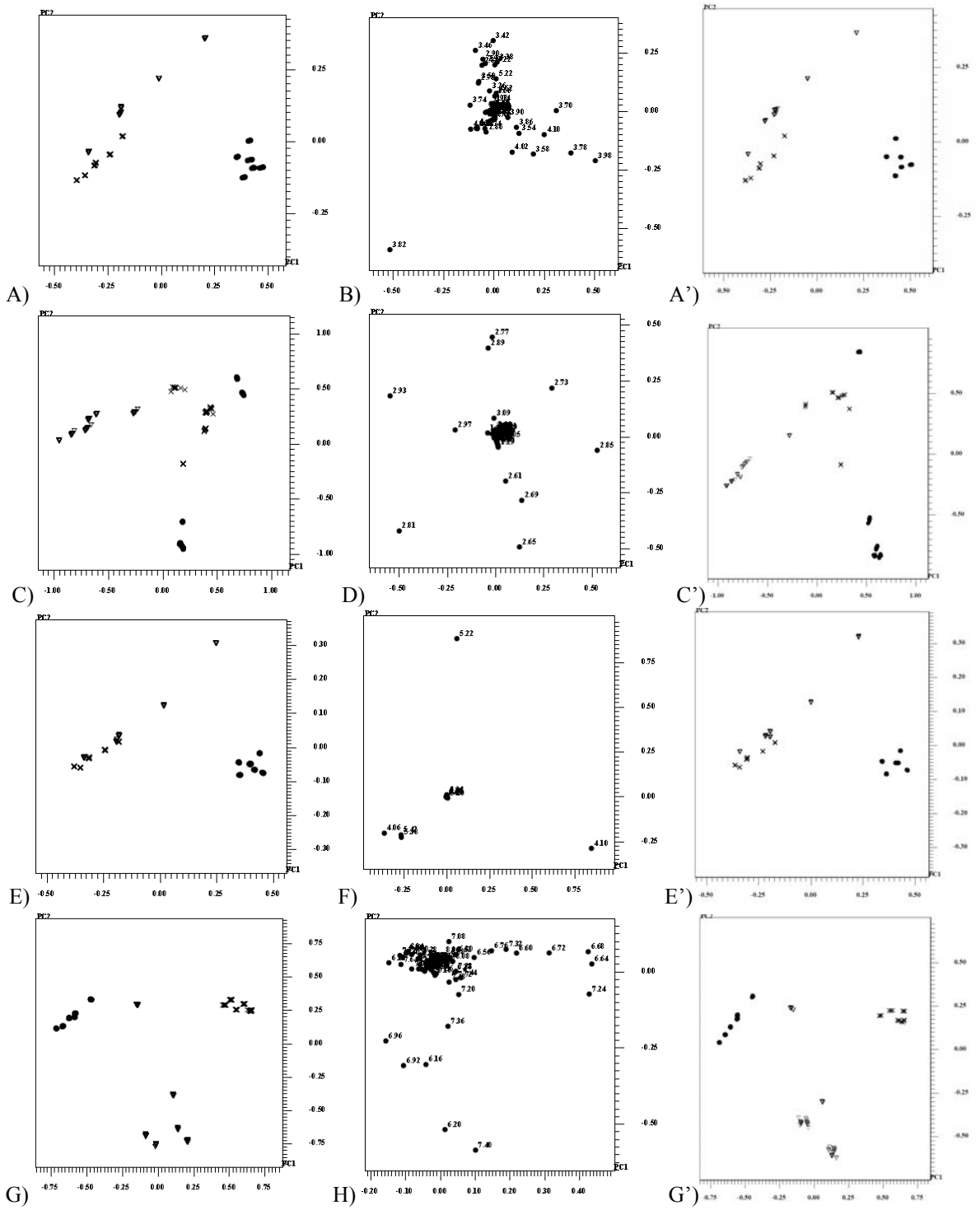

Fig. 2 – Score and loadings plots derived from fruit juices NMR spectra. The plots based on: A) and B) the entire spectrum excluding the ethanol and water regions (1.15-1.20, 3.60-3.70 and 4.69-5.00 ppm); C) and D) aliphatic region 0.50-3.15 ppm, exclusion region 1.14-1.20 ppm; E) and F) sugars signals in the region 4.07-5.80 ppm, exclusion region 4.12-5.13 ppm; G) and H) aromatic region 6.00-9.30 ppm. A', C', E', G' represent scores plots for freeze dried samples of the corresponding regions. **X** – orange juices, ▼ – grapefruit juices and ● – apple juices.

By performing the PCA analysis on the signals from aromatic region (Fig. 2G), we obtained a very good clustering according to the fruits types. Furthermore, the six sub-classes from each fruit were very well differentiated. From the loadings plots (Fig. 2H) the main compound contributing to the separation of grapefruit juices is naringin. In the case of orange juices the separation is based on tyrosine and an unknown large signal (6.50-6.80 ppm) most probably belonging to a phenolic

compound. The PCA analysis was based on the signals in the region 6.0-9.30 ppm, without any exclusion.

By reviewing the plots in Fig. 2, one can conclude that sugar metabolites (Fig. 2E,F) can clearly discriminate between citrus and apple. The low field region 6.0-9.0 ppm (Fig. 2G,H) is adding the supplementary data needed for further differentiating between citrus fruits.

When comparing fresh and freeze-dried scores plots (Fig. 2) for the whole spectra (A/A') or for various spectral regions (C/C', E/E', G/G') one can conclude that the pattern is similar, although not identical, and that the power of classification is maintained. Thus, in spite of small changes during freeze drying process, one may use this technique for obtaining samples with higher concentrations or for longer storage before NMR analysis. The smaller discriminating power of the PCA on the NMR data from the aliphatic region of the freeze dried samples may be due to the loss of some volatile compounds.

EXPERIMENTAL

Sample preparation

Fresh juices were prepared in the laboratory using an electrical fruit juicer. Oranges and grapefruits were pilled before being squeezed. Apples were squeezed as a whole. The obtained juice (250 mL from each fruit type) was centrifuged for 30 minutes at 6000 rotations per minute in order to remove any solid particles. 60 mL from each fresh juice were kept at -80 °C until NMR analysis and the remaining juice was divided in small plastic cups and freeze-dried.

Prior to NMR analysis, the juice samples were allowed to thaw at room temperature for 30 minutes. For the NMR analysis 900 μL of juice was mixed with 100 μL of 50 mM sodium 3-(trimethylsilyl)-[2,2,3,3-d4]-1-propionate (TSP) in D_2O solution. 600 μL of sample were transferred into a 5 mm NMR tube and subjected to the NMR analysis.

NMR measurements

^{1}H NMR spectra were acquired at room temperature, on a Bruker Avance III 400 MHz spectrometer, operating at 400.13 MHz, using a 5 mm inverse detection probe equipped with gradients on the *z*-axis. The spectra have been recorded with the noesy presaturation pulse sequence using 32 scans, 30 s relaxation delay, 4 s acquisition time, 8223 Hz spectral window, collecting 64 K data points, with a resolution of 0.12 Hz. An exponential line broadening factor of 0.3 Hz was used in post-acquisition FID processing. The chemical shifts are reported as δ values (ppm) referred to TSP (0.0 ppm) as internal standard.

Statistical processing

The PCA analysis was performed with AMIX 3.8 software (Bruker-Biospin, Rheinstetten).

CONCLUSIONS

Fast discrimination of three types of fruits (apples, grapefruits and oranges) was achieved based on ^{1}H NMR spectra. When applying statistical processing on three different spectral regions, sugars' region discriminates well between fruits. The best discrimination is achieved based on the aromatic region, whereas the aliphatic region is the less discriminating one. When comparing the results on pure juice with those on freeze-dried samples, the discrimination is comparable with the exception of the aliphatic region. Thus after freeze, drying the aliphatic region becomes even less discriminating, this result indicating that some volatile aliphatic metabolites may be lost together with the water during freeze drying.

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