Use of High-Field ^{1}H NMR Spectroscopy for the Analysis of Liquid Foods

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Proton NMR (600 MHz) is shown to be a potentially very valuable method for the analysis of low-molecular weight compounds in fluid foodstuffs. Very high levels of water signal suppression may be achieved, and a very large dynamic range is available. Thus, signals from a very large range of compounds in fruit juices and vinegars were obtained. Typically, within acquisition times of 7−14 min, many compounds with concentrations on the order of micromoles per milliliter could be observed. Partial spectral assignment using one- and two-dimensional methods was carried out and a semiquantitative comparison of NMR and chromatographic methods made.

Keywords: *NMR; fruit juice; analysis*

INTRODUCTION

Over recent years, the application of high-resolution proton (^{1}H) NMR to biological fluids has proved immensely valuable as an analytical technique (Nicholson *et al.*, 1995a,b). It has been possible to follow subtle changes in mammalian metabolism as the result of the administration of drugs or toxins by examination of the proton NMR spectra of body fluids such as plasma or urine (Nicholson *et al.*, 1995a). With the advent of ultra-high-field (600 and 750 MHz) spectrometers, very high levels of sensitivity and resolution are possible (Spraul and Hofmann, 1995; Nicholson *et al.*, 1995b), with detection levels routinely in the micromoles per milliliter range. In this paper, these techniques are applied to the analysis of fluid foods in order to explore their application to the improved measurement of quality and the origination of raw materials. Currently, such analyses as exist for samples such as fruit juices [see for example van Gorsel *et al.* (1992)] are partial. The high-resolution solution state NMR spectrum is by contrast a complete representation of all the proton-containing low-molecular weight components of the sample and may contain information on a very large number ($>10^3$) of compounds. Clearly, with such large numbers of resonances, a complete assignment is not easy and a strategy of selecting assignment tasks must be based on those resonances which appear to be markers for quality or origin. While it is true that some high-resolution NMR spectra of fluids in foods have been published (Eads and Bryant, 1986), these have been at relatively low field, and the number of resonances observed were relatively few. In this paper, we report on the 600 MHz spectra of a variety of fruit juices and vinegar and assign some of the resonances observed using one- and two-dimensional (1D and 2D) spectra.

MATERIALS AND METHODS

NMR experiments were carried out on a Bruker DRX 600 NMR spectrometer operating at 600.14 MHz for protons.

Samples were obtained from retail sources; those containing particulate matter were filtered through a 50 μm filter into a 5 mm NMR tube. Typically, 0.5 mL of sample was used. To this was added 0.05 mL of D_2O containing 1% TSP (trimethylsylyl [2,2,3,3-$^2H_4$]propionate). No additional treatment was used. The D_2O was used as a source of the field frequency lock signal, and TSP served as an internal chemical shift standard at $\delta = 0.00$. Care was taken to ensure that the samples were well-equilibrated at the probe temperature (303 K) before spectra were collected. Typically, 1D spectra were obtained using the following pulse sequence for water presaturation and suppression:

$$90-t_1-90-t_m-90-\text{acquire FID}-\text{RD}$$

FID represents acquisition of the free induction decay. RD is the recycle delay, typically 3 s. t_1 is 3 μs, and t_m is on the order of 100−150 ms. During the period t_m, the water resonance is irradiated with appropriate phase cycling. This sequence, called NOESYPRESAT, can result (Nicholson *et al.*, 1995b) in attenuation of the water signal by a factor of up to 10^5. In some cases, the irradiation is applied at two different frequencies in order to reduce signals from two intense peaks. Normally, for 1D spectra, 128 or 256 transients were accumulated with 64K data points. Typical acquisition times were therefore on the order of 7−14 min.

2D COSY spectra were obtained using pulsed field gradients with a 2 s water presaturation period which was also used for the relaxation delay. Sixteen scans of 4K data points were acquired for each of 300 increments. Magnitude mode plots were produced using unshifted sine bell functions in both dimensions.

RESULTS AND DISCUSSION

A full spectrum of bottled red grape juice is shown in Figure 1A; at this level of vertical amplification, the signals from the sugars are apparent together with a signal from the triplet of ethanol at $\delta = 1.17$ ppm and the TSP reference at $\delta = 0$ ppm. It should be noted that on this scale the water signal is completely suppressed. It becomes visible at 4.8 ppm in Figure 1B

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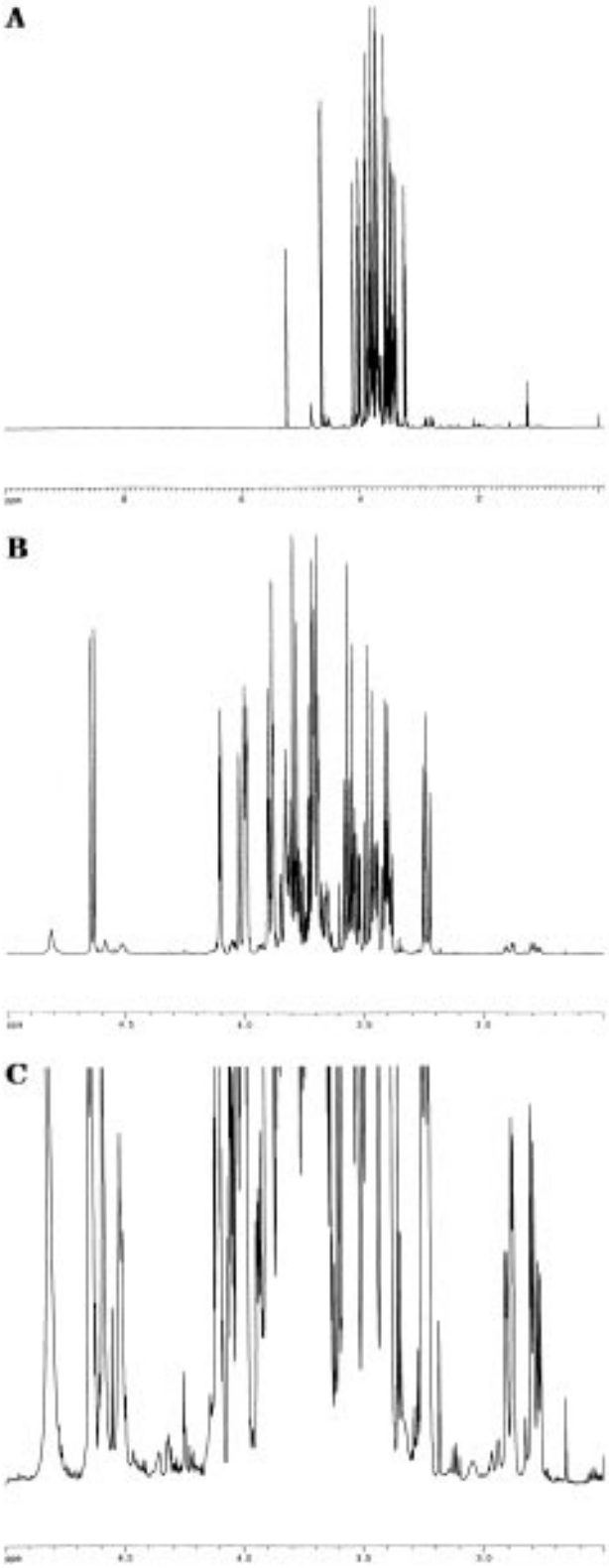

Figure 1. Partial spectra of grape juice at (A) 1 times vertical amplification, (B) 12 times vertical amplification, and (C) 264 times vertical amplification.

which has a vertical expansion of 12 times compared to Figure 1A and is limited to the 2.5−5 ppm region. However, the water peak is very small compared to many of the other resonances. Suppression of the water peak to these low levels is essential if good spectra of dilute species are to be observed. At a vertical expansion of 264 times (Figure 1C), electronic noise is not visible and there are many more signals becoming apparent.

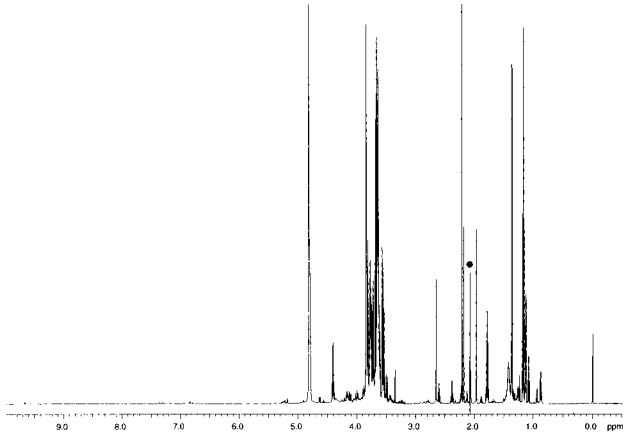

Figure 2. Spectrum of a vinegar sample showing the effects of double irradiation for suppression of water and acetic acid signals.

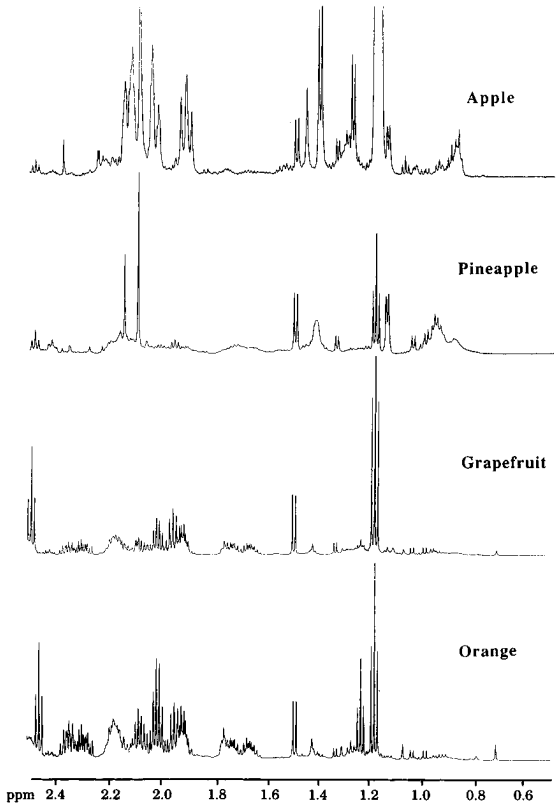

Figure 3. Partial spectra of four different fruit juices.

In the case of vinegar as well as the large water peak, there is a large acetic acid peak at $\delta = 2.1$. Using the double irradiation presaturation sequence, this can also be readily suppressed as shown in Figure 2.

It is clear that it is possible to obtain well-resolved selectively suppressed spectra from food-related fluids. However, the question that must then be addressed is how sensitive to the nature of the fluid is the NMR spectrum? It is clear that for fruit juices the spectra will be sensitive to the sugar composition. However, much more subtle information is available; Figure 3 illustrates the spectra from four different fruit juices in the region 0.5−2.5 ppm. From simple inspection, it is obvious that there are differences in the spectra. This is the case even for the two citrus fruit juice samples. Although quantitative comparisons between spectra must be approached with care, relative intensities within spectra may be used as a guide to relative

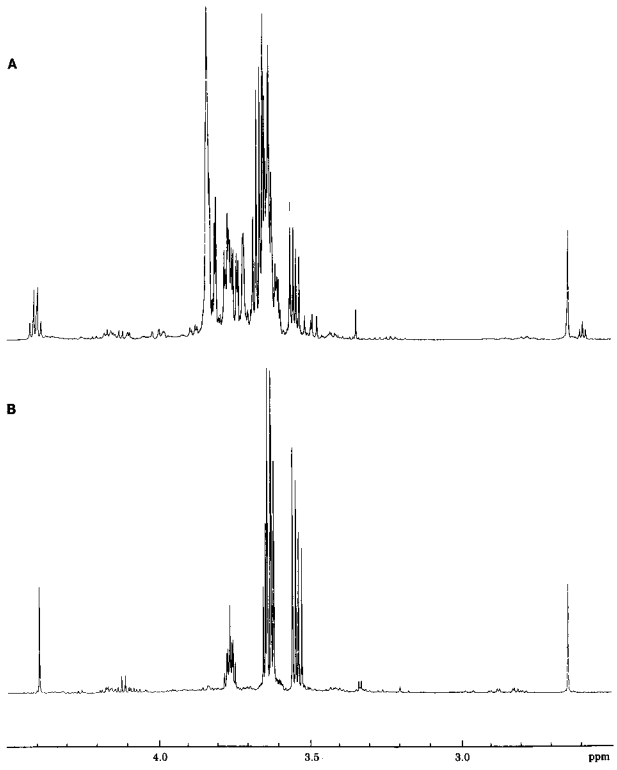

Figure 4. (A) Spectrum of wine vinegar and (B) the same spectral region from fruit vinegar.

concentrations of species within the sample (see below). The region shown in Figure 3 arises mainly from amino and organic acid signals, although a strong ethanol signal at $\delta = 1.17$ is apparent. In the orange and grapefruit spectra, the doublet from alanine at $\delta = 1.47$ is readily apparent and of about equal intensity in both spectra. Differences in other signal intensities relative to these reference intensities may be used as indications of differences in content. Starting at the low chemical shift end, there is strong intensity difference in the unassigned singlet at $\delta = 0.7$. Differences are also apparent in the cluster of peaks around the two valine doublets centered at $\delta = 1.0$. Particularly noticeable in this group is the relatively strong signal in orange at $\delta = 1.07$. Some differences are apparent in the ethanol triplet; however, some care is needed in the interpretation of this as the intensity of this peak could be strongly affected by microbial spoilage. In orange, a second unassigned triplet is present at $\delta = 1.22$ and there are some clear differences in the peak cluster from $\delta = 1.22$ to 1.45. The signals from citrulline centered around $\delta = 1.7$ and 1.95 are more intense in orange than in grapefruit as are arginine signals at $\delta = 2.0$ and the clusters from $\delta = 2.1$ to 2.4. The arising triplet γ-aminobutyric acid at about $\delta = 2.5$ has the same intensity in both samples. These marked differences in the distribution of intensities demonstrate that the method has considerable potential for authentication. A further illustration of the potential for authentication is given in Figure 4. This shows two partial spectra from samples of vinegar. Figure 4A is a sample of wine vinegar and Figure 4B from a sample of fruit vinegar, although no assignments have so far been made. Differences in the spectra are readily apparent, once again illustrating the potential of the technique in authentication problems.

The problem of authentication does not necessarily require that the spectra be assigned, merely that

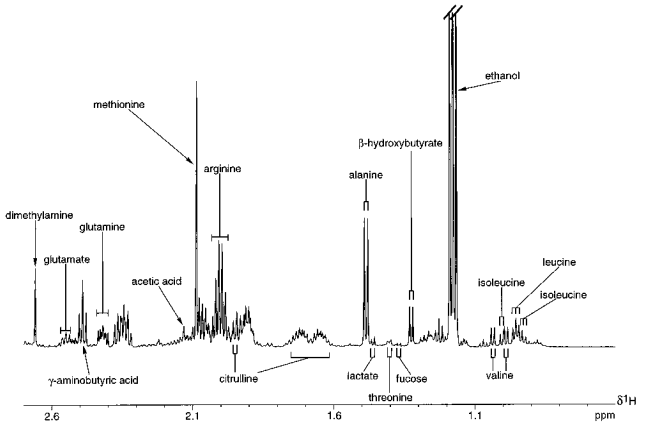

Figure 5. Partial assignment of a one-dimensional spectrum of grape juice.

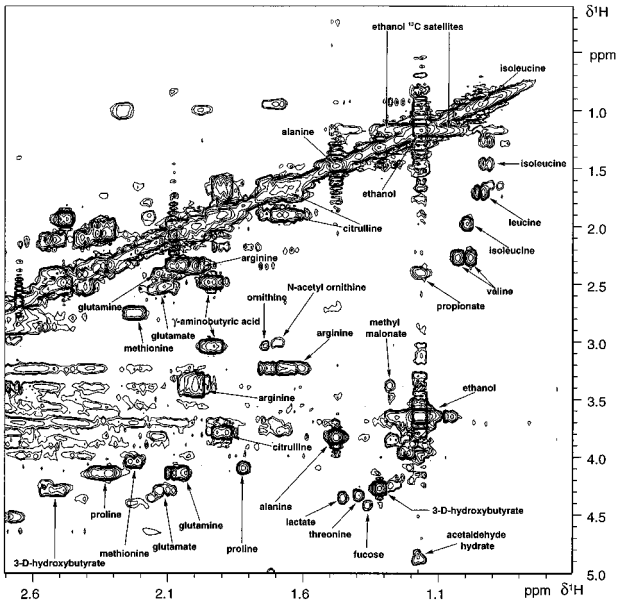

Figure 6. Part of the 2D COSY spectrum of grape juice with partial assignment.

spectral differences can be quantified by some suitable method of multivariate statistics. The use of pattern recognition techniques is already well-developed in other biofluids (Nicholson *et al.*, 1995a). If, however, the technique is to be used for analysis, then some degree of spectral assignment is required. The simplest approach to assignment is to use the one-dimensional spectrum and assign on the basis of chemical shift and multiplicity. This approach is illustrated for the spectrum of bottled red grape juice in Figure 5. It has a number of disadvantages. The first of these is that the spectrum is very crowded and there are many overlapping peaks. The second is that the assignment is based on assumptions about the sorts of compounds which are likely to be present. Typically, it is not possible to observe all the resonances arising from one molecule, and assignment is often made on the basis of the coincidence of multiplicity and chemical shift of one peak. As can be seen in Figure 5, these problems result in only a very limited assignment of the spectrum. A more satisfactory approach is to use two- or more-dimensional NMR methods. These have the advantage that the spectral information is spread out in two dimensions and that, provided the correct form of connectivity is used, peaks which are overlapping in one dimension will be separated in two. The use of 2D methods is illustrated in Figure 6. The spectrum is for

Table 1. Relative Amino Acid Concentrations As Determined in Grape Juice by NMR and HPLC[a]

amino acid	concn[a] (μmol/mL)	NMR signal intensity (arbitrary units)[b]	normalized NMR intensity[c]	rel intensity (NMR)	rel intensity (published)[a]
valine	0.40	14	4.6	0.13	0.21
alanine	1.9	104	35	1.0	1.0
arginine	3.7	128	64	1.8	1.9
methionine	0.08	102	34	0.97	0.04

[a] Data calculated from van Gorsel *et al*. (1992). [b] Calculated on the basis of the total height of all the peaks in the multiplet. [c] Calculated by dividing signal intensity by the number of protons contributing to the signal.

Table 2. Comparison of Relative Signal Intensities Measured by NMR with Calculations from Published Data

	rel intensity in orange		rel intensity in grapefruit	
amino acid	NMR	published[a]	NMR	published[a]
valine	0.14	0.09 (0.3)	0.10	0.17
alanine	1.0	1.0 (0.3)	1.0	1.0
arginine	4.1	2.6 (1.0)	1.4	2.7
γ-aminobutyric	1.0	1.9 (0.2)	0.84	1.8

[a] Figures in parentheses are standard deviations calculated from published data. Where none are shown, only one published value was found. Data from Burroughs (1970).

the same sample as in Figure 5 and is a portion of a contour plot of a homonuclear proton COSY spectrum. The COSY method correlates the chemical shifts of scalar coupled nuclei. The one-dimensional spectrum is represented by the diagonal, and the cross-peaks appear at positions whose coordinates are determined by the chemical shifts of the scalar coupled nuclei. Peaks are now fixed by two chemical shift parameters instead of one, and the certainty of assignment is increased. In addition, the second dimension results in the spreading of peaks which overlap in one dimension; for example, in the peak cluster at $\delta = 2$, this overlap results in the signals from α-aminobutyric acid and glutamate becoming visible. This effect is particularly noticeable close to the large ethanol peak, where the previously hidden propionate and acetaldehyde peaks become visible. The use of two-dimensional methods thus increases the available spectral information. This is at the cost of increased acquisition time, but the penalty is not very great, considering the extra amount of information obtained. Two-dimensional spectra could also be of considerable value in authentication since they result in significantly increased spectral information content.

The final problem to be overcome in the analysis of foods by NMR is the problem of quantification. In principle, provided suitably long relaxation delays are used, one-dimensional spectra should be quantitative in the sense that the magnetization associated with a specific resonance is proportional to the numbers of nuclei contributing to that resonance. In general, a resonance will be split into more than one peak by scalar coupling. Magnetization can be measured by measurement of the area under a peak. It is clear from inspection of one-dimensional spectra that the measurement of area is not straightforward as the peaks are not well-separated. Further separation by two-dimensional methods does not help as the areas under peaks in 2D spectra do not have relative intensities which are proportional to the numbers of nuclei contributing to the resonance.

Even if at this stage accurate quantitative measurement has not been developed, semiquantitative inferences can be made. It is clear from inspection of the orange and grapefruit spectra in Figure 3 that the resonances arising from the ethanol methyl triplet at about $\delta = 1.17$ are significantly larger than those arising from the alanine methyl doublets at about $\delta = 1.47$. The peak widths of both resonances are approximately the same so that an estimate of the relative numbers of nuclei can be obtained by measurement of relative peak height. In the example of orange juice, the ratio of ethanol to alanine is about 3.48.

Using this approach, it is possible to compare amino acid ratios observed in NMR with those that have been published. In Table 1, this has been done for grape juice. The number of comparisons is limited by the number of compounds measured in the literature and the number of resonances that are amenable to the simple measurement technique described. The intensities are used in a ratio with the intensity of the alanine peak since it appears that this amino acid has a relatively low variability in concentration compared to others (Burroughs, 1970). The mean ratio of alanine to arginine for 15 grape varieties is 2.54 with a standard deviation of 0.77 [data calculated from Burroughs (1970)]. Thus, the ratios of 1.8 and 1.9 observed from the NMR data and the HPLC data of van Gorsel *et al*. (1992) fall within the expected range. Burroughs also reports three additional grape varieties in which the ratio is greater than 5; these have not been included in the calculation. While data are not available for the variation in valine concentration, it seems reasonable to assume that the difference in ratios between the HPLC and NMR methods falls within the natural variation of samples. In the case of methionine, the discrepancy does seem to be outside what could be regarded as a natural variation. Presently, the cause of the discrepancy is not clear. However, it is clear from inspection of the other spectra of juices as shown in Figure 3 that the peak assigned to methionine is not always present in the signal and is thus not an electronic or chemical artifact. It is also worth noting that van Gorsel *et al*. (1992) reported a level of methionine which is significantly higher in grape juice than in all the other juices that they examined. For comparison with the grape juice data, similar calculations have been made for orange juice and grapefruit juice (Table 2). In these samples, a methionine signal is not present. However, data are available from our results and published sources (Burroughs, 1970) for γ-aminobutyric acid which has been included. The orders of magnitude obtained by both methods are similar, but the relative NMR intensities for the γ-aminobutyric acid are about half that expected from the published data. Further investigation of a range of samples will be required to demonstrate whether this is a result of biological variation or genuine differences in sensitivity. One advantage of NMR is that it is able to measure a large number of samples rapidly and thus observe the inherent variability in products. The treatment of NMR spectra with pattern recognition methods would also be valuable in sample classification. Such studies are being currently undertaken in our laboratories.

CONCLUSIONS

High-field NMR has been shown to be a potentially valuable method for the nondestructive analysis of low-molecular weight compounds in foods. Spectra may be obtained routinely and simply in a short period of time. Sensitivity is very high, and the method has the advantage that it surveys a large range of components. The technique could have applications in the authentication of food products, in quality control of food and agricultural products, and in food chemistry.

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