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High-Field Proton NMR Studies of Apple Juices

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High-field (11.7 and 14 T) proton high-resolution NMR spectra of apple juices obtained from a variety of cultivars are reported and partial spectral assignments are made. There are significant spectral differences between cultivars, which may be of value in identifying the origins of apple juices. The results also indicate that the method is likely to be of value in the authentication of fruit juices. Careful spectral analysis shows that some differences arise simply as a result of the differences in the pH of the juices and that microbiological and oxidative effects must be taken into account. Care must therefore be exercised in the application of multivariate methods to the data as spurious or trivial correlations may be obtained. It is concluded that the richness of the spectra and the ease with which they may be obtained indicate that high-field proton NMR will prove valuable not only in speciation and authentication studies, but also in the analysis of biochemical changes occurring in fruits and their juices. © **1997 John Wiley & Sons, Ltd.**

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INTRODUCTION

High-resolution proton NMR has shown considerable potential for the analysis of biological fluids.[1,2] Fruit juices lend themselves very well to analysis by high-resolution proton NMR and are also a focus of concern for problems of authenticity and purity. In this paper we explore further the potential of NMR for juice analysis by concentrating on the spectra of apple juices and considering the effects of cultivar variations on juice spectra. From a survey of the literature[3–10] reporting the results of the use of conventional techniques such as high-performance liquid chromatography it is clear that variations in the distribution of sugars and amino acids are likely to be found in different cultivars. NMR is ideal for the survey analysis of a large number of samples since in a very short time (typically 10–20 min) information about the presence and relative concentration of a very large number of chemical species may be obtained.[1] When dealing with large data sets it is tempting to resort immediately to multivariate methods. It is clear that the use of multivariate methods can be extremely powerful and that they will eventually be the method of choice for handling the data obtained by NMR. However, before moving to that stage we wish to consider the spectra in some detail so as to understand the origins of the variations that may lead to discrimination by multivariate statistics.

EXPERIMENTAL

Apples were collected from orchards in England and Germany in Autumn 1994. Ten cultivars were represented. Juices were prepared shortly afterwards from the whole apples using an electrical mincer. The extracted juices were stored at $-20\,^{\circ}C$. Typical storage times until use were 6 months. These juices will be referred to as the 1994 set. After thawing, a small quantity of each juice was centrifuged to remove suspended matter, and D_2O containing 0.1% TSP [sodium salt of 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid] was added to each sample to bring the concentration of TSP to 0.01%. Samples (0.7 ml) were placed in 5 mm o.d. NMR tubes. D_2O served as the field frequency lock and all spectra were referenced to the signal from TSP at 0.00 ppm. In a few cases spectra were obtained from freshly prepared apple juices within about 20 min of the juice preparation. The juices were prepared by hand (from the flesh only) using a garlic press. Spectra of these samples were remeasured over a period of several hours to observe changes occurring under different conditions (with or without prior heat treatment and with or without exposure to air).

^{1}H NMR spectra were recorded at 500 or 600 MHz using a Bruker DRX-500 or DRX-600 spectrometer. Spectra of the 1994 set (about 60 samples) were recorded

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on the DRX-500 in two overnight runs using an automatic sample changer. Spectra of the freshly prepared juices were measured at 600 MHz. Data were accumulated for 160 scans with a recycle time of 6 s; 64K data points were acquired with spectral widths of 8333 Hz (500 MHz) or 9980 Hz (600 MHz). Water suppression was effected by use of the NOESYPRESAT sequence.[1,2] The acquisition time was 16 min per sample.

RESULTS AND DISCUSSION

The spectra are most easily discussed in terms of three regions: 0.8–2.7, 2.7–5.5 and 5.5–8.5 ppm. For each region an overview will be presented using 500 MHz spectra of seven cultivars from the 1994 set. Detailed assignments will then be illustrated using 600 MHz spectra of two freshly squeezed juices (Granny Smith and Boscop).

0.8–2.7 ppm region

Figure 1 shows the spectra obtained at 500 MHz for seven apple varieties in the region 0.8–2.7 ppm. From visual inspection of this region it is clear that there is a significant variation between varieties. Some signals appear in the spectra of all varieties and other signals only for certain varieties. There are obviously large variations in relative intensities between spectra. A tentative assignment of the region for the two freshly squeezed apple juices is shown in Fig. 2 and Table 1. A full assignment awaits detailed interpretation of 2D NMR spectra, but the main contributors to the signals in this region are quinic and chlorogenic acids, amino acids and alcohols (ethanol and propanol). A number of the amino acids were assigned in a previous study[1] of grape juice. The most distinctive feature of the apple juice spectra in this region is the complex set of multiplets between 1.9 and 2.3 ppm. These arise mainly from quinic acid, which, in apple juice, occurs both in the free form and as part of chlorogenic acid (5′-caffeoylquinic acid). Figure 2 illustrates that resonances from the free and bound forms of quinic acid are clearly resolved. The Boscop juice contains comparable amounts of quinic and chlorogenic acids whereas Granny Smith juice contains relatively little chlorogenic acid. Signals from ethanol, lactic acid and propanol are found in many of the spectra. In the stored samples, the amounts of ethanol and lactic acid may be enhanced by microbiological action (see below), but the freshly squeezed

Table 1. NMR signal assignments for Boscop apple juice

Compound	Chemical shift (ppm), multiplicity and *J* value (Hz)
Propanol	0.89 t 7.2; 1.51 m
Leu	0.94 t unresolved
Ile	0.95 t unresolved; 1.00 d unresolved
Val	0.98 d 7.2; 1.03 d 7.2
Ethanol	1.17 t 7.2
U1[a]	1.26 d 6.6
Lactic acid	1.32 d 6.6
U2[a]	1.39 d 6.8
Citramalic acid	1.45 s
Ala	1.48 d 7.2
GABA	1.93 t 7.2; 2.47 t 7.2
Acetate	2.07 s
Glu	2.54 dd 7, 11
Asp	2.92 overlap; 2.97 dd 4, 16
Asn	2.87 overlap; 2.95 dd 4, 16
Citric acid	2.83 d 16; 2.88 overlap
Succinic acid	2.65 s
Quinic acid	1.90 dd 10, 11; 2.02 m; 2.07 m; 2.12 m
Chlorogenic	2.07 m; 2.14 m; 2.20 m; 2.23 m; 5.30 ddd 4, 9, 10
Malic acid	2.77 dd 7, 16; 2.89 dd 4, 16; 4.40 dd 4, 7
β-Glucose	3.25 dd 8, 8; 4.64 d 8
β-Galactose	3.32 dd 8, 10; 4.58 d 8
Fructose	4.11 d 4
Sucrose	4.22 d 9; 5.41 d 4
Tartaric acid	4.56 s
α-Galactose	5.20 d 4
α-Glucose	5.23 d 4
Phloretin	6.05 s
Epicatechin	6.10 d 2; 6.11 d 2; 7.05 s; 6.93 s; 6.95 s
Phloridzin	6.14 d 2; 6.29 d 2; 6.82 d 8
Chlorogenic	6.38 d 16; 7.13 dd 8, 1.8; 7.20 d 1.8; 7.64 overlap; 6.96 overlap
pCoumaric acid	6.49 d 16; 7.39 d 16
Polyphenol	6.92 broad; 7.61 broad
Formic acid	8.34 s

[a] Unknowns.

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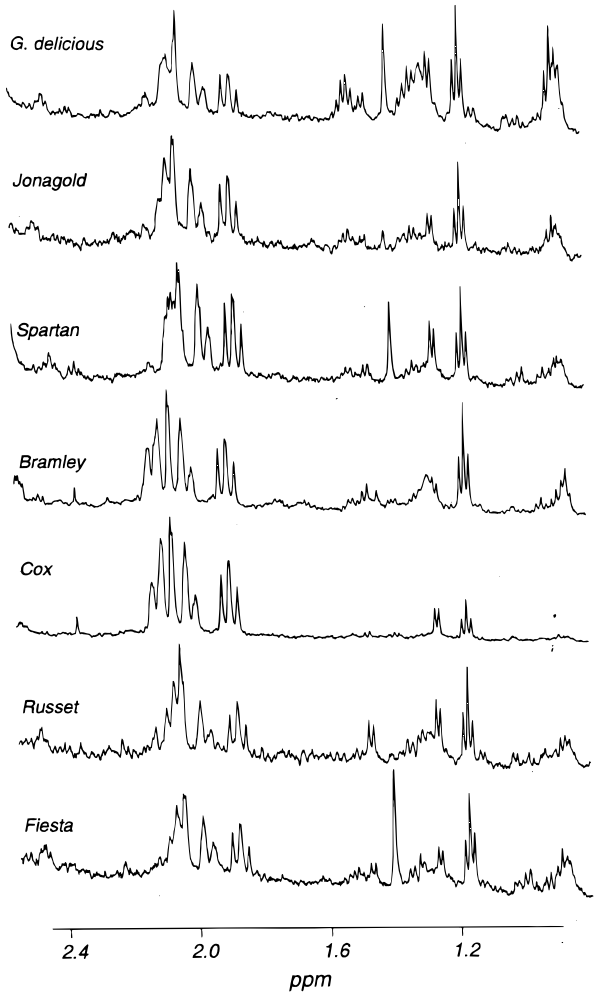

Figure 1. 500 MHz ^{1}H NMR spectra of seven cultivars from the 1994 set of apple juices (0.8–2.5 ppm region).

juices also contain ethanol, lactic acid and, in Boscop juice, propanol (triplet at 0.89 ppm). Variability of the propanol content can be seen from Figs 1 and 2. Following exposure of Boscop juice to air (3 h) a marked decrease in the intensity of the ethanol and propanol signals was noted, accompanied by an increase in the formate and acetate peaks. Other chemical changes are discussed below. The strongest amino acid signals, from asparagine, lie just outside this region at *ca.* 2.9 ppm. They have a degree of overlap (depending on pH; see below) with the very strong neighbouring signals of malic acid.

Consistent with previous studies using conventional methods,[3,4] there is a great variation in the pattern of the amino acids for different cultivars. Burroughs[3] reported sixfold variations in the alanine content of apple juices depending on variety. Inspection of the variation of the intensity of the doublet peak of alanine (at 4.8 ppm) in Fig. 1 shows at least this variation in intensity. In the case of the Cox sample there appears to be a generally low level of amino acids. Some caution is required in the interpretation of these data, however, since it has been shown[10] that ripening can change amino acid concentrations by an order of magnitude. Hence the effects observed in the Cox sample may be due to a ripening effects.

The main signals yet to be assigned in this region are two doublets at 1.26 ppm (labelled K) and 1.39 ppm (labelled I), but as Fig. 2 shows, their importance varies considerably from sample to sample.

2.7–5.5 ppm region: effects of pH and microbiological activity

There are a number of possible origins of spectral variation which must be considered before the differences that are observed can be confidently assigned to species effects. It may be that the differences are agronomic in origin rather than due to cultivar effects. Degree of ripeness will also have a role to play. For the samples examined here, exposure to microbiological activity is obviously a factor, but one reflecting the specific sample preparation and storage conditions employed. Even though the spectral differences may arise from genuine cultivar differences, it is well known that some apparent changes in the spectra come from differences in sample pH. Close inspection of Fig. 1 shows that although some of the multiplet structures are obviously of identical origin, their positions are slightly displaced from spectrum to spectrum. The samples in Fig. 1 have a pH range from 3.31 to 4.42. It seems likely, therefore, that the shifts observed are the result of pH changes. However, it is clear that the intensity differences seen cannot be explained on that basis and that there are genuine differences in chemical composition between the samples. The effects of pH can be clearly seen in Figs 3 and 4 by considering the changes observed in the malate peaks (labelled E in Fig. 4) which occur as a double doublet around 4.4 ppm and as two double doublets centred around 2.8 ppm. The positions of both sets of resonances change with pH over the range of acidities observed in the samples. Figure 5 shows a plot of the observed shift of the malate low-field multiplet *vs.* sample pH for a group of samples representing different varieties.

Molecules whose state of ionization does not change in the pH range of interest show no chemical shift effects with pH. This applies to most of the sugars where the chemical shifts, but not relative intensities, remain constant in all the samples (Fig. 3). Generally, a particular cultivar will tend to occur within a particular pH range when ripe. If this is true then the malate shift will be an indicator of cultivar type. However, this is only a result of the pH effects. In the statistical analysis of data it is important not merely to seek correlations but to seek meaningful correlations that cannot be obtained in simpler ways. Fuleki *et al.*[5] have reported the malic acid content of a range of apple cultivars together with the pH of the juices. Figure 6 is a plot of their data and shows that the major determinant of the pH is the malic acid content. A simple pH measurement would therefore be sufficient to indicate malate content. If malate were the only indicator of apple variety, it therefore follows that measurement of the NMR intensity and shift of the malate resonance would be redundant for purposes of seeking correlations between chemical composition and cultivar type. There is, of course, considerable overlap in the pH ranges and malate levels of the different apple varieties, and the pH

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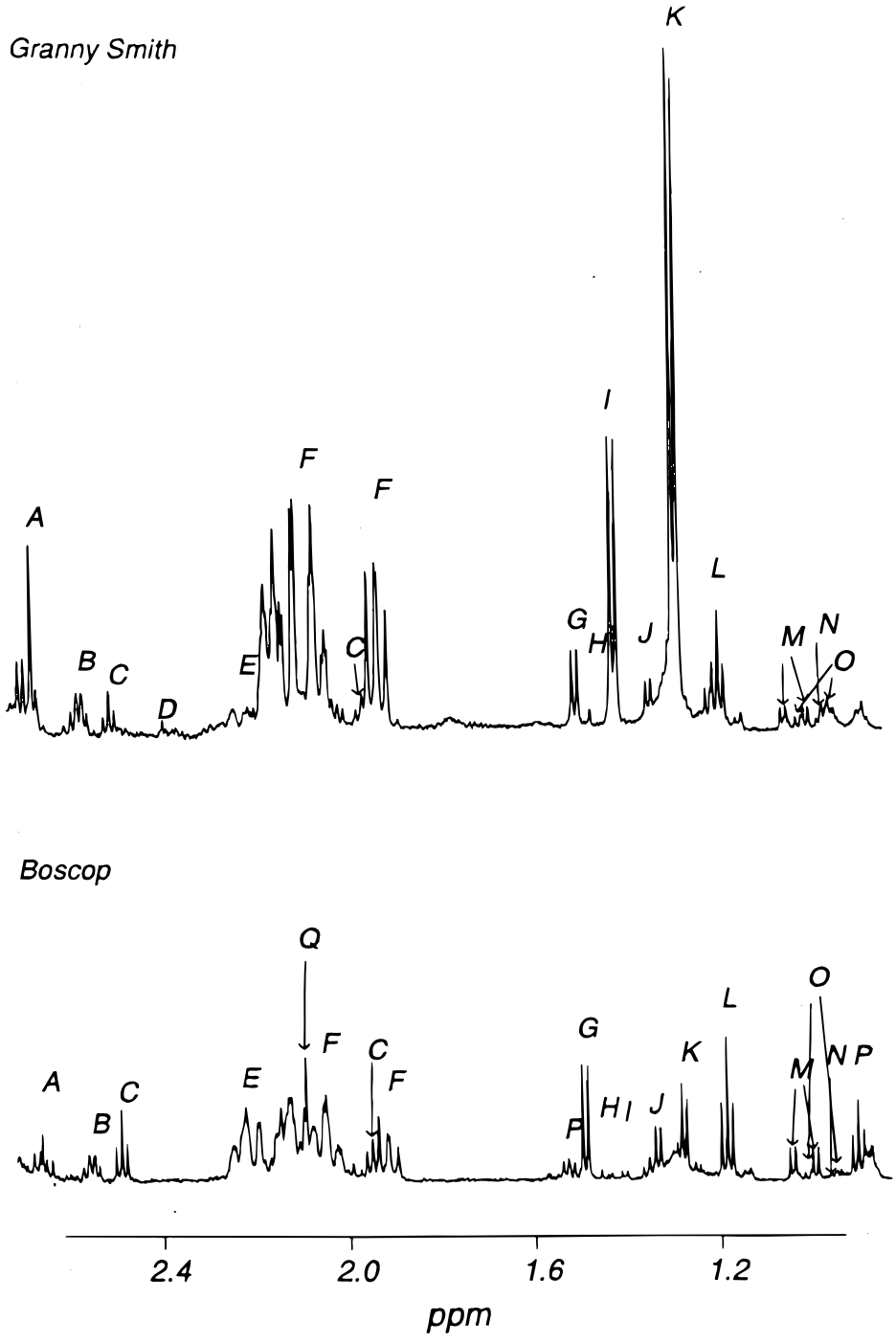

Figure 2. 600 MHz ^{1}H NMR spectra of two freshly squeezed apple juices (0.8–2.5 ppm region). Spectra are plotted with a vertical gain of *ca*. 100 with respect to Fig. 4. Key to assignments: A, succinic acid; B, glutamic acid; C, γ-aminobutyric acid; D, proline (or hydroxymethylproline); E, chlorogenic acid; F, quinic acid; G, alanine; H, citramalic acid; I, unknown; J, lactic acid; K, unknown; L, ethanol; M, valine; N, leucine; O, isoleucine; P, propanol; Q, acetic acid.

is a quantity that can easily be altered. The strength of NMR is that it measures many intensities and shifts simultaneously and malate is only one of many indicators that has to be used.

The sugar region contains resonances arising mainly from sucrose, glucose and fructose. Inspection of the spectra in Figs 3 and 4 shows that the relative intensities of the resonances arising from each of these species varies from cultivar to cultivar. It is particularly noticeable that the intensities of the resonances arising from the anomeric protons of glucose and sucrose show clear variations in their relative intensities. There is evidence from conventional analytical methods that three factors are of importance: natural variations due to differences in cultivars, growing season, etc.,[6] the effects of microbiological action[7] and acid hydrolysis of sucrose on storage.[8] The last two factors are very dependent on storage conditions. Noticeable hydrolysis of sucrose, over a relatively short time-scale (1 week at room temperature), has been observed by us in NMR studies

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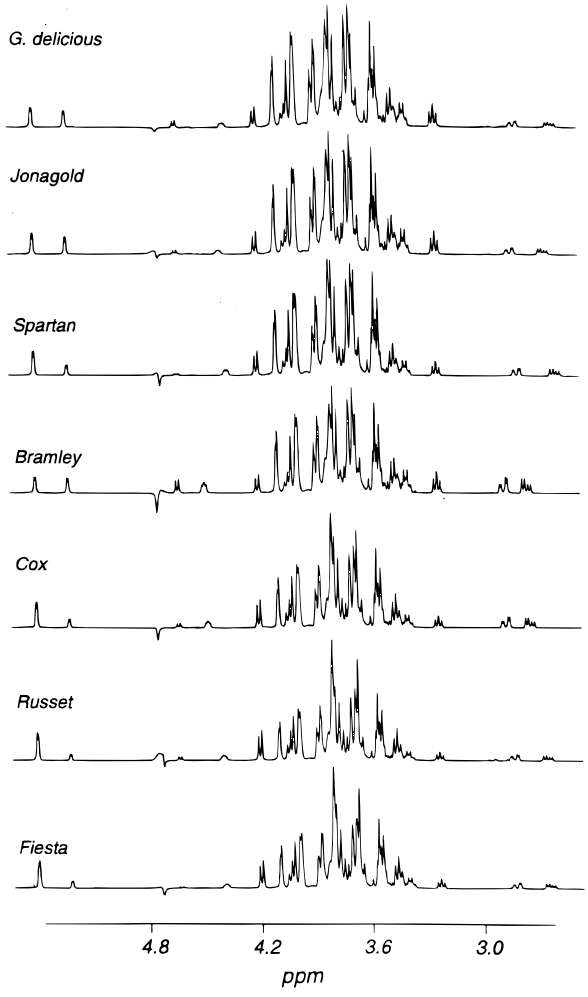

Figure 3. 500 MHz ^{1}H NMR spectra of the 1994 set (same samples as in Fig. 1) (2.7–5.5 ppm region).

of sugar–malic acid mixtures. In about one third of the stored samples examined the signals of ethanol and, in some cases, lactic and acetic acid were much stronger (10–100-fold) than in freshly squeezed juices. Most of the additional ethanol arises from the action of yeasts, although moulds and bacteria may also be important. The use of simple sugar ratios to establish cultivar type needs to be approached with some caution if microbiological activity is indicated by the presence of ethanol or the other compounds. Badly affected samples were excluded from the quantitative treatment described below.

5.5–8.5 ppm region

Inspection of the aromatic region shows differences between and within cultivars. An example of variation between cultivars is shown in Fig. 7. These variations are consistent with those reported in the literature.[9] Most of the spectra are dominated by two intense broad peaks, but the absolute intensity of these signals varies from sample to sample. In a number of samples, a variety of other less intense and narrower peaks are apparent. The broad peaks are assigned to condensed polyphenol species. A partial assignment of the narrow resonances observed in the freshly squeezed Boscop and Granny Smith juices is given in Fig. 8 and Table 1. Resonances were mostly identified by comparison with reference phenolic compounds known to be present in apple juice.[9] A number of differences are illustrated in Fig. 8: in the ratio of polyphenols to monomeric phenolic species, in the levels of chlorogenic acid (already noted in the discussion of the high-field region) and in the chlorogenic acid to epicatechin ratio.

The origins of the differences in the spectra observed in Fig. 7 are related to the activity of polyphenoloxidase and exposure to oxygen.[11] No precautions were taken to limit enzyme activity during the preparation of the samples shown in Fig. 7, so it is likely that the changes have proceeded to their limit. The effects of these reactions on the phenolic region of the spectrum were studied by following the changes with time of a sample of juice freshly squeezed from the cultivar Granny Smith which was exposed to air. Spectra obtained 17 min, 4 h and 8 h after squeezing are shown in Fig. 9. After only 17 min a considerable amount of condensed aromatic species is already observed, but a number of weaker narrower resonances indicate the presence of significant quantities of free epicatechin and chlorogenic acid and some minor phenolic compounds. After 4 h of exposure to air, the peaks assigned to epicatechin and the other phenolic compounds have decreased significantly compared with the main broad peaks. After 8 h of exposure, almost all the narrower peaks are lost. The concerted effects of polyphenoloxidase and oxidation by oxygen show that, in this case, all epicatechin and phenolic compounds are involved in the chain oxidative reactions that lead to the formation of polymeric condensation products. In some samples (Fig. 7), narrow resonances, mainly of chlorogenic acid, remain after reaction is complete. This appeared to be a characteristic of particular samples, not of any particular cultivar.

The role of polyphenoloxidase was seen by repeating the experiment after having heated the sample at 75 °C for 30 s. This procedure deactivates the enzyme, as shown by the small spectral changes after 7 h of exposure to air [Fig. 9(D)]. Comparison with Fig. 9(C) clearly shows that most of the epicatechin and chlorogenic acid remain unreacted in the absence of polyphenoloxidase activity. However, a slight decrease in intensity of the epicatechin peaks is observed compared with the freshly squeezed juice [Fig. 9(A)]. This must due to non-enzymatic reactions. We conclude that the analysis of the low-field spectral region may prove to be very powerful for the investigation of polyphenoloxidase activity and the chemistry of aromatic species in fruit juices. It should also provide a distinctive fingerprint for the detection of adulteration.

Cultivar effects: quantitative comparison

As a guide to possible effects of cultivar on the chemical composition, selected multiplets were integrated. The selected multiplets were ones that could be clearly identified and had no overlap with neighbouring signals.

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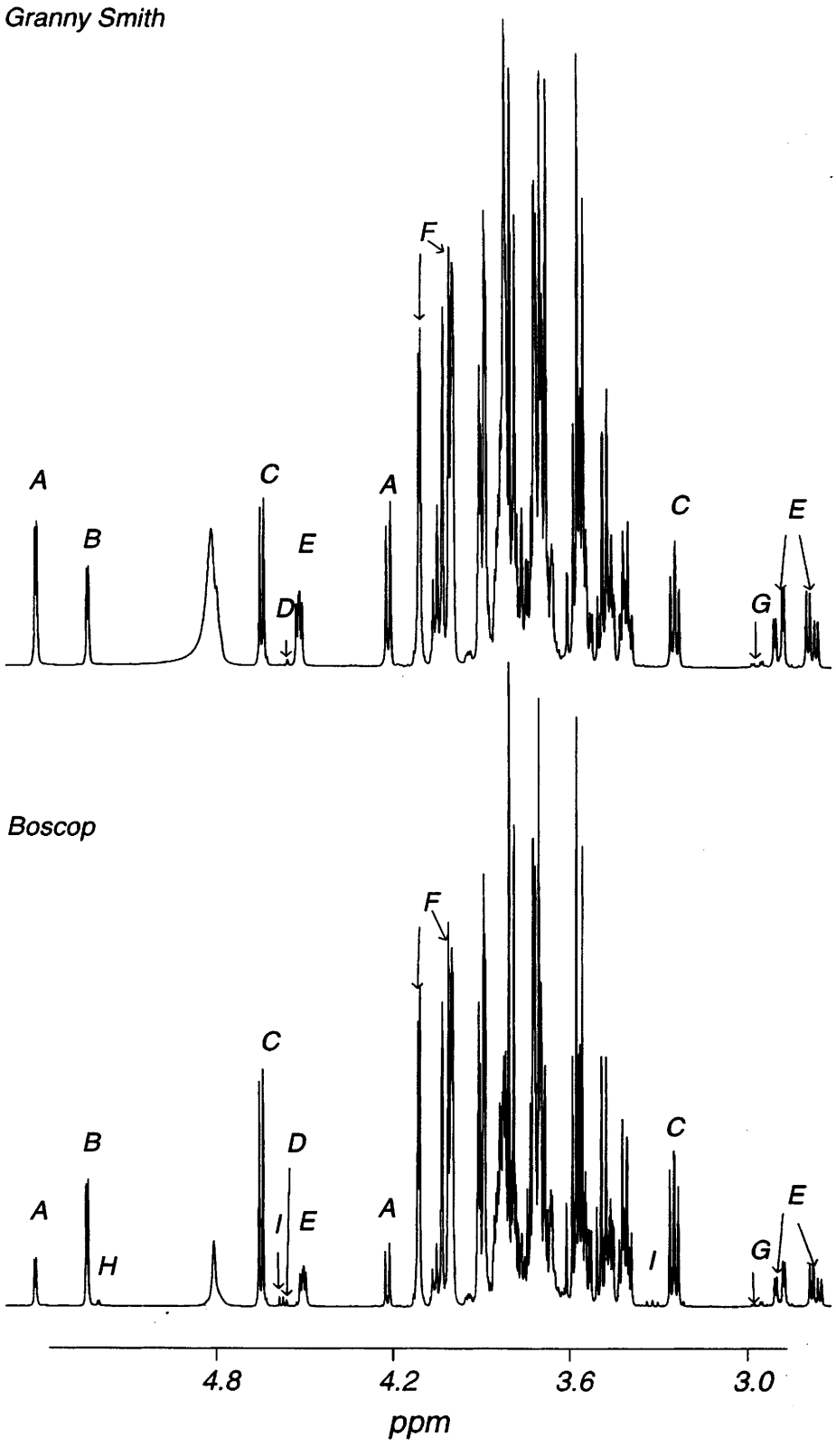

Figure 4. 600 MHz ^{1}H NMR spectra of freshly squeezed juices (same samples as in Fig. 2) (2.7–5.5 ppm region). Key to assignments: A, sucrose; B, α-glucose; C, β-glucose; D, tartaric acid; E, malic acid; F, fructose; G, asparagine; H, α-galactose; I, β-galactose. In the main carbohydrate region only a few well resolved multiplets have been labelled.

This was done for the four varieties for which reasonable numbers of samples were available: Cox, Bramley, Russet and Spartan. The results are given in Table 2. The signals chosen for integration (chemical shifts in parentheses) were polyphenols (broad line, δ 6.92), sucrose (d, δ 4.22), fructose (d, δ 4.11), glucose (t, δ 3.25), malic acid (dd, δ *ca.* 2.77) and quinic acid (dd, δ 1.90). The integrals were normalized by dividing by the total

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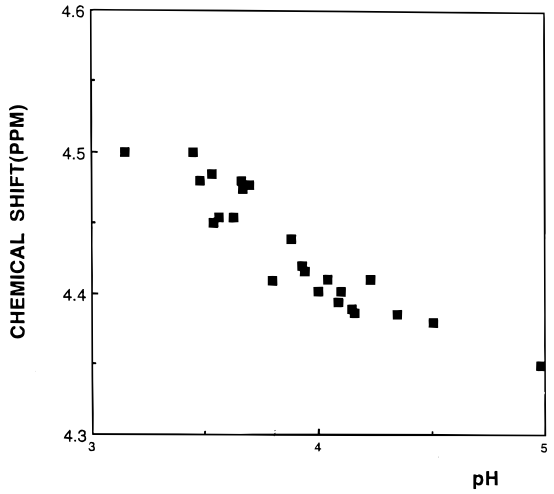

Figure 5. Plot of the chemical shift of the malate double doublet *vs*. sample pH.

integral for the three sugars in each sample. At this stage we have not attempted to determine the true molar ratios (some of the signals integrated arose from only one anomeric form of the sugar) or the absolute

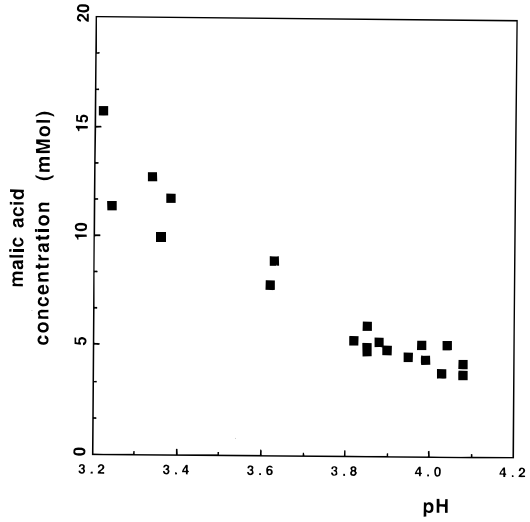

Figure 6. Plot of malic acid content *vs*. juice pH. Data from tables given in Ref. 4.

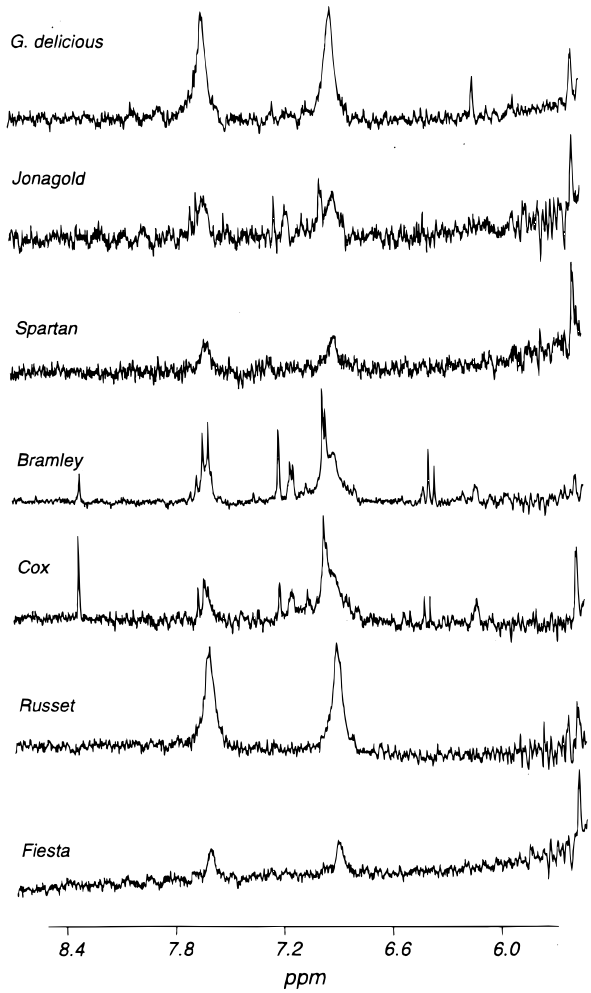

Figure 7. 500 MHz ^{1}H NMR spectra of the 1994 set (same samples as in Fig. 1) (5.8–8.5 ppm region).

concentrations. Calibration methods are being developed which will allow this to be done for a wide range of fruit juice constituents, including compounds (e.g. sorbitol) which do not have any non-overlapped multiplets. Some of the features in Table 2 are as expected, e.g. the high malic acid to sugar ratio in Bramleys would be predicted from the pH. There also appear to be significant differences in the sugar ratios, although the caveats mentioned above should be borne in mind.

Table 2. Average composition of apple juices by variety from NMR analysis (expressed as integral ratios)

			Integral ratios[b,c]					
Variety	*N*[a]	pH	P/T	Suc/T	Fru/T	Glc/T	MA/T	QA/T
Cox	15	4.1 ± 0.3	0.011 ± 0.004	0.30 ± 0.07	0.51 ± 0.04	0.19 ± 0.04	0.13 ± 0.06	0.008 ± 0.004
Bramley	11	3.5 ± 0.2	0.008 ± 0.004	0.17 ± 0.08	0.58 ± 0.05	0.26 ± 0.04	0.26 ± 0.06	0.009 ± 0.002
Russet	7	4.1 ± 0.2	0.011 ± 0.004	0.27 ± 0.09	0.51 ± 0.03	0.22 ± 0.06	0.10 ± 0.02	0.003 ± 0.002
Spartan	9	4.3 ± 0.2	0.004 ± 0.004	0.09 ± 0.07	0.66 ± 0.03	0.25 ± 0.04	0.09 ± 0.02	0.007 ± 0.002

[a] Number of samples.
[b] Not molar ratios; see text.
[c] P = polyphenol, Suc = sucrose, Fru = fructose, Glc = glucose, MA = malic acid, QA = quinic acid, T = Suc + Fru + Glc. For signals integrated, see text.

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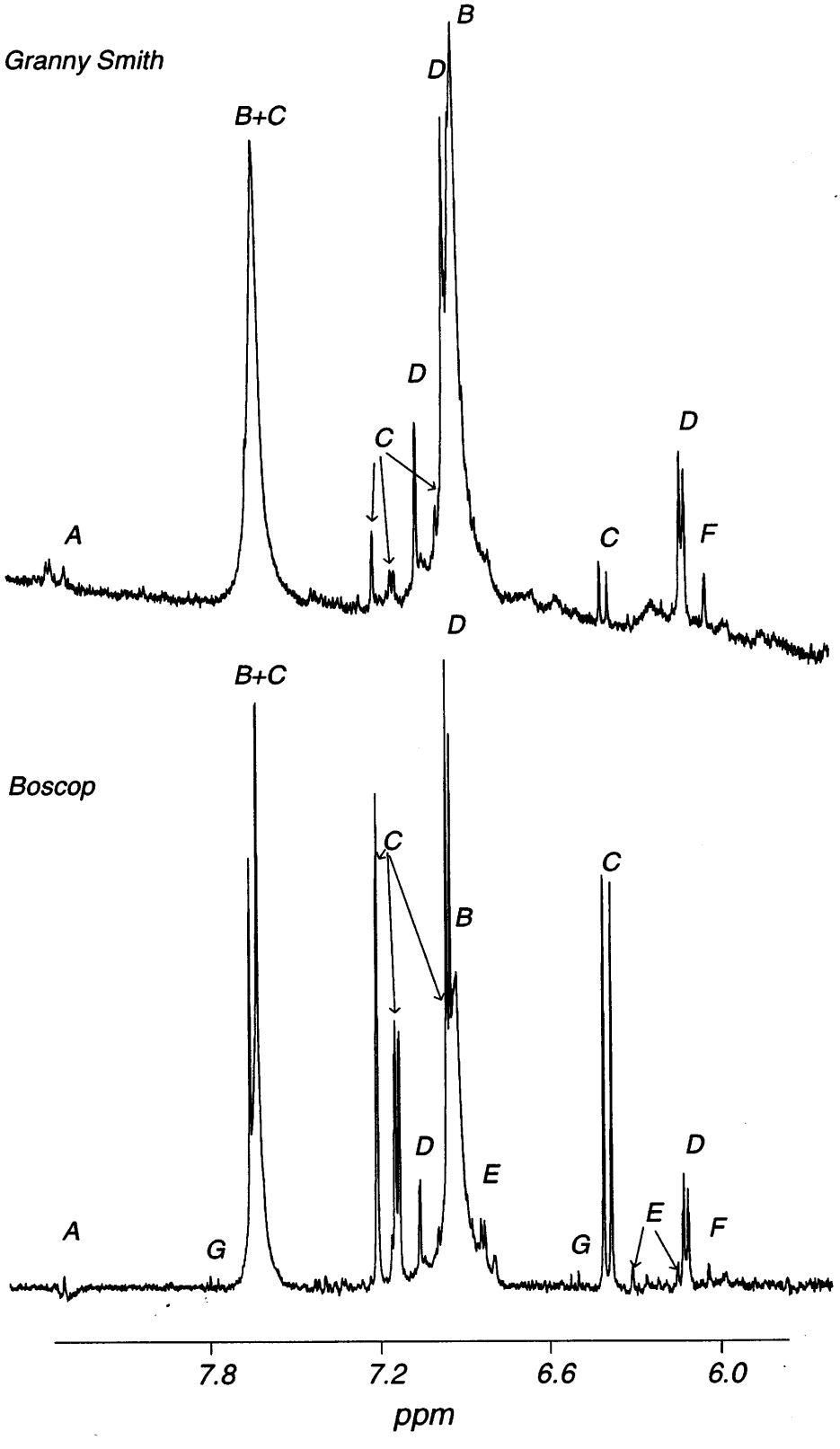

Figure 8. 600 MHz ^{1}H NMR spectra of freshly squeezed juices (same samples as in Fig. 2) (0.8–2.5 ppm region). Spectra are plotted with a vertical gain of *ca*. 350 with respect to Fig. 4. Key to assignments: A, formic acid; B, polyphenols; C, chlorogenic acid; D, epicatechin; E, phloridzin; F, phloretin; G, *p*-coumaric acid.

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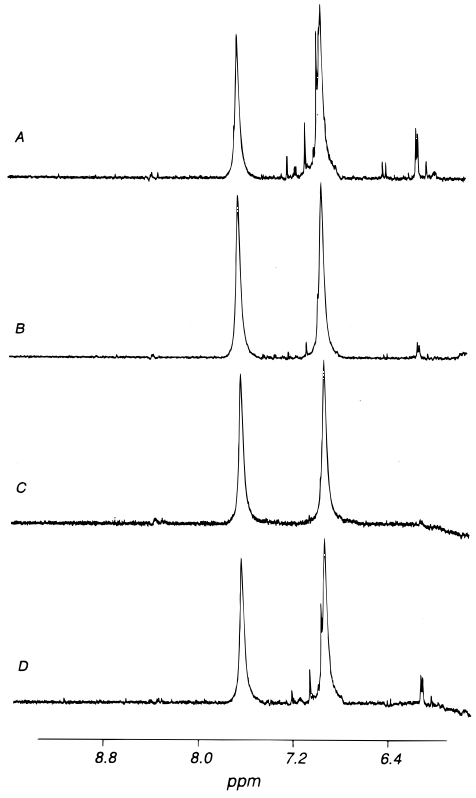

Figure 9. Phenolic region of the 600 MHz ^{1}H NMR spectra of apple juices of the Granny Smith cultivar: (A) 17 min after squeezing, (B) after 4 h and (C) after 8 h of exposure to air; (D) after 7 h of exposure to air after polyphenoloxidase deactivation.

For the other components, polyphenols are present at a lower level in Spartan than in the other varieties, and the quinic acid content is lowest for the Russets. A full multivariate analysis of the spectral data will be reported elsewhere.[12]

CONCLUSION

The high-field proton NMR spectra of fruit juices reveal a richness and complexity of information. The spectra are sensitive to a number of factors, among which are cultivar type, microbiological activity and enzymic activity. Although not specifically investigated here, it seems very likely that agronomic history and degree of ripeness will also affect the spectra. The rapidity with which information can be obtained about a large number of compounds within the juice indicates that high-field proton NMR is unique in its ability to survey comprehensively the chemistry of a large number of samples. It therefore has considerable advantages over conventional methods of analysis. Not withstanding these advantages, it is clear that extreme care must be taken in the interpretation of spectra as the differences observed arise from a number of historical factors. Currently the spectral assignments are incomplete and it is a high priority to assign as many additional resonances as possible. This will require the use of multidimensional methods and the careful preparation of standard materials.

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REFERENCES

1. P. S. Belton, I. Delgadillo, E. Holmes, A. Nicholls, J. K. Nicholson and M. Spraul, *J. Agric. Food Chem.* **44**, 1483 (1996).
2. J. K. Nicholson, P. J. D. Foxall, E. Holmes, G. H. Nield and J. Lindon, in *Magnetic Resonance in Food Science*, edited by P. S. Belton, I. Delgadillo, A. M. Gil and G. A. Webb, p. 177. Royal Society of Chemistry, Cambridge (1995).
3. L. F. Burroughs, *J. Sci. Food Agric.* **8**, 122 (1957).
4. H. J. Bielig and H.-J. Hofsommer, *Flüss Obst* **49**, 50 (1982).
5. T. Fuleki, E. Pelayo and R. B. Palabay, *J. Agric. Food Chem.* **43**, 598 (1995).
6. T. Fuleki, E. Pelayo and R. B. Palabay, *J. Agric. Food Chem.* **42**, 1266 (1994).
7. H. H. Dittrich, *Flüss Obst* **49**, 312 (1982).
8. L. R. Mattick and J. C. Moyer, *J. Assoc. Off. Anal. Chem.* **66**, 1251 (1983).
9. G. A. Spanos and R. E. Wrolstad, *J. Agric. Food Chem.* **40**, 1478 (1992).
10. J. Ackermann, M. Fischer and R. Amado, *J. Agric. Food Chem.* **40**, 1131 (1992).
11. J. J. Nicolas, F. C. Richard-Forget, P. M. Groupy, M.-J. Amiot and S. Y. Aubert, *CRC Crit. Rev. Food Sci. Nutr.* **34**, 109 (1994).
12. P. S. Belton, I. J. Colquhoun, E. K. Kemsley, I. Delgadillo, P. Roma, M. J. Dennis, M. Sharman, E. Holmes, J. K. Nicholson and M. Spraul, *Food Chem.* in press.

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