High-Resolution Proton NMR Spectroscopy of Milk, Orange Juice, and Apple Juice with Efficient Suppression of the Water Peak

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Soluble components of natural aqueous fluids can be analyzed by high-resolution proton nuclear magnetic resonance spectroscopy using a new method that suppresses the water proton peak to very low levels. The method exploits the selective effects of a paramagnetic reagent, added at low concentration, on water proton transverse relaxation rate (T_2^{-1}), by acquiring a spin–echo spectrum of the solutes after water magnetization has decayed in the transverse plane. The method eliminates the dynamic range problem, reduces the water peak up to 10^5-fold, eliminates base-line roll and other spectral artifacts associated with the intense water peak, and permits observation with excellent sensitivity and resolution of solute resonances otherwise obscured by water. Proton spectra of milk, orange juice, and apple juice that demonstrate these features are presented.

INTRODUCTION

Standard methods of analysis of low molecular weight (up to 10^3 Da) solutes in milk, in liquids derived from plant tissue, and in other aqueous products of agriculture usually include some degree of fractionation and purification before measurement of the solute of interest. There is obvious advantage to analytical methods that measure directly, without fractionation or partial purification. High-resolution proton nuclear magnetic resonance (NMR) has the potential to fill this role for many interesting solutes. A major obstacle in Fourier transform proton NMR, however, has been that, due to the high concentration of water in most natural fluids, the water proton signal overwhelms the NMR spectrum, obscuring solute proton resonances that lie close to the water resonance, causing nonlinear response in detection circuitry and creating a dynamic range problem (the signal digitization may not be capable of discriminating solute peaks, whose intensities are often less than $^1/_{10000}$th the intensity of the water peak).

Techniques of several types have been developed to ameliorate these problems (these have been recently restated in Hore (1983)). Several techniques involve radiofrequency pulse strategies to minimize the water peak. In most cases these techniques leave at least some region of the spectrum unobservable because of selective nonexcitation at one or several frequencies (Hore, 1983). Other techniques exploit the difference when it exists between the *longitudinal* relaxation time (T_1) of water protons and that of solute protons. Recently the fortuitous difference in *transverse* relaxation time has been used (Rabenstein and Isab, 1979; Rabenstein, 1984). In this paper we report application of a very general method of water peak suppression based on selective *control* of solvent transverse relaxation rate by using a paramagnetic reagent. Subsequent detection with a spin–echo pulse sequence produces a high-resolution proton spectrum of the principal organic solutes, without interference from the water resonance (Bryant and Eads, 1985).

The method described here is for implementation on modern Fourier transform NMR spectrometers, with no modification of hardware or software. Sample preparation is trivial. We will discuss the salient features of paramagnetically induced solvent relaxation and the rationale of spin–echo detection.

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Our purpose is simply to introduce the method and demonstrate its power when applied to a few food products. Its potential for quantitative chemical analysis is clear. Although we do not attempt to analyze these spectra, some approaches for extracting composition information are suggested.

The chemical and theoretical basis of the method are discussed more fully elsewhere (Eads et al., 1986).

EXPERIMENTAL SECTION

Materials. Pasteurized, homogenized whole milk was purchased locally (Deryke Dairy, Rochester, NY; expiry May 28, 1985). Orange juice was freshly squeezed from a Sunkist Valencia (California) orange. The juice was centrifuged at 1000*g* for 5 min to remove pulp, and the resulting serum, still somewhat turbid, was used without further treatment. Apple juice purchased locally was labeled as unfiltered, fresh-pressed juice, with no sugar or water added and with less than 0.1% potassium sorbate added as a preservative. Deuterium oxide (^2H_2O, 99.9 atom % ^2H) and 3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt (TSS), were obtained from Aldrich. Manganese chloride was reagent grade (ACS).

Preparation of NMR Samples. Samples contain about 10% by volume of ^2H_2O to provide a lock signal for field frequency stabilization; about 0.2–2% of a saturated aqueous solution of TSS, whose high-field trimethyl proton resonance is used as an internal chemical shift reference, assigned a value of 0 ppm in the spectrum; and an appropriate amount of paramagnetic reagent, in this case the hexaaquomanganese ion that forms upon dissolution of $MnCl_2$ in water, present at about 200 μM final concentration. A 10 mM $MnCl_2$ solution is used for additions; it is prepared freshly from 1.0 M refrigerated stock solution.

Samples were prepared by pipetting the TSS, ^2H_2O, $MnCl_2$, and test fluids, in that order, directly into a 5-mm NMR tube (Wilmad #507pp) to obtain a final volume of 0.500 mL. The tube was capped and mixed by inversion three or four times. It is important to use the same kind of NMR tubes, to fill them to the same volume, and to position them reproducibly. NMR spectra were obtained as soon as 10 min, and always within 2 h of sample preparation.

^{1}H NMR Spectra. Spectra were obtained at ambient temperature (28 ± 2 °C) on an IBM WP 270 SY spectrometer (operating frequency 270 MHz, ^1H) using a manufacturer-supplied two-channel (^1H and ^2H) 5-mm probe. External field homogeneity was obtained by adjusting magnet shim coils while observing the free induction decay of residual protons in a sample of ^2H_2O spinning

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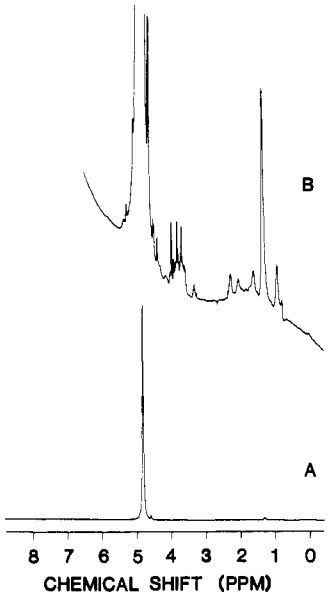

Figure 1. (A) Single-pulse ^{1}H NMR spectrum of whole cow's milk; (B) 128× vertical expansion of (A). Sample composition and spectral acquisition parameters are given in the text.

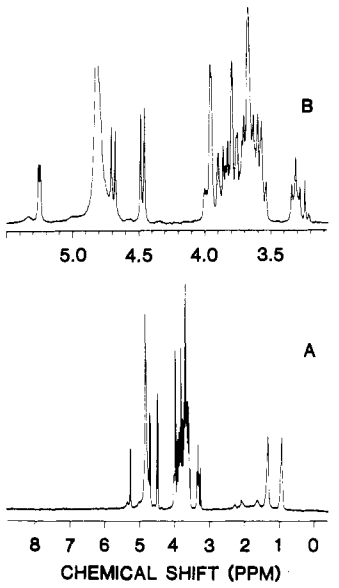

Figure 2. Spin–echo spectrum of whole cow's milk containing 200 μM $MnCl_2$: (A) full spectrum; (B) expansion of the carbohydrate region, dominated by lactose. The peak at 4.8 ppm is due to ^1H_2O.

at 30 ± 1 Hz. The resulting line width obtained was typically 0.50 ± 0.05 Hz; under these conditions, a resolution of 0.15–0.2 Hz could be observed in subsequent spectra of organic solutes. 90 and 180° pulse widths were determined and set. Single-pulse spectra of aqueous samples (e.g., Figure 1) (D–pulse–acquisition) were obtained by attenuating by 8–12 dB both the transmitted radiofrequency pulse power into the probe and the rf signal coming from the probe. The result is that the pulse angle is actually much less than 90°. An unattenuated pulse would produce a very intense magnetization due to ^1H_2O, resulting in radiation damping, an artifactual broadening of resonances (Abragam, 1961). An unattenuated rf signal would overwhelm the analog-to-digital converter resulting in a "clipped" fid and subsequent base-line distortions in the spectrum. Spin–echo spectra (Figures 2–4) were obtained with attenuators removed, by executing the spin–echo train pulse sequence (D–90_x°–(τ–180_y–τ)$_n$–acquisition) where τ is typically 0.6 ms and n is adjusted to be large

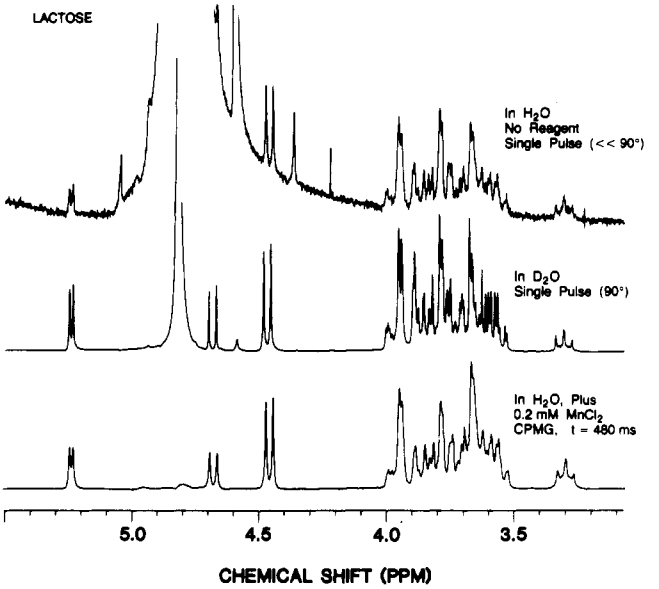

Figure 3. Proton NMR spectra of lactose, taken under different conditions: top; single pulse in water with tip angle less than 90°; middle, lactose dissolved in D_2O using a 90° pulse; bottom, spin–echo spectrum obtained after a Carr–Purcell–Meiboom–Gill echo train at 480 ms, as described in the text, using 200 μM $MnCl_2$ as the relaxation reagent in H_2O.

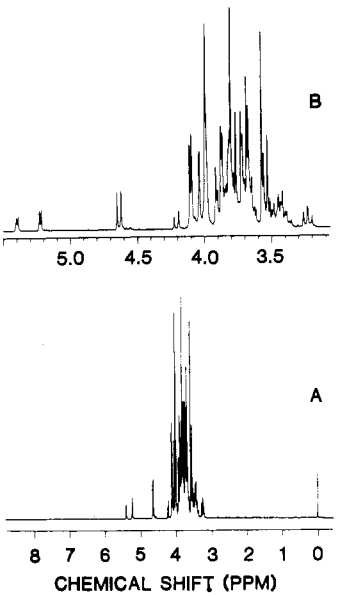

Figure 4. Spin–echo spectrum of apple juice containing 200 μM $MnCl_2$: (A) full spectrum; (B) expansion of the carbohydrate region.

enough to reduce or eliminate the water peak: n is typically 360–400 in the experiments reported here. This pulse sequence is the Meiboom–Gill modification of the Carr–Purcell spin echo sequence (Carr and Purcell, 1954; Meiboom and Gill, 1958), usually used to generate a series of echoes for determination of T_2 but used here to allow rapid water proton relaxation to occur before beginning sampling an echo, which is a free induction decay (time domain) subsequently transformed into a spectrum (frequency domain). Pulse widths of 90 and 180° were 4.5 and 9.8 μs, respectively, with the 90° pulse being phase cycled, consistent with quadrature detection. D is a delay allowing return of magnetization to equilibrium; a value of 5 s was found to be sufficient to avoid saturation of any resonance. Sweep width was 2500 Hz, and 16K data points were collected. A total of 64 scans was acquired in each spectrum shown. Signal-to-noise ratio was enhanced by ap-

plication of an exponential multiplication (EM) of the fid, corresponding to 0.4-Hz line broadening. Otherwise no base-line adjustments or data manipulations were applied.

RESULTS AND DISCUSSION

Paramagnetic ions such as Mn^{2+} affect solvent nuclear magnetic relaxation rates T_1^{-1} and T_2^{-1} in a way that depends on the magnetic field strength, such that at high field (>30 MHz) the transverse rate T_2^{-1} becomes very large compared to the longitudinal rate (see, e.g., Dwek (1973)). The efficiency of relaxation depends on rapid chemical exchange of the water molecule or of water protons between the paramagnetic environment and the bulk solution. Thus, a solute species not sampling that environment is affected only very weakly.

The essence of this method is to establish a large difference between solvent and solute T_2 by adding a paramagnetic reagent, flipping all proton spins into the xy plane, allowing the transverse magnetization of water to decay quickly while solute magnetization remains, and then acquiring the solute spectrum. Several types of reagents may be employed, and other detection schemes are possible (Bryant and Eads, 1985; Eads et al., 1986).

Milk. The single-pulse ^1H NMR spectrum of whole milk is shown in Figure 1. Whole milk is 88% water, or about 97 M in protons. Thus, the ^1H_2O signal dominates. Observation of some solute resonances is possible in the expanded spectrum; however, severe base-line distortions occur, solute resonances near the water peak are not detectable, and spinning side bands from the intense water peak complicate spectral interpretation. The spectrum was obtainable on a normal-size sample only by attenuation of both the radiofrequency pulse going into the probe and the resonance signal from the sample, in order to avoid the artifactual line broadening due to radiation damping and base-line rolls due to data clipping that occur when the data system receives an overwhelming signal.

Application of the solvent suppression scheme relieves these distortions. Figure 2 shows the spin–echo spectrum of milk to which 200 μM $MnCl_2$ is added. Peaks previously obscured by water are now clearly visible. Excellent sensitivity and resolution are maintained. Lactose, which is the most abundant low molecular weight solute in milk (about 4.5% by weight, or 135 mM; Swaisgood, 1985) contributes in the region from 3.2 to 5 ppm. Lactose spectra are shown in Figure 3 for comparison. Resonances due to the aliphatic protons of milk fat appear in the region from 0.5 to 2.5 ppm. All protons in the sample lose magnetization due to T_2 relaxation while being held in the transverse plane by the multiple 180° flips. The water protons decay very quickly compared to solute protons, allowing reduction of the water peak. Obviously, the method is less well suited for solutes that, because of their size or association with large particles, also have short T_2's. This is the case for some aliphatic protons of fat, which decay faster than the lactose protons. This can serve as the basis of further spectral editing, since the value of n is easily adjusted. Thus, the relative intensities of lactose and fat will depend on the value of n used in the spin–echo pulse sequence.

Overall, resolution is not degraded by addition of Mn and use of the spin–echo sequence, since lactose resonance line widths are the same in the spin–echo spectra (Figure 2) as they are in the single-pulse spectrum (Figure 1) and in a single-pulse spectrum obtained without Mn (not shown).

Apple Juice. The highly detailed spin–echo spectrum of apple juice containing 200 μM manganese ion is shown in Figure 4. Most resonances for this sample fall within the range from 3.1 to 5.5 ppm. Apple juice generally contains a high concentration of sugars (about 11 g/100 g), primarily fructose (about 6 g/100 g or 340 mM), sucrose (about 3.8 g/100 g or 110 mM), and glucose (about 1.2 g/100 g or 65 mM); other principal solutes are malic acid (about 0.5 g/100 g or 40 mM) and ascorbic acid (about 1 mg/100 g or 6 mM) (Smock and Newbert, 1950; Watt and Merrill, 1963). These values may vary widely with species, location, maturity, processing, etc. We may tentatively identify resonances in Figure 4 due to fructose, sucrose, and glucose on the basis of visual comparison with spectra of the pure compounds. Resonances due to malic and ascorbic acids are not seen in this spectrum, although they might be observable, if they are present, with further signal averaging, since each has unique resonances upfield of the sugar peaks.

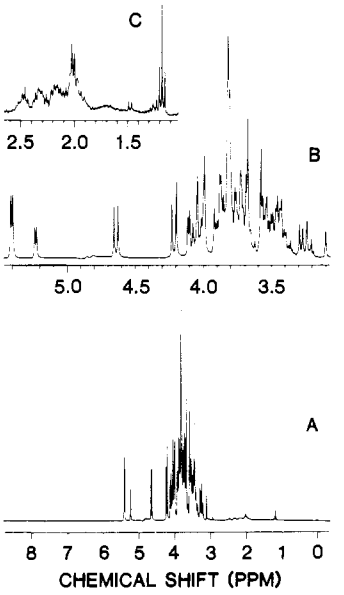

Figure 5. Spin–echo spectrum of orange juice containing 200 μM $MnCl_2$: (A) full spectrum; (B) expansion of the carbohydrate region; (C) expansion of the organic acid region, 15× vertical expansion.

Orange Juice. Freshly squeezed orange juice also contains fairly large amounts of dissolved sugars (about 8 g/100 g), which in Valencias include primarily sucrose (about 5 g/100 g or 150 mM) and about 2.5 g/100 g of fructose and 2.5 g/100 g of glucose (Braverman, 1949; Watt and Merril, 1963; McCready, 1977). Other major soluble components include citric acid (about 1.2 g/100 or 60 mM) and volatiles, primarily ethanol, which may reach up to 20 mM (Lund et al., 1981). Other components such as phenolics, amino acids, and various flavors are present at a few millimolar or less (Vandercook et al., 1975) and would not be expected to appear in these spectra under these conditions. It is possible to tentatively identify resonances belonging to sucrose and fructose, which dominate observed intensity, and also glucose, in the spin–echo spectrum of orange juice (Figure 5). The complex high-field resonances shown in Figure 5C are characteristic of organic acids, but a clear identification of citrate is not evident.

Although we have made no attempt to extract it, it is clear that the proton spectra presented contain information not only about identity and concentration of major solutes but also about their chemical state as well, since proton chemical shifts and resonance splittings can be sensitive to ion-binding equilibria, pH, temperature, etc.

Our chosen samples contain greater than 0.1 M solutes; thus, the residual water peak is very small compared to solute peaks. In more dilute solutions, the size of the

residual water peak relative to the solute peaks increases. Nonetheless we have easily detected glucose in human cerebrospinal fluid (normal concentration of glucose 64 mg/100 g or 3.5 mM) and at higher concentrations in other body fluids (Eads et al., 1986). Thus, the method is applicable to more dilute systems. In addition aqueous slurries and semisolids may be studied to advantage with this method, as has been shown with spin–echo spectra of minced and whole brain tissue (Eads et al., 1986) and in other tissues and cells (Rabenstein, 1984) whose natural water proton T_2 may sometimes be short enough that a paramagnetic reagent need not be added.

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Selective Removal of Bitter Compounds from Grapefruit Juice and from Aqueous Solution with Cyclodextrin Polymers and with Amberlite XAD-4

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Several polymers were prepared from α-, β-, and γ-cyclodextrin with various cross-linking agents and evaluated for capacities to remove bitter compounds. Naringin and limonin removal was tested in grapefruit juice, as well as removal of these plus caffeine in aqueous solution. They were compared with Amberlite XAD-4 for capacity to remove these compounds from aqueous solution. None of the cyclodextrin polymers removed caffeine. The capacity of cyclodextrin monomer to form inclusion compounds with naringin and limonin was determined with a YM2 membrane and with undissolved cyclodextrin which did not remove any of either compound. The polymers appeared to have about 10 times the capacity of the monomer for formation of inclusion complexes with these bitter components. Of nine media tested, two commercially prepared β-cyclodextrin polymers showed the greatest capacities to remove limonin and naringin combined.

Some processed citrus juices have excessive bitterness that adversely affects the flavor and therefore the marketability of products made from these juices. In California, bitterness occurs mainly in processed navel orange juice; 60 million gallons were processed during the 1982–1983 season (Citrus Fruit Industry Statistical Bulletin, 1984), and about half of that was bitter enough for its flavor to be adversely affected. In Florida, excessive bitterness is a major problem in early-season grapefruit juice, potentially affecting about 6.4 million gallons of single-strength juice. Bitter juices are often blended with less bitter juice so that an even larger portion of the processed juice products are affected by these bitter juices (Shaw, 1985). A process is needed to decrease the bitterness of these juices that does not add anything to the juice or remove desirable juice components.

Several methods have been developed recently to remove the bitter components naringin, limonin, and nomilin from citrus juice by treatment of the juice with insoluble polymers (Johnson and Chandler, 1982; Puri, 1984; Shaw and Wilson, 1983) or immobilized dead bacteria (Hasegawa et al., 1985). None of these processes are currently being used commerically to debitter citrus juices, although some of the insoluble polymers have been approved for food use in the United States.

In earlier studies, we found polymers made from β-cyclodextrin to be effective in removing limonin and nomilin from navel orange juice and in removing these bitter compounds as well as naringin from grapefruit juice (Shaw, 1985; Shaw and Wilson, 1983; 1985; Shaw et al., 1984). Before studies are carried out on pilot plant scale with grapefruit juice, the most effective of several possible polymers of cyclodextrin at debittering citrus juices needs

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