shaking. They observed an excellent correlation for hydrogen reduced and electrolytic iron samples, however, when the solubility was determined for 1 min. Their results for three carbonyl iron samples having particles less than 10 μm in size were not consistent with those for the above two types of powders. This was attributed to the unique chemical and physical properties of the carbonyl powders. From our results (Table II), however, it is evident that at shorter times the differences between solubilities of different powders are maximized whereas they are reduced at longer times. It is, therefore, essential to employ an optimal time for the solubility test. Ninety minutes is suggested above on a physiological basis.

The variation in the solubility of four iron powders determined in triplicate on 3 days is indicated by the results given in Table III. The hydrogen reduced iron powder which had the largest median particle size and the lowest solubility showed the maximum coefficient of variation of 12.0% on one day and also the highest overall coefficient of variation (7.8%). The corresponding values for the other three powders of acceptable quality did not exceed 3%.

From these results, and from data reported previously by others, it is concluded that iron powder used as an iron source should meet the following specifications to ensure acceptable bioavailability. (1) The iron content of the powder should not be less than 96%. (2) At least 95% of the powder should pass through a 325 mesh sieve having a pore size of 44 μm. (3) At least 90% of the weight of the powder should be soluble iron, as determined in triplicate by the method described.

ACKNOWLEDGMENT

The authors would like to thank John Hall, Food Statistics and Operational Planning, Health Protection Branch for statistical analysis of the data. They are indebted to J. Leroux and A. B. C. Davey, Physics Section, Environmental Health Directorate for the particle size analysis of the samples of reduced iron. The competent technical assistance of C. Desloges is gratefully acknowledged.

LITERATURE CITED

Amine, E. K., Hegsted, D. M., *J. Nutr.*, **101**, 927 (1971).
Association of Official Analytical Chemists, "Official Methods of Analysis", 12th ed, Washington, D.C., 1975, p 22–23.
Chapman, D. G., Campbell, J. A., *Br. J. Nutr.* **2**, 117 (1957).
Cook, J. D., Minnich, V., Moore, C. V., Rasmussen, A., Bradley, W. B., *Am. J. Clin. Nutr.*, **26**, 861 (1973).
Farris, E. J., Griffith, J. Q., Ed., "The Rat in Laboratory Investigation", 2nd ed, J. B. Lippincott Co., Philadelphia, Pa., 1949, p 431.
Fritz, J. C., Pla, G. W., Rollinson, C. L., *Baker's Dig.* **39**, 47 (1975).
Hart, H. V., *J. Sci. Food Agric.* **22**, 354 (1971).
Health and Welfare Canada, "The Food and Drugs Act and Regulations", B.13.002 and B.13.022, Information Canada, Ottawa, 1975, pp 60 and 63.
Hinton, J. J. C., Carter, J. E., Moron, T., *J. Food Technol.* **2**, 129 (1967).
Höglund, S., Reizenstein, P., *Blood* **34**, 469 (1969).
Ministry of Agriculture, Fisheries and Food, U.K., "Second Report on Bread and Flour", Her Majesty's Stationery Office, London, 1974, p 149.
Motzok, I., Pennell, M. D., Davies, M. I., Ross, H. V., *J. Assoc. Off. Anal. Chem.* **58**, 99 (1975).
National Research Council, "Food Chemical Codex", 2nd ed., National Academy of Sciences, Washington, D.C., 1972, p 394.
Oser, B. L., Ed., "Hawk's Physiological Chemistry", 14th ed, McGraw-Hill, New York, N.Y., 1965, p 473.
Pennell, M. D., Wiens, W. D., Rasper, J., Motzok, I., *J. Food Sci.* **40**, 879 (1975).
Pla, G. W., Fritz, J. C., Rollinson, C. L., *J. Assoc. Off. Anal. Chem.* **59**, 582 (1976).
Pla, G. W., Harrison, B. N., Fritz, J. C., *J. Assoc. Off. Anal. Chem.* **56**, 1369 (1973).
Rios, E., Hunter, R. E., Cook, J. D., Smith, N. J., Finch, C. A., *Pediatrics* **55**, 686 (1975).
Shah, B. G., Belonje, B., *Nutr. Rep. Int.* **7**, 151 (1973a).
Shah, B. G., Belonje, B., *Can. Inst. Food Sci. Technol. J.* **6**, 37 (1973b).
U.S. Pharmacopeia XIX, United States Pharmacopeial Convention Inc., Rockville, Md., 1975, p 765.

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Nuclear Magnetic Resonance Analysis of Bulk Food Additive Chemicals. 1. Food Chemicals Codex Chemicals, Group 1

John W. Turczan,* Bruce A. Goldwitz, and Thomas Medwick

A number of representative food additive chemicals that are listed in the Food Chemicals Codex and do not have analytically useful ultraviolet (UV) chromophores have been studied by nuclear magnetic resonance (NMR). The value of the latter technique has been investigated both in qualitative and quantitative analyses. Conditions for the analyses have been established in that appropriate solvents and internal standards have been selected. Results indicate that the qualitative identification is specific and that the quantitative measurements are reproducible to within 1.5% relative standard deviation.

Prior to 1958, it was incumbent upon food processors to provide detailed procurement specifications when ordering bulk food chemicals from primary manufacturers or distributors. In 1958, the Industry Liaison Panel and other sources requested from the Food Protection Committee of the National Academy of Sciences that a Food Chemicals Codex (FCC) be produced, comparable in many respects to the drug compendia, the United States Pharmacopeia (USP) (1974), and the National Formulary (NF) (1974). As a result, the first bound copy of the FCC (1976) was published in 1966.

The FCC was given quasi-legal recognition, a state which

Food and Drug Administration, Department of Health, Education, and Welfare, New York, New York 11232.

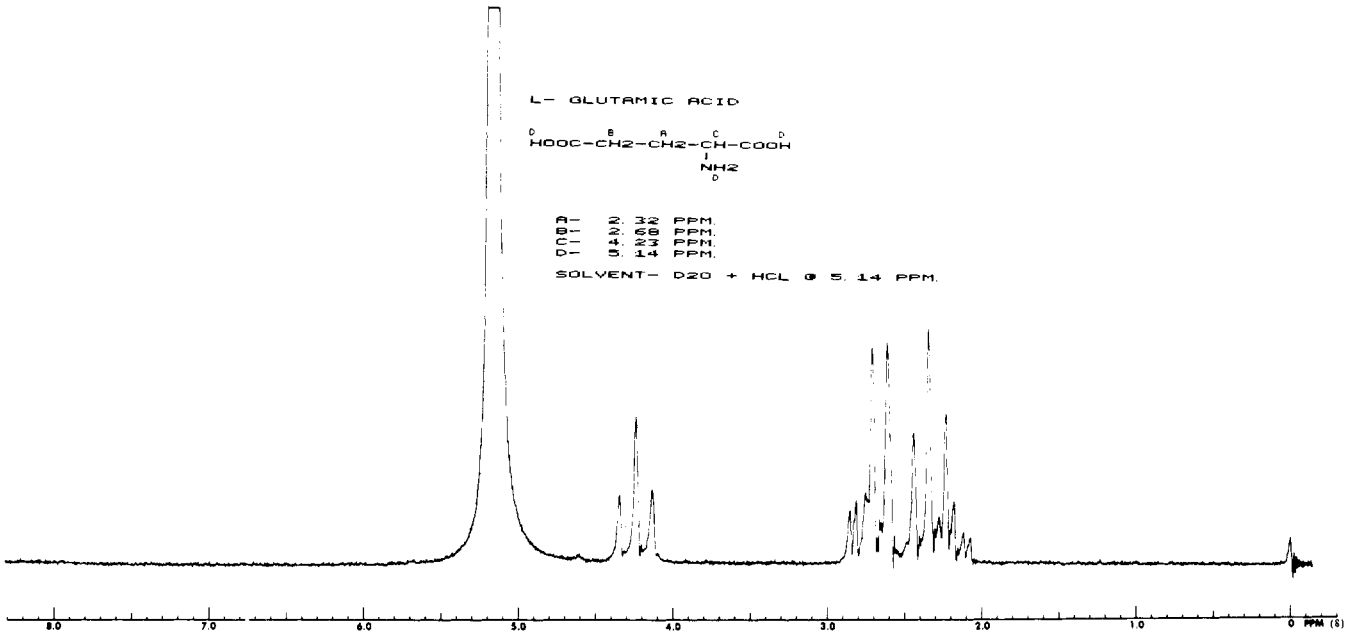

Figure 1. L-Glutamic acid; solvent, D_2O + HCl at 5.14 ppm.

lasted till FCC specifications were officially recognized by the Food and Drug Administration in the publication of the June 25, 1971 *Federal Register*, in the section entitled, "Definitions and Procedural and Interpretative Regulations". This regulation relates to the eligibility of substances for classification as GRAS (generally recognized as safe) materials.

The aim of the FCC is to define the food grade chemicals in terms of identity and purity specifications based on the elements of safety and good manufacturing practices.

Some 639 monographs for food additive materials are listed in the FCC (2d edition) (1972), where various physical methods have been used for the characterization of some of these substances. Of all the methods used to date in the FCC, gas and thin-layer chromatography have probably been the most helpful in identifying substances by virtue of relative retention times and R_f values, which can be compared with those of reference chemicals. While chromatography is helpful in the identification of these chemicals by utilizing relative mobilities, its utility for distinguishing between different compounds, and their impurities, if any, is much reduced and invariably laborious.

The infancy of the FCC, now in its second edition as of 1972, would suggest that the analytical procedures found in this compendium deserve careful review. This paper describes a study made to assess the value of nuclear magnetic resonance (NMR) as a means of qualitative and quantitative analysis of chemicals in the FCC. The nuclear magnetic resonance technique which is well established for the identification and quantitation of organic compounds is both specific and rapid, and will permit relatively decisive and easy characterization of many chemicals.

EXPERIMENTAL SECTION

Apparatus and Reagents. (1) Varian A-60 NMR spectrometer equipped with a V-6031 variable temperature probe having a six-turn insert. All spectra were recorded using a sweep width of 500 Hz and a sweep time of 250 s, at a probe temperature of 40 °C. The δ scale was used throughout. (2) FCC chemicals: all compounds were obtained from various firms and were used without further purification. (3) Internal standards: (a) *tert*-butyl alcohol, Matheson Coleman and Bell, Norwood, Ohio; (b) dimethyl sulfoxide, Fisher Scientific Co., Fairlawn, N.J.; (c) succinic acid, Fisher Scientific Co., Fairlawn, N.J.; (d) benzyl benzoate, Merck & Co., Inc., Rahway, N.J. (4) Reference standards: (a) sodium 4,4-dimethyl-4-silapentanesulfonate (DSS), Nuclear Magnetic Resonance Specialties, New Kensington, Pa.; (b) tetramethylsilane (Me_4Si), Merck Sharpe and Dohme Canada Ltd., Kirkland, Quebec, Canada. (5) Solvents: (a) deuterium oxide, 99.7 atom % D, Merck & Co., Inc., Rahway, N.J.; (b) carbon tetrachloride, Spectro grade, Fisher Scientific Co., Fairlawn, N.J.

Procedure. Weigh accurately in those instances where the analytical material is a volatile liquid, the use of a "microflex tube" in determining the analytical mass is convenient (Turczan and Medwick, 1976) 5–6 ^{1}H NMR mequiv (the term ^{1}H NMR mequiv, as used here, refers to the number obtained by dividing the formula weight in milligrams of a compound by the number of protons that give rise to the area to be integrated) of the sample and the internal standard into a 25-ml glass-stoppered conical flask and then add 2–3 ml of the appropriate solvent (in those cases where deuterium oxide or deuterium oxide with HCl is the solvent, DSS reference standard should not be used when quantitative analysis is to be carried out since peaks from DSS occur at field positions where integration may be necessary). Stopper and shake the flask for 2 min. Transfer approximately 0.4 ml of this solution into an NMR tube. Place the tube in an NMR spectrometer and obtain the spectrum, adjusting the spin rate so that no spinning sidebands occur in the region to be integrated. Integrate the peaks of interest at least five times.

The amount (in milligrams) of FCC substance found per weight taken for analysis may be calculated as follows:

$$\% \text{ found} = \left(\frac{A_{spl}}{A_{std}}\right)\left(\frac{\text{MW}_{spl}/\text{no. of protons}}{\text{MW}_{std}/\text{no. of protons}}\right)\left(\frac{\text{mg of int. std.}}{\text{mg of sample}}\right) \times 100$$

where A = integral area ascribable to the material indicated by subscript, MW = molecular weight of the material

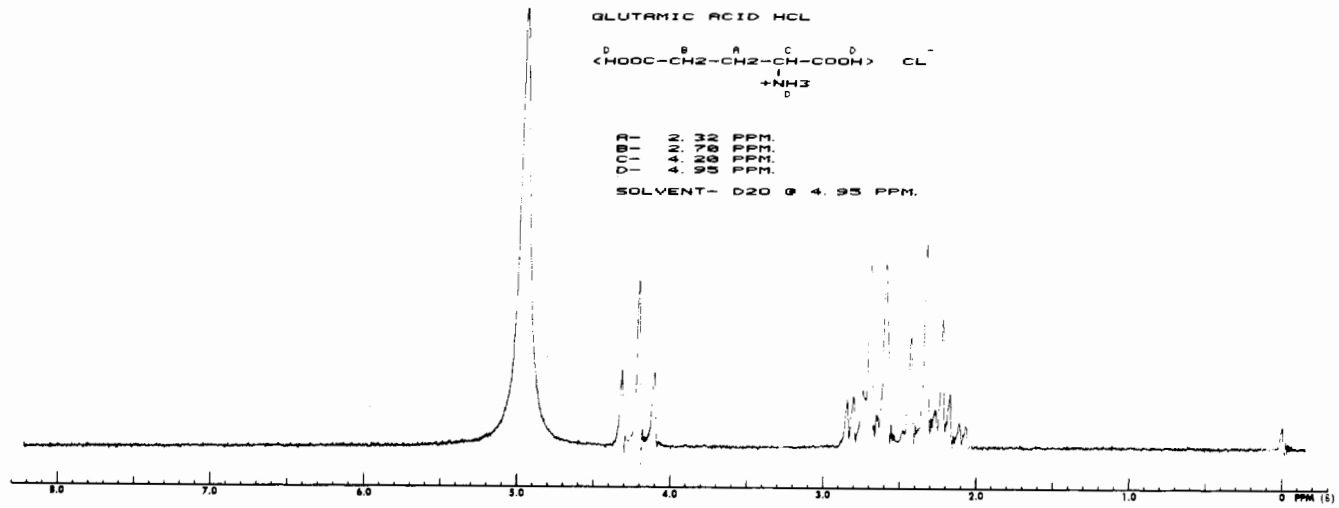

Figure 2. Glutamic acid-HCl; solvent, D_2O at 4.95 ppm.

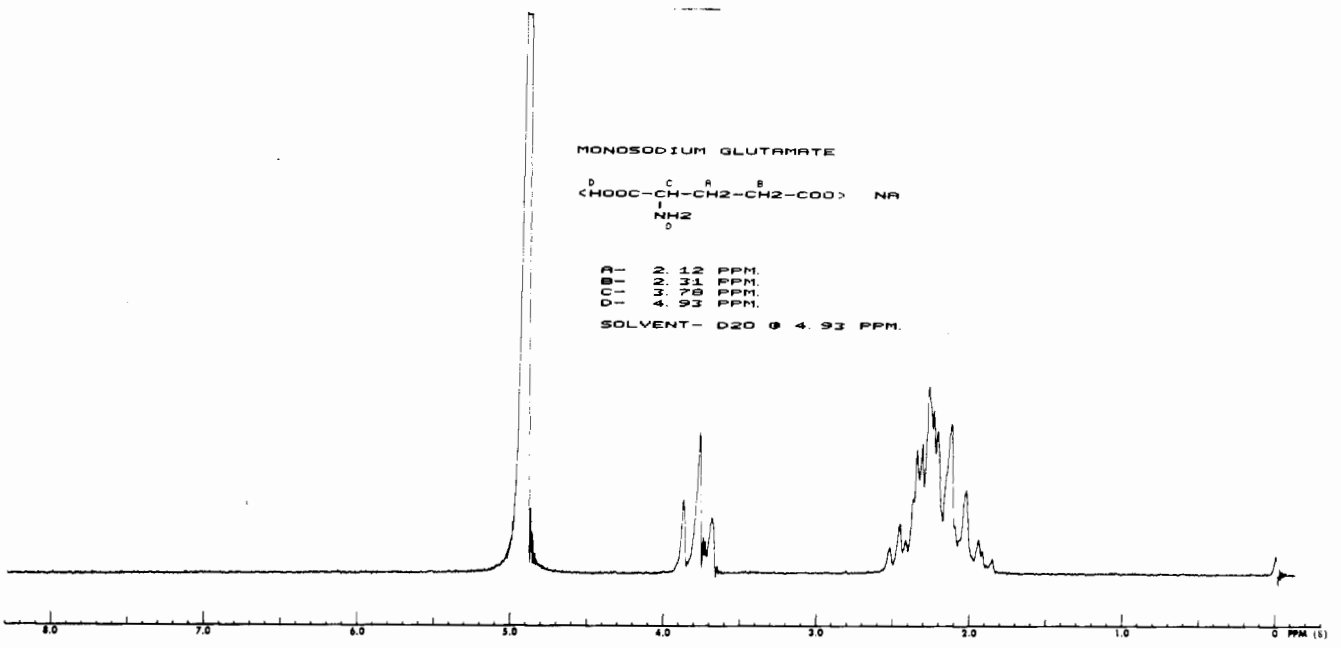

Figure 3. Monosodium glutamate; solvent, D_2O at 4.93 ppm.

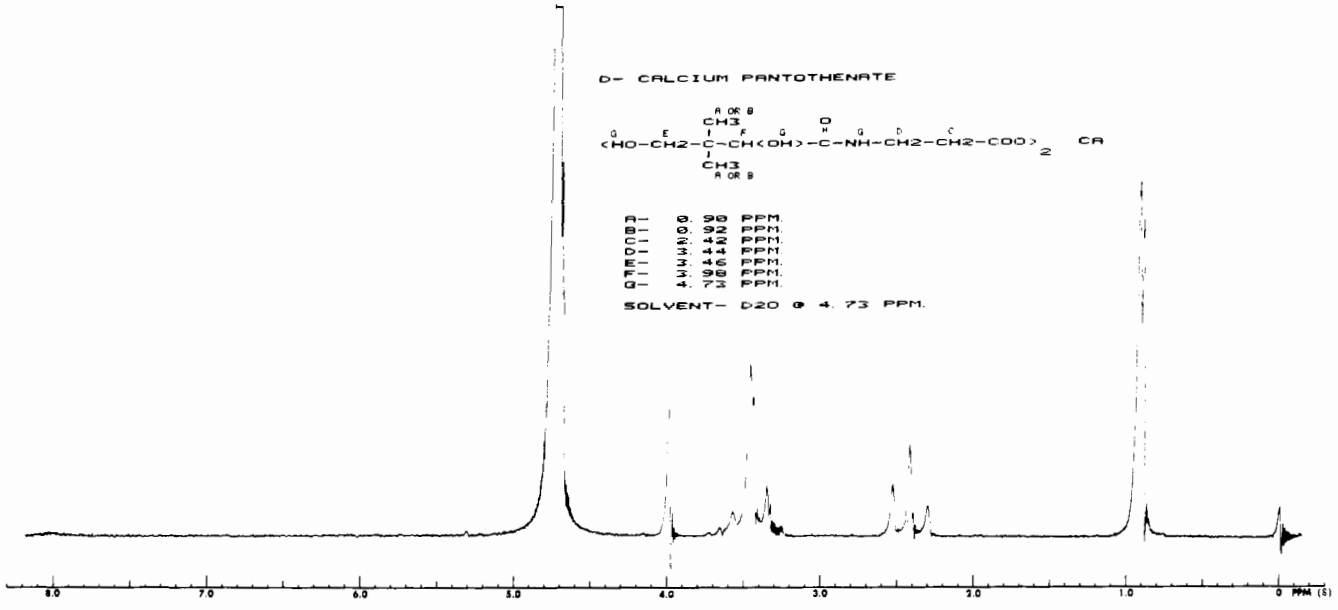

Figure 4. Calcium D-pantothenate; solvent, D_2O at 4.73 ppm.

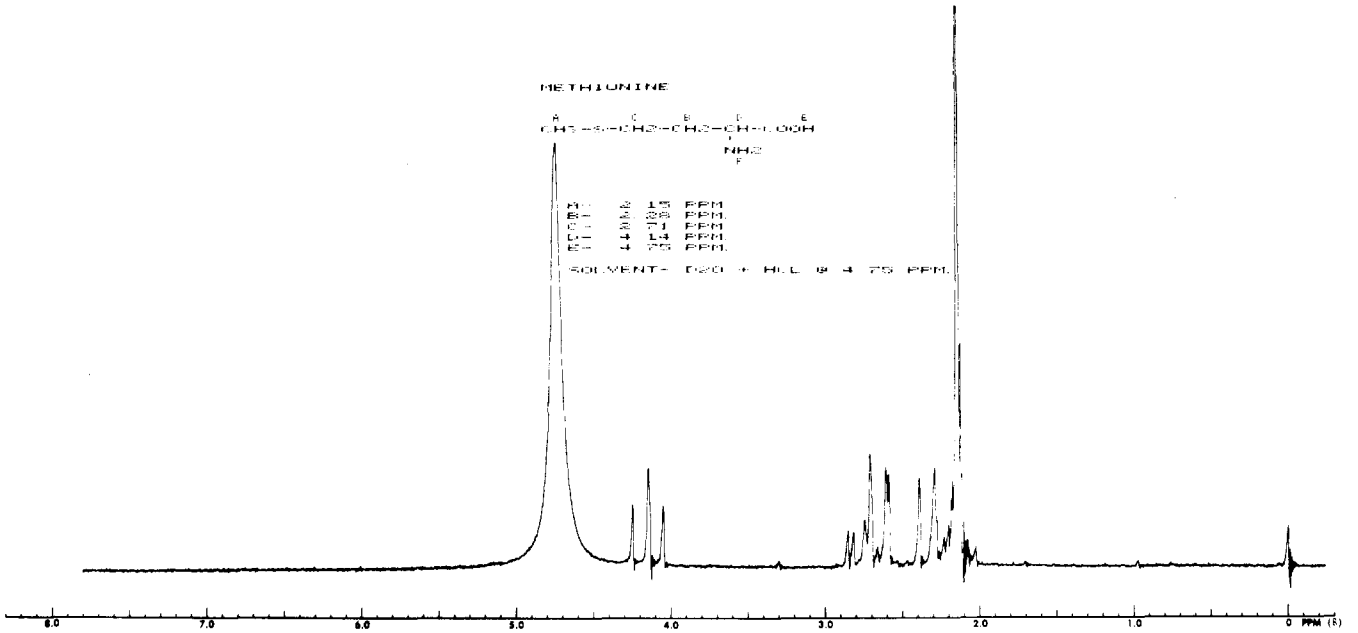

Figure 5. Methionine; solvent, D_2O + HCl at 4.75 ppm.

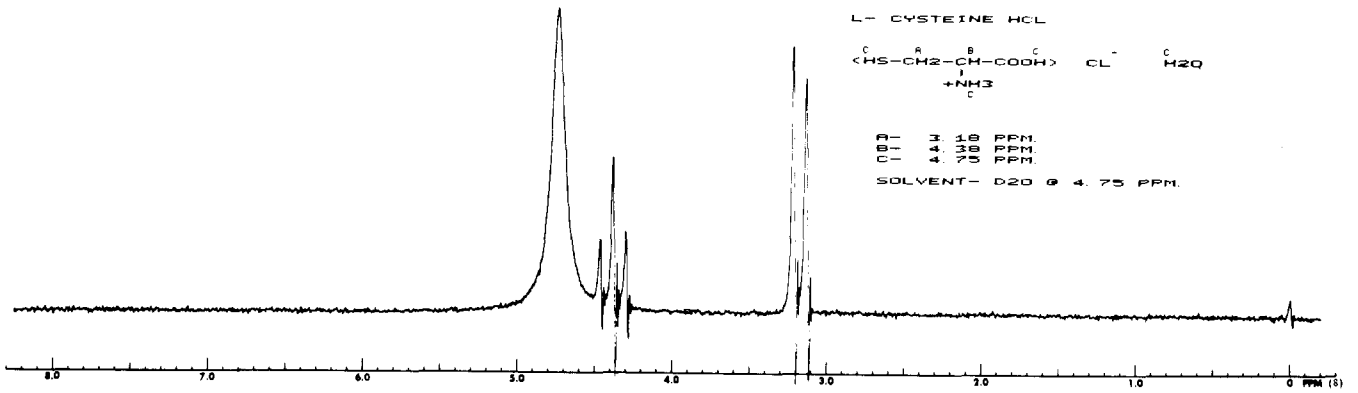

Figure 6. L-Cysteine-HCl; solvent, D_2O at 4.75 ppm.

Table I. NMR Quantitative Analysis of Some FCC Chemicals

Figure no.	Compound (FCC)	Mol wt	No. of protons of interest	Region of interest, ppm, δ	Int std[a]	Solvent	NMR assay Av[b]	NMR assay ±SD[b]
1	L-Glutamic acid	147.13	4	2.0-3.0	1	D_2O + HCl	99.2	0.45
2	L-Glutamic acid-HCl	183.59	4	2.0-3.0	1	D_2O	100.2	0.57
3	Monosodium L-glutamate	187.13	4	1.8-2.7	1	D_2O	99.6	0.60
4	Calcium D-pantothenate	476.55	12	0.8-1.1	1	D_2O	99.3	0.38
5	L-Methionine	149.21	7	1.8-3.0	1	D_2O + HCl	99.6	0.61
6	L-Cysteine-HCl	175.64	2	3.0-3.3	3	D_2O	99.7	0.55
7	Diethylpyrocarbonate[c]	162.14	6	1.1-1.6	2	CCl_4	100.3	0.44
8	Calcium lactate	308.30	6	1.2-1.5	3	D_2O	99.9	0.50
9	Calcium propionate	186.22	4	1.9-2.5	2	D_2O	100.6	0.71
10	Citric acid	192.13	4	2.6-3.3	1	D_2O	100.9	0.35
11	Calcium citrate	570.51	8	2.6-3.4	1	D_2O + HCl	101.4	0.40
12	Potassium citrate	324.42	4	2.2-2.9	1	D_2O	101.2	0.55
13	Ethyl formate	74.08	2	3.9-4.5	4	CCl_4	98.1	0.76
14	Ethyl propionate	102.13	2	3.8-4.4	4	CCl_4	99.1	0.52
15	Ethyl butyrate	116.16	2	3.8-4.4	4	CCl_4	99.4	0.62
16	Malic acid	134.09	2	2.5-3.3	1	D_2O	100.0	0.64
17	Aminoacetic acid (glycine)	75.07	2	3.6	2	D_2O	99.8	0.64

[a] The internal standards used together with the regions of interest in δ, ppm, with respect to either Me_4Si or DSS, for each are: (1) *tert*-butyl alcohol, 1.3; (2) dimethyl sulfoxide, 2.75; (3) succinic acid, 2.65; (4) benzyl benzoate, 5.3. [b] The average (av) and standard deviations (SD) are based on three determinations. [c] Nonofficial chemical.

indicated by subscript, spl = sample, and std = internal standard.

RESULTS AND DISCUSSION

The NMR spectra of 17 FCC compounds are shown in Figures 1–17. The structure, solvent used, and chemical-shift assignments for the protons on each compound are also included on each spectrum.

The list of FCC compounds includes some amino acids, organic acids, salts of organic acids, ethyl esters, and a vitamin as is shown in Table I. Table I also shows for each compound the region (in δ parts per million) used for

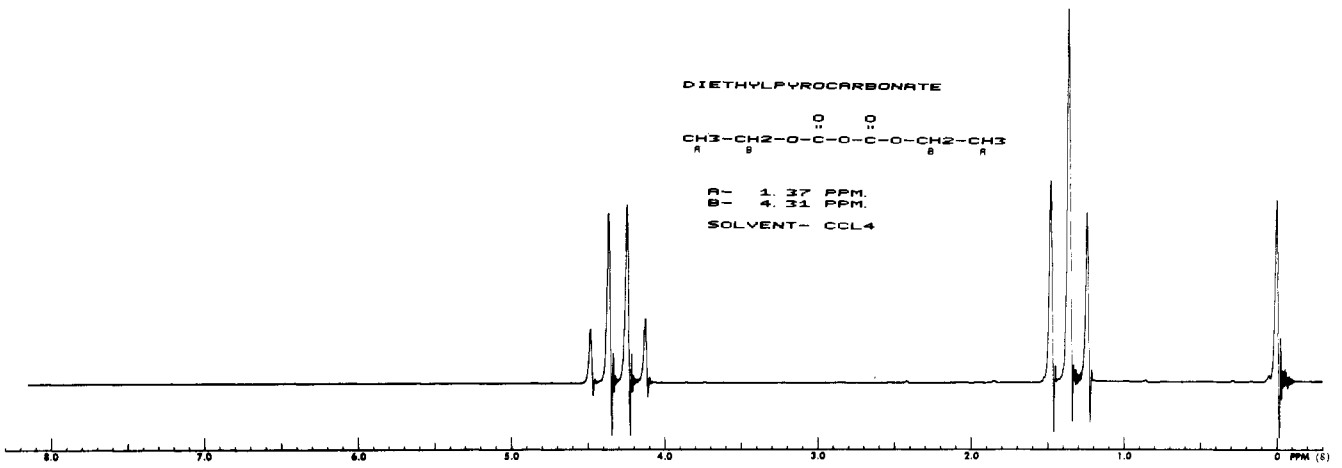

Figure 7. Diethyl pyrocarbonate; solvent, CCl_4.

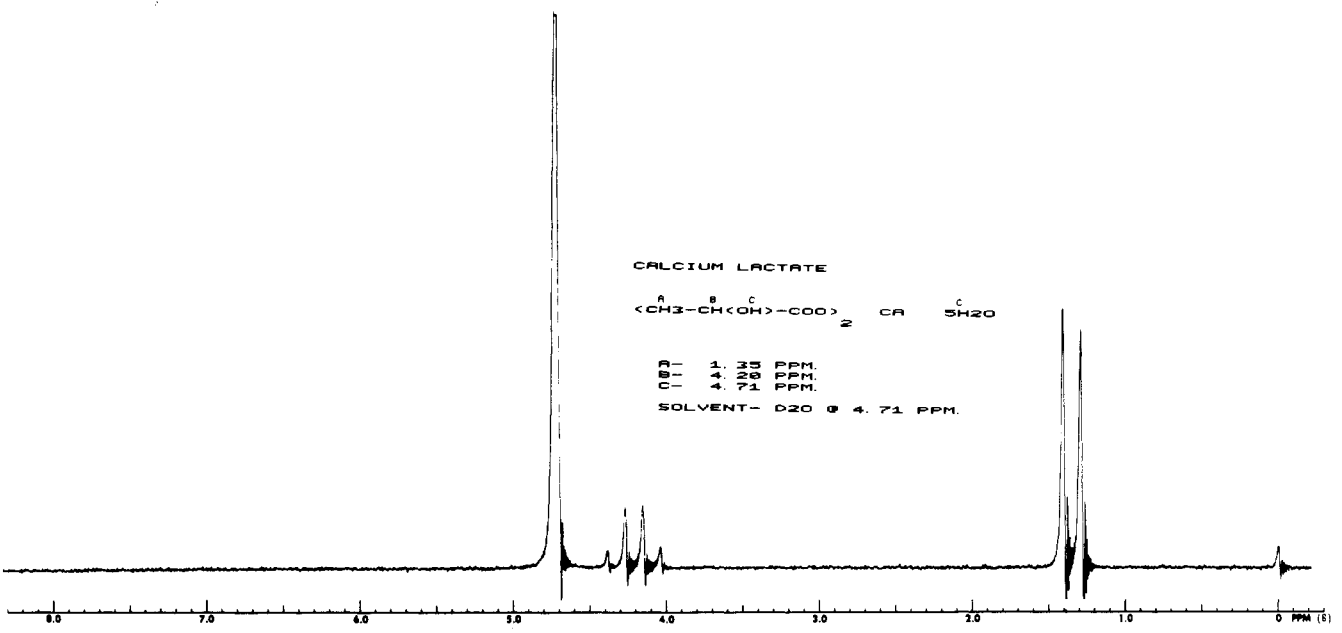

Figure 8. Calcium lactate; solvent, D_2O at 4.71 ppm.

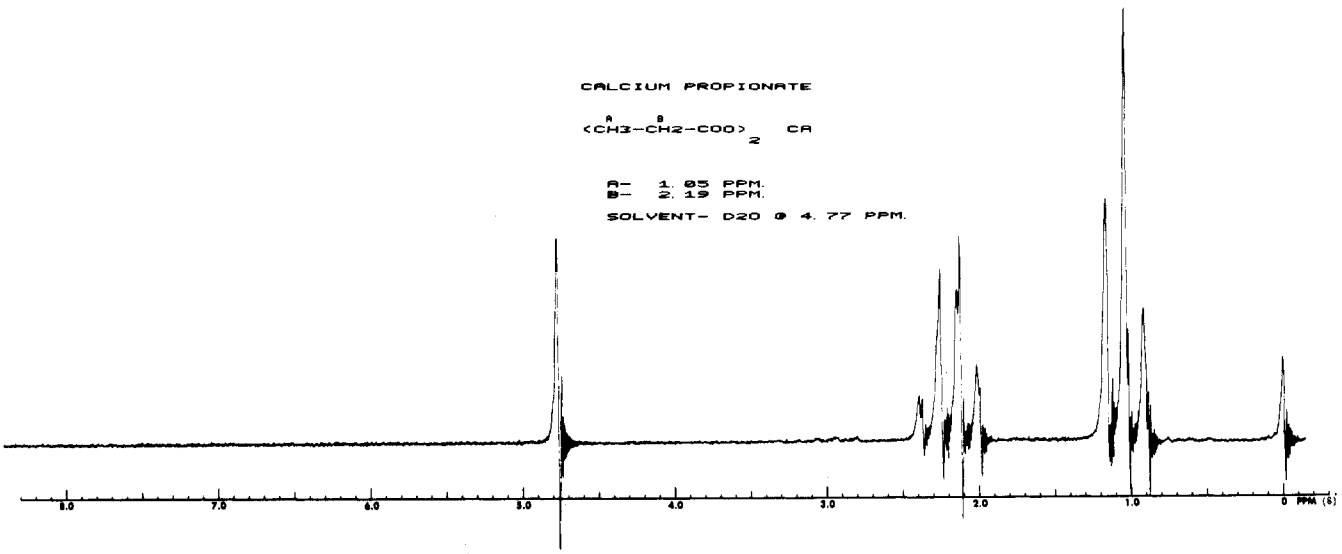

Figure 9. Calcium propionate; solvent, D_2O at 4.77 ppm.

quantitation, the number of protons each region represents, the internal standard, the solvent, and the analytical results in terms of the average value and standard deviation based on three determinations.

The probable assignments of peaks to structural groups of the compounds studied were based on intensities of

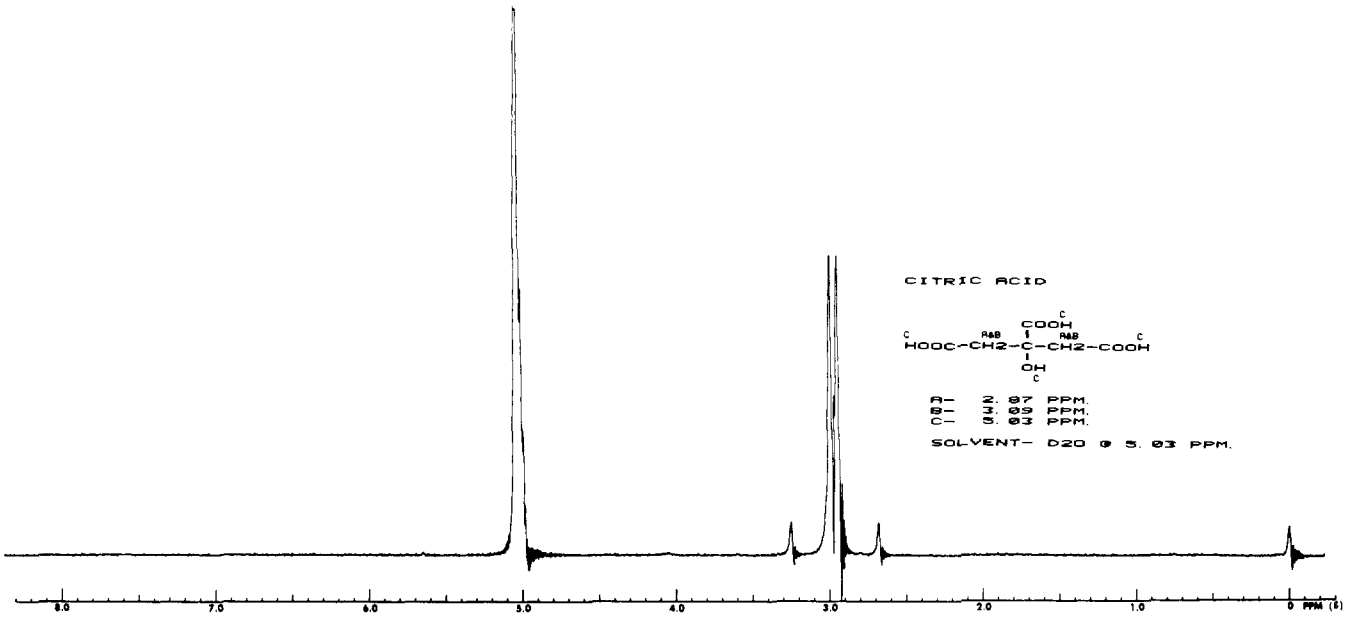

Figure 10. Citric acid; solvent, D_2O at 5.03 ppm.

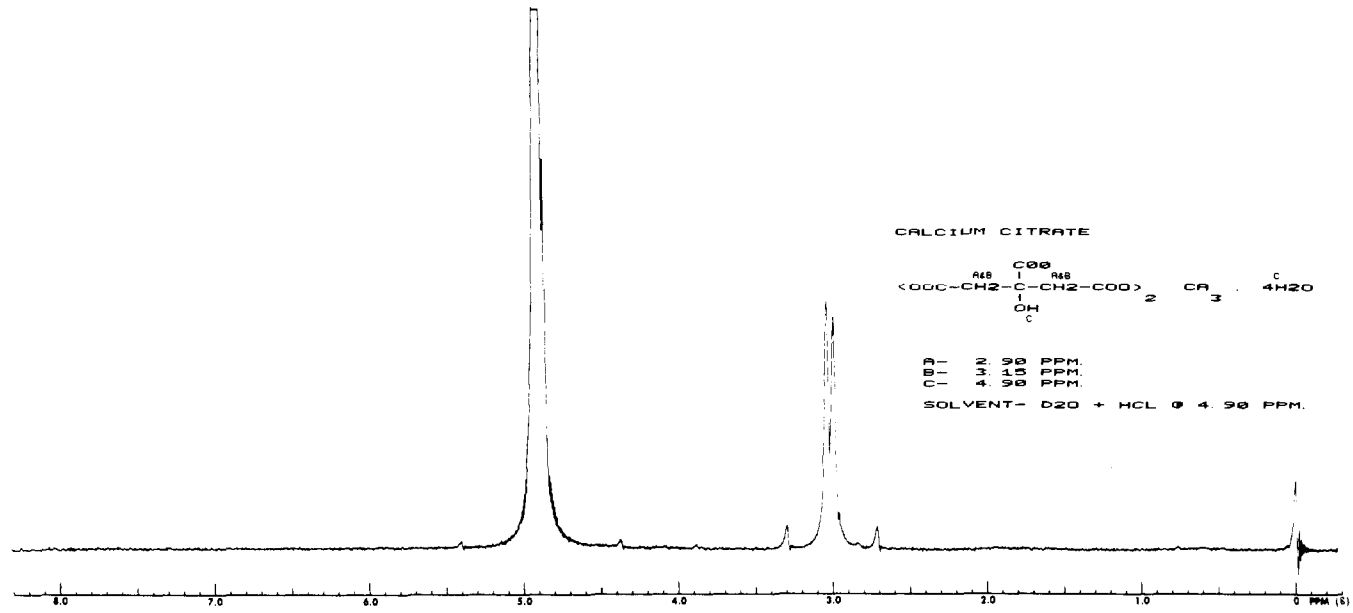

Figure 11. Calcium citrate; solvent, D_2O + HCl at 4.90 ppm.

absorption peaks and their predicted splitting, as well as on the already existing correlation (Gutowsky, 1954; High Resolution NMR Spectra Catalog, 1963; Becker, 1969) between the shift and shielding of various groups. In most cases, the relative magnitude of the peaks would obviously permit only one solution.

In the case of labile protons such as NH_2, OH, SH, and COOH, the chemical shifts that are specified on the figures are the same as for the solvent impurity HDO reflecting the exchange of these labile hydrogens with the solvent. Since the rate of exchange of labile protons between solute and solvent can be relatively slow, evidence of peak broadening such as in Figure 6 may be seen (Becker, 1969).

In contrast to other amino acids summarized in Table I, methionine and glutamic acid have complex spectra. Their spectra in terms of peak chemical shifts as well as peak area establish that the downfield resonances are due to the α proton and that the upfield multiplets are due to the β and γ protons. The two spectra are quite similar, but for a sharp prominent singlet at 2.15 ppm which can be attributed to the terminal *S*-methyl group on methionine. This methyl peak is unique among all those occurring in the NMR spectra of natural amino acids in that there are no neighboring protons to split it, and therefore its spectrum is easily differentiated from the very similar spectrum of glutamic acid. Hydrochloric acid was used to help dissolve both L-glutamic acid and L-methionine.

The asymmetric α carbon in amino acids gives rise to complexities which should be understood if the NMR spectra are to be intelligently used for analytical purposes. Because the α carbon is asymmetric, the protons of the β carbon would be expected to be nonequivalent, resulting in an NMR spectrum representative of an ABX system and not an A_2X system. However, as can be seen from the two spectra, the α carbon proton appears to be a triplet which implies that the two β protons are essentially equivalent under acidic conditions. Furthermore, under similar conditions, the NMR spectrum of L-cysteine-HCl

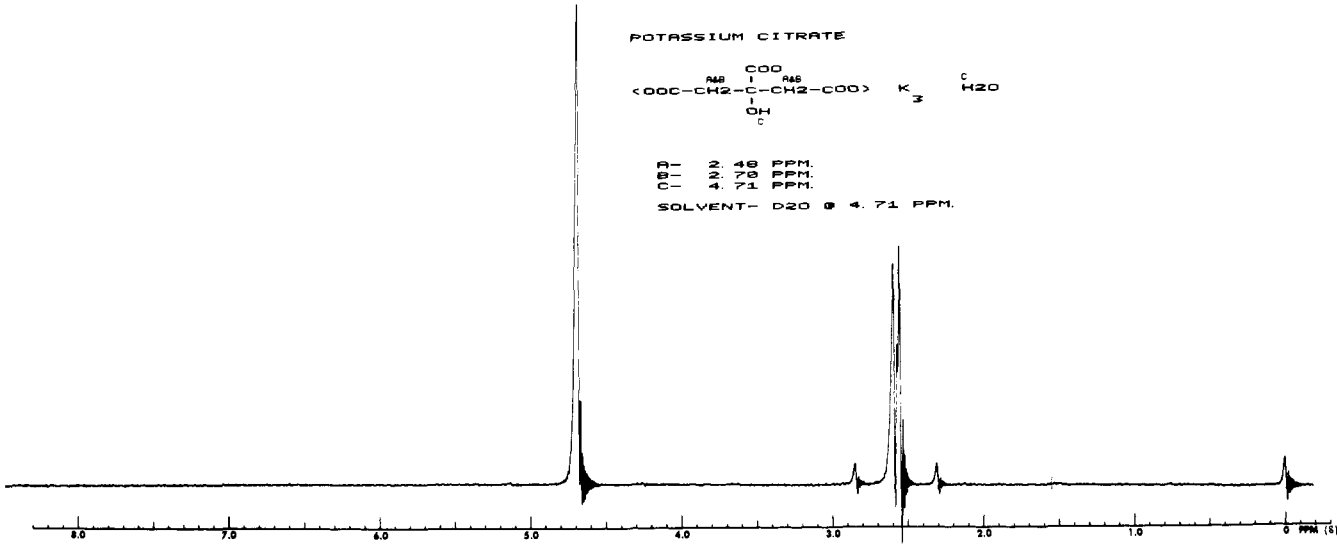

Figure 12. Potassium citrate; solvent, D_2O at 4.71 ppm.

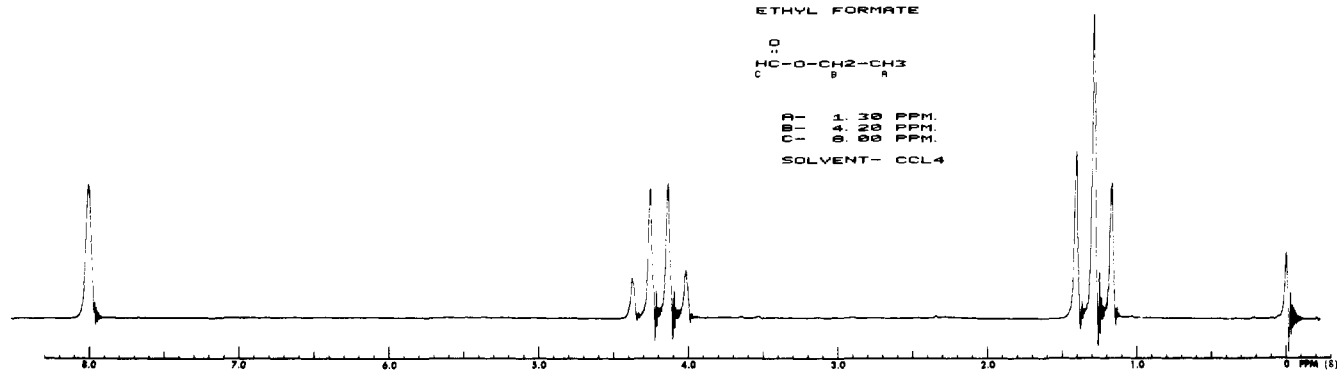

Figure 13. Ethyl formate; solvent, CCl_4.

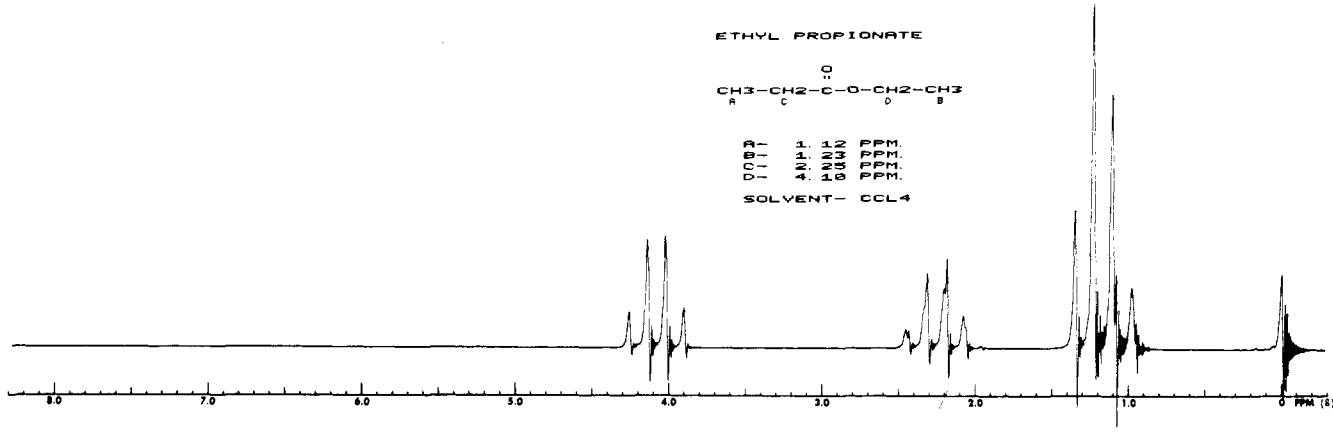

Figure 14. Ethyl propionate; solvent, CCl_4.

also appears to be of an A_2X system, although the protons are expected to be nonequivalent. This kind of behavior indicates that solution conditions are an important variable in achieving reproducible spectra. From the described observation it seems clear that acidic conditions, whereby the carboxylic acid is largely undissociated and the amino function is protonated, lead to the simplest system. However, an increase in pH produces a corresponding increase in shift between the β protons, and the resulting spectrum changes to that of a nonequivalent three-spin (ABX) system. The same occurs in the spectrum of L-glutamic acid. Apparently, when the carboxyl group is not dissociated, the two β-methylene protons will be magnetically equivalent giving rise to an A_2X NMR spectrum as was observed in the acidic solution of L-cysteine-HCl or L-glutamic acid. However, when the carboxyl is as the carboxylate, the resulting AB pattern is a more complex spectrum that confirms the actual nonequivalence of the β protons producing a chemical-shift difference and distinguishable vicinal coupling constants of the two methylene protons. For more information concerning the interpretation of complex multiple proton spin–spin interactions, the reader is referred to standard works on NMR (e.g., Becker, 1969).

Two of the compounds studied warrant some additional comment. Malic acid, because of its asymmetric α carbon, shows a spectrum similar to that of L-cysteine. The centers of the two portions of the ABX system are at 2.9 ppm for

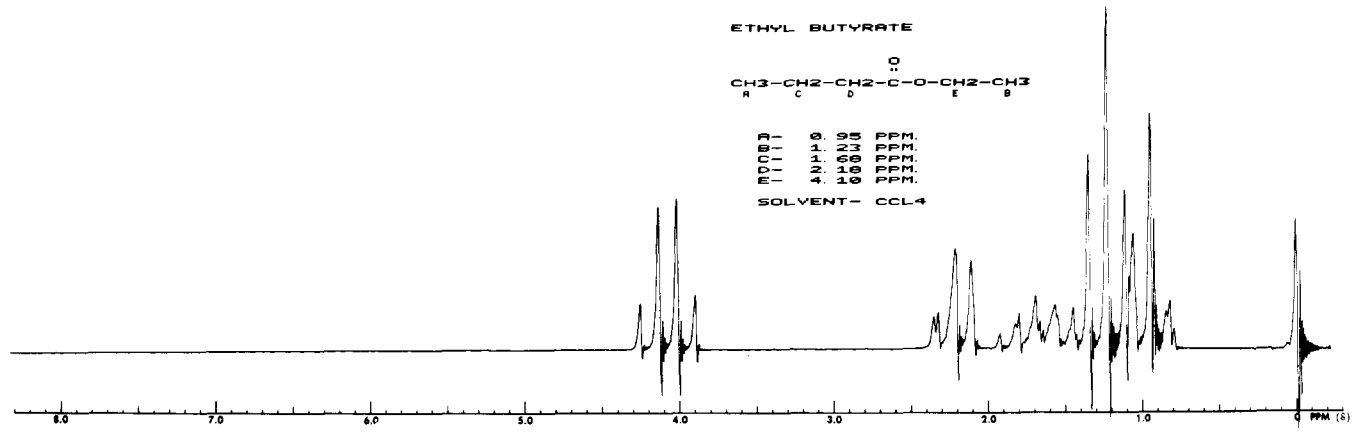

Figure 15. Ethyl butyrate; solvent, CCl_4.

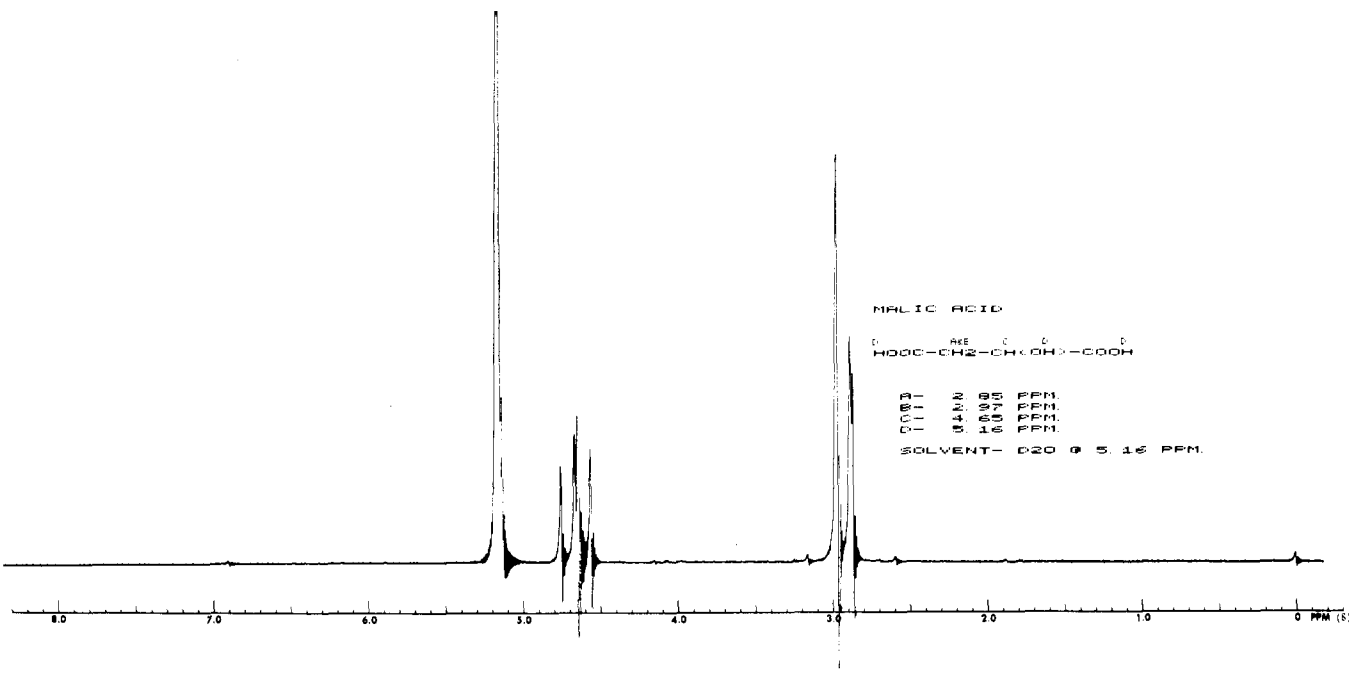

Figure 16. Malic acid; solvent, D_2O at 5.16 ppm.

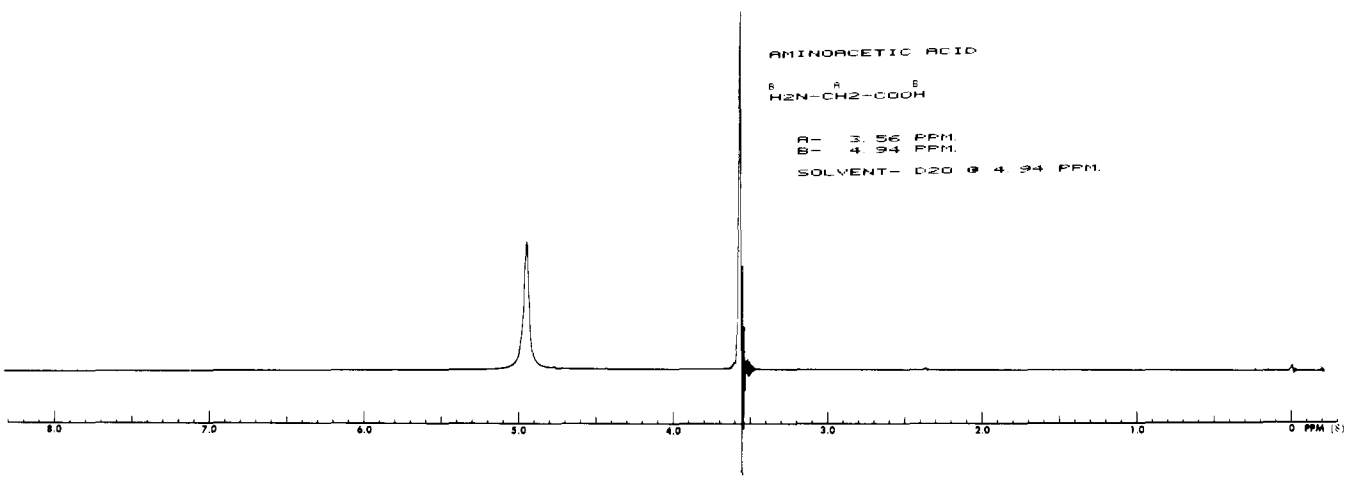

Figure 17. Aminoacetic acid; solvent, D_2O at 4.94 ppm.

the A and B signals and at 4.65 ppm for the X portion. In addition, the nature of the NMR scan offers the possibility of monitoring the presence of both maleic and fumaric acid, unsaturated dicarboxylic acids that are possible impurities in malic acid. In fact, fumaric acid is seen in Figure 16 as a very small peak at about 6.9 ppm. Maleic acid, not evident in Figure 16, would be manifest at about 6.3 ppm.

As indicated by the chemical-shift assignments in Figure 14, the spectrum of ethyl propionate is characterized by

a pair of overlapping triplets in the range 0.9–1.4 ppm.

The measurement of citrate salts rests on the NMR spectrum of the citrate ion. This is seen in the potassium citrate spectrum, a case where the salt is water soluble. In the case of calcium citrate it was necessary to dissolve the salt in an acidic medium and the spectrum is essentially that of citric acid, the species to be expected under these solution conditions. Attention is called to Figure 11 where low intensity spinning side bands are situated symmetrically about 0.52 ppm from the solvent peak.

The differences in the spectra recorded for the citrates and citric acid are useful. The NMR spectrum for citric acid shows signals from both methylene groups as two superimposed AB systems in the 2–3-ppm region due to the nonequivalence of the two protons of each methylene group. These are the signals available for integration and quantitative measurement. It is important to note that when the citrate spectrum is compared with citric acid, the methylene signal center is shifted downfield by about 20 Hz in the case of the acid. This difference provides a way for differentiation between samples of citric acid and its salts, but not in a mixture.

Table I presents a summary of the analytical results compiled in the study of all 17 compounds. It is noted that the internal standard and solvent are indicated for each compound. The assay results indicate that the maximum uncertainty experienced is less than 0.8%. The average values are all as might be expected for pure chemicals.

Some comment seems appropriate with regard to the process by which a particular resonance peak or cluster of resonance peaks is chosen for quantitative measurement. First, the analytically significant moiety giving rise to the resonance peak or peaks should be stable under analytical conditions and not be disturbed by proton exchange processes during the time of analysis. Second, in general the strongest resonance peak or multiplet is chosen provided that it is an independent signal standing at least 0.5 ppm if possible from any other signal. In many cases, a molecule may provide more than one analytically useful resonance signal. Use of the strongest signal clearly results in the most sensitive measurement since the ^1H NMR equivalent weight is smallest. In those instances where the strongest resonance is interfered with, an alternative choice is made. Third, the internal standard should be a compound that possesses a strong resonance signal, preferably a singlet, in the proximity of the chosen resonance signal of the specimen compound undergoing analysis.

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LITERATURE CITED

Becker, E. D., "High Resolution NMR—Theory and Chemical Applications", Academic Press, New York, N.Y., 1969.

Food Chemicals Codex, 1st ed, National Academy of Sciences–National Research Council, Washington, D.C., 1966.

Food Chemicals Codex, 2d ed, National Academy of Sciences–National Research Council, Washington, D.C., 1972.

Gutowsky, H. S., *J. Am. Chem. Soc.* **75**, 4567 (1953).

"High Resolution NMR Spectra Catalog", Vol. 1 and 2, Varian Associates, Palo Alto, Calif., 1963.

National Formulary, 14th ed, Mack Publishing Co., Easton, Pa., 1974.

Turczan, J. W., Medwick, T., *J. Pharm. Sci.* **65**, 235 (1976).

United States Pharmacopeia, 19th Revision, Mack Publishing Co., Easton, Pa., 1974.

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Browning Determination in Citrus Products

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Previous methods for browning determination in citrus products suffer from serious accuracy drawbacks, especially while measuring minor differences, resulting from various processing treatment of juices and concentrates. The main inaccuracy and scattering of results were caused by carotenoid interference. A modified clarification procedure is proposed: pulp removal by centrifugation, succeeded by 1:1 dilution with ethyl alcohol for floculation of remnant cloud particles and filtration through Whatman No. 42 filter paper. By this procedure carotenoid interference was eliminated and good repeatability was obtained.

Various processed food products are susceptible to browning deterioration occurring during processing and storage. Due to browning reactions miscellaneous chemical changes develop, resulting in undesirable flavors (Hodge, 1967), nutrient losses (Labuza, 1972), and formation of brown color pigments (Reynolds, 1965). Several methods were applied for the measurement of visual browning changes in food products (Hendel et al., 1950, 1955; Notter et al., 1958; Stephenson et al., 1958). These methods are based on three main steps: (1) extraction of soluble color materials in case of solid foods; (2) clarification of the resulting extract, or direct clarification in liquid foods; (3) colorimetric measurement of the clarified extract. The methods proposed in the literature differ in the extraction–clarification step, and in the wavelength used for the optical density measurement.

Widely used clarification methods in citrus juices and citrus products were based upon the dilution of single strength juice with an equal volume of acetone (Curl, 1949; Joslyn, 1957; Bakal and Mannheim, 1966) or alcohol

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